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Molecular identification and antimicrobial resistance of *Escherichia fergusonii* and *Escherichia coli* from dairy cattle with diarrhoea

U. Parin[1]*, S. Kirkan[1], S.S. Arslan[2], H.T. Yuksel[1]

[1]Faculty of Veterinary Medicine, Adnan Menderes University, Aydin, Turkey
[2]Institute of Health Sciences, Adnan Menderes University, Aydin, Turkey

*Corresponding author: uparin@adu.edu.tr

ABSTRACT: The aim of this study was to determine the incidence of *Escherichia fergusonii* in dairy cattle with clinical signs of diarrhoea. The specimens were obtained from three different farms in Denizli province of Turkey, between August 2016 and December 2016. Rectal contents of 57 Holstein-friesian dairy cattle with diarrhoea were collected from farms located in the Aegean Region (Denizli province, Turkey). Rectal swabs were inoculated into enrichment, differential and selective culture media. A total of 49 (86%) *Escherichia* spp. were isolated by phenotypic identification from 57 rectal swab samples. Presumptive *E. fergusonii* isolates were tested with the API 20E identification kit and all isolates (100%) were identified as *E. coli*. Primers targeting specific *E. coli* and *E. fergusonii* and genes, including the beta-glucuronidase enzyme, conserved hypothetical cellulose synthase protein and regulator of cellulose synthase and hypothetical protein, putative transcriptional activator for multiple antibiotic resistance were used for detection and differentiation of *E. coli* and *E. fergusonii*. Thirteen of the 49 *E. coli*-verified isolates were identified as *E. fergusonii* after duplex PCR using EFER 13- and EFER YP-specific primers. Confirmation of strain identity was conducted using Sanger sequencing analysis. The rates of antibiotic resistance of *E. fergusonii* to penicillin G and erythromycin were 100% and 77%, respectively. In conclusion, field strains of *E. fergusonii* were detected in cattle with diarrhoea in Turkey, and the strains were found to be resistant to multiple antibiotics.

Keywords: bovine; cow; *E. fergusonii*; *E. coli*; identification; PCR; antibiotics

Escherichia fergusonii bacterium was first proposed as a new species 65 years after the formal classification of *Escherichia coli*. Since 1985, there has been an increasing number of case reports in animals. While *E. fergusonii* has also been associated with Ferguson's disease in humans, only a very few studies have been reported. *E. fergusonii* is pathogenic in humans and animals, but causes diseases only infrequently (Savini et al. 2008). *E. fergusonii* has been isolated in cases of wound infection, urinary tract infection, bacteraemia, diarrhoea, endophthalmitis and pleuritis in humans. It has also been isolated from domestic animal faeces and greens (Hariharan et al. 2007; Weiss et al. 2011). Data obtained from the various sources available show that *E. fergusonii* can currently only be isolated as a dominant member of the aerobic microbial flora. The bacterium was not identified in birds, fish, frogs or reptiles (Oh et al. 2012).

With respect to veterinary medicine, Bain and Green (1999) reported that *E. fergusonii* causes various clinical indications in cattle and sheep, such as abortus, diarrhoea and mastitis symptoms. Herraez et al. (2005) isolated *E. fergusonii* from septicaemia cases, fibrino-necrotic typhlitis, fibrinous peritonitis, blood, spleen, liver specimens and multifocal serosal haemorrhages in ostriches. These authors reported that stress plays an important role in enteric diseases in ostriches, and multifactorial factors frequently play a role in the

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aetiology. Subsequently, it was reported that *E. fergusonii* showed a septicaemic distribution in goats and caused diarrhoea (Hariharan et al. 2007).

Phenotypic characterisation, DNA hybridisation and molecular phylogenetic data suggest that *E. fergusonii* may be classified within the genus *Escherichia*. *E. fergusonii* has been proposed as a new species in the *Enterobacteriaceae* family and *Escherichia* genus. *E. fergusonii* is closely related to *E. coli* based on DNA hybridisation, but there are some phenotypic differences that allow differentiation from other species: *E. fergusonii* does not ferment lactose, sucrose, raffinose or sorbitol, which helps distinguish it from *E. coli.* Although *E. fergusonii* is isolated from specimens such as blood, urine and faeces, its clinical significance has been underestimated and has not been taken into account in the literature. The food chain and environment may be contaminated by colonisation of potentially pathogenic *E. fergusonii* (Rayamajhi et al. 2011) in the cattle gut. Current diagnostic methods for identification of *E. fergusonii* consist of inoculation into selective and differential media and biochemical identification methods such as API 20E. The conventional identification of *E. fergusonii* is time-consuming and commercial biochemical identification methods cannot discriminate *E. fergusonii* and other *Enterobacteriaceae* species, since *E. fergusonii* is occasionally regarded as *E. coli* (Chandra et al. 2013). We hypothesised that the differences between *E. fergusonii* and *E. coli* can be diagnosed based on the detection of highly conserved *E. fergusonii* genes in genetic material isolated from dairy cows. Therefore, the aim of our study was to standardise a method which can be employed in field diagnosis for detection of potentially pathogenic and multidrug-resistant *E. fergusonii* from dairy cattle in Turkey.

Genome sequencing followed by discriminative analysis permits the comprehensive determination of relationships among bacterial species and allows the identification of target genes involved in antibiotic resistance and virulence. According to the best of our knowledge, ours is the first report to describe the isolation of *E. fergusonii* from dairy cattle in Turkey.

MATERIAL AND METHODS

Collection of specimens and bacterial isolation. The specimens were obtained from three different farms in Denizli province of Turkey, a significant area for dairy production, between August 2016 and December 2016. The farms were at least 35 km away from each other. A total of 57 Holstein-Friesian dairy cows aged between two and five years were used for sample collection. All of the cows were in the lactation period. Rectal swab samples were collected aseptically from each animal showing clinical symptoms of diarrhoea. All of the farms used conventional dairy practices approved in Turkey. The specimen collection method was approved by the local Animal Ethics Committee of Adnan Menderes University (document No. 64583101/2016/50). The rectal swab samples were inoculated into 5 ml of tryptic soy broth (Merck Millipore®, Germany) and incubated at 37 °C for 24 h. Ten microlitres of each culture in TSB were inoculated onto Simmons citrate agar (Oxoid®, UK) containing 4% adonitol (SCA) as described before (Foster et al. 2010) and eosin methylene blue agar for identification of *E. coli*. The agar plates were incubated at 37 °C for 24 h. Adonitol-positive yellow colonies were transferred to sorbitol MacConkey agar (SMAC) and incubated at 37 °C for 24 h (Wragg et al. 2009; Oh et al. 2012). After Gram staining, Gram-negative small coccobacilli were subjected to further identification. The presumptive *Escherichia* spp. colonies were applied to API 20E test strips (bioMerieux®, France) according to the manufacturer's recommendations.

Bacterial strains. *E. coli* ATCC® 25922 and *E. fergusoniii* ATCC® 35469 were obtained from the American Type Culture Collection (ATCC). The list of bacterial strains used to ensure the specificity of the PCR assay is given in Table 1.

Table 1. The list of bacterial strains used in this study

Microorganism	Origin
Escherichia fergusonii ATCC 35469	American Type Culture Collection
Escherichia coli ATCC 25922	American Type Culture Collection
Escherichia coli O157:H7 RHFS 232	Refik Saydam Public Health Institute/Turkey
Salmonella enterica serovar Enteritidis ATCC 13076	American Type Culture Collection
Salmonella enterica serovar Typhimurium ATCC 14028	American Type Culture Collection
Staphylococcus aureus ATCC 25923	American Type Culture Collection

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Staphylococcus aureus ATCC 25923® was used as a negative control.

Primers. Primers for a putative toxin gene of *E. coli* (*uidA*-F: 5' ATGCCAGTCCAGCGTTTTT GC 3', *uidA*-R: 5' AAAGTGTGGGTCAATAATC AGGAAGTG 3') (1487 bp) (Chandra et al. 2013), a gene encoding a conserved hypothetical cellulose synthase protein (*yliE*) of *E. fergusonii* (EFER 13-F: 5' GGGCATAAATCTGGTTGGC 3', EFER 13-R: 5' CGGGCATACCATAACAATCG 3') (233 bp) and a putative transcriptional activator for a multiple antibiotic resistance gene (*EFER_1569*) (EFER YP-F: 5' GCAATATACAGGACACAGTGTCG 3', EFER YP-R: 5' CTATGAAGGGAAGGGTAGGAGC 3') (432 bp) (Simmons et al. 2014) were ordered from Macrogen® (Republic of Korea). The first primer pair that is specific for the *uidA* gene, which is present in most *E. coli* strains (Mahaeux et al. 2009), were also used as a control for *E. fergusonii* primers. The other two primer pairs that are specific to highly conserved genes in *E. fergusonii* were selected according to NCBI BLAST results of close to 100% coverage with respect to the finished genome of *E. fergusonii* ATCC 35469 (GenBank accession no. CU928158), as described before (Chandra et al. 2013).

DNA isolation. DNA extraction from isolates was performed using a DNA Extraction Kit (Fermentas®, Lithuania) according to the manufacturer's specifications. The concentrations of DNA obtained using the kit were determined with a micro-volume spectrophotometer (ProNano PN-913, Maestrogen®, Taiwan).

PCR. PCR mixtures were prepared in a total volume of 25 µl, containing a final concentration 1 × Taq enzyme buffer solution, 100 mM KCl, 1.5 mM magnesium chloride ($MgCl_2$), 0.5 mM dNTPs, 0.2 µM of each primer and Taq DNA polymerase 5 (Genet Bio® Exprime Taq DNA Polymerase, Republic of Korea) for identification of *uidA* gene-specific products in *E. coli* isolates. The duplex PCR for *E. fergusonii*-specific products contained 6.5 µl of ddH_2O, 12.5 µl of Accustart™ PCR SuperMix (Quantabio®, Beverly, USA) and 1.25 µl of each primer in a total volume of 25 µl per reaction. Thermal cycling conditions were as described previously (Chandra et al. 2013; Simmons et al. 2014). The PCR products were loaded on a 2% Tris-EDTA buffer agarose electrophoresis gel stained with 3 µl ethidium bromide. The bands were compared to a GeneRuler 100-bp DNA ladder (Fermentas®, Canada) and a 1-kb DNA ladder (Fermentas®, Canada) to determine the size of amplicons.

Sanger sequencing analysis. Purified PCR products (~50 ng) were prepared for sequencing. Samples were analysed using the Applied Biosystems PRISM® 310 Genetic Analyzer in accordance with the protocol of the BigDye terminator method (Applied Biosystems®, USA). Nucleotide sequences of PCR products were analysed using Standard Nucleotide BLAST® NCBI Genomic Reference Sequences (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Antibiotic susceptibility test. The disc diffusion method was applied using Mueller-Hinton Agar (Merck Millipore®, Germany) to determine the antibiotic susceptibilities of *E. fergusonii* and *E. coli* isolates. The antimicrobial agents used in the antibiotic susceptibility testing were penicillin G, oxytetracycline, cefoperazone, trimethoprim-sulfamethoxazole, erythromycin, amoxicillin-clavulanic acid, gentamycin and florfenicol.

RESULTS

Phenotypic identification and biochemical characterisation

From the 57 rectal swab samples examined in this study, a total of 49 (86%) *Escherichia* spp. were isolated by phenotypic identification. Presumptive *E. fergusonii* isolates were tested using the API 20E identification kit and all isolates (100%) were identified as *E. coli*. Seven different biochemical profiles were recorded with the API 20E identification test. An identification accuracy of 73.5% was assumed to be confirmative for *E. coli*; however, *E. fergusonii* isolates could not be identified by API 20E biochemical profiling.

Genotypic identification

Forty-nine isolates identified as *E. coli* by the API 20E identification system exhibited expression of the *uidA* gene and were confirmed as *E. coli* (Figure 1). However, 13 of the 49 *E. coli*-verified isolates were identified as *E. fergusonii* after duplex PCR using EFER 13- and EFER YP-specific primers (Figure 2).

https://doi.org/10.17221/156/2017-VETMED

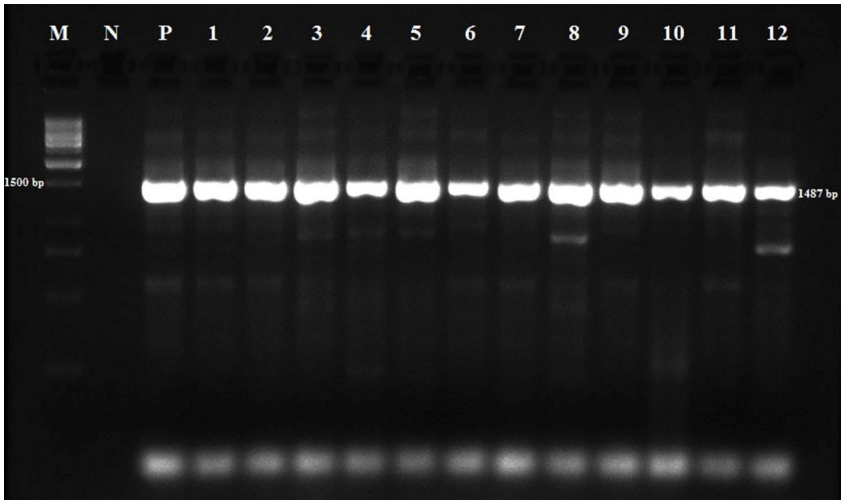

Figure 1. PCR results for the *E. coli*-specific *uidA* gene
M = 1500 bp DNA ladder, N = negative control (*S. aureus* ATCC® 25923), P = positive control (*E. coli* ATCC® 25922), 1–12 = *uidA* gene-positive samples

Antibiotic susceptibility of *E. fergusonii* and *E. coli* isolates

The results of antibiotic susceptibility of *E. coli* and *E. fergusonii* isolates are shown in Table 2.

Sequence analysis

Sequence analysis revealed that the products of field isolates exhibited 98% alignment with the *E. fergusonii* ATCC® 35469 *yliE* conserved hypothetical protein (GenBank accession No. CU928158.2) (Figure 3).

PCR specificity

The specificity of PCR analysis was determined by running the protocol with a broader range of *Enterobacteriaceae* strains: *Escherichia coli* ATCC® 25922, *Escherichia coli* O157:H7 RHFS 232, *Salmonella enterica* serovar Enteritidis ATCC® 13076, *Salmonella enterica* serovar Typhimurium ATCC® 14028 and *Staphylococcus aureus* ATCC® 25923 (Figure 3). The results indicate that EFER YP primers are specific only for *E. fergusonii* standard strains and field isolates.

DISCUSSION

While the origin and distribution of *E. coli* is the subject of intense research, little is known about the ecology of other *Escherichia* species such as *E. fergusonii*. Since the first identification of the bacterium in 1985, *E. fergusonii* has been described as an important opportunistic pathogen in both animals and humans. Transfer of genetic material between species of *E. fergusonii*, *E. coli* and *Shigella* sp. was

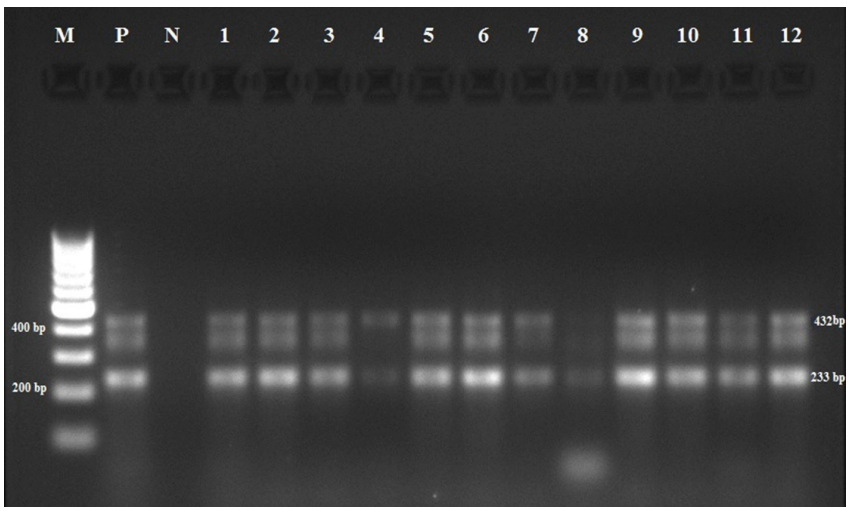

Figure 2. PCR results for EFER 13- and EFER YP-specific genes
M = 100 bp DNA ladder, N = negative control (*S. aureus* ATCC® 25923), P = positive control (*E. fergusonii* ATCC® 35469), 1–12 = EFER 13- and EFER YP-gene positive samples

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Table 2. Antibiotic susceptibility results of *E. coli* and *E. fergusonii* isolates

Antimicrobial agent	*E. coli* (*n* = 36)		*E. fergusonii* (*n* = 13)	
	n of resistant strains	%	*n* of resistant strains	%
Amoxicillin-clavulanic acid	16	44.4	1	7.6
Cefoperazone	22	61.1	2	15.3
Gentamycin	24	66.6	1	7.6
Oxytetracycline	31	86.1	3	23
Trimethoprim-sulfamethoxazole	27	75	7	53.8
Penicillin G	36	100	13	100
Erythromycin	36	100	10	76.9
Florfenicol	23	63.8	1	7.6

observed and more resistant strains of *E. fergusonii* and *E. coli* have been described. Antimicrobial resistance has also been reported against various existing therapeutic antimicrobials (Rayamajhi et al. 2011) and a large number of antibiotic-resistant *E. fergusonii* strains have been identified (Forgetta et al. 2012).

Current identification methods for *E. fergusonii* include the use of differential and selective media as well as biochemical profiling methods such as API 20E. The use of citrate adonitol and sorbitol MacConkey media is an important part of the culture process; however, the interpretation of results may be difficult, especially when using specimens with complex bacterial flora such as rectum content (Foster et al. 2010; Oh et al. 2012). The API 20E identification system is based on 20 different biochemical tests that greatly increase the accuracy of correct identification. However, the use of biochemical tests is dependent on the use of a purified bacterial isolate, and samples with high bacterial biodiversity can lead to erroneous results. For this reason, the use of PCR primers specific to highly conserved genes in *E. fergusonii* strains can provide for fast, simple and accurate identification of *E. fergusonii*. In our study, all 49 isolates were identified as *E. coli* based on their API 20 E profiles; however, molecular identification tests established that 13 isolates of these isolates were, in fact, *E. fergusonii*. In the duplex PCR test conducted in this study, both EFER 13 primer- and EFER YP primer-specific products were detected in all presumptive *E. fergusonii* isolates. Thus, the use of duplex PCR allowed a higher level of specificity for the detection of the emerging multidrug-resistant pathogen *E. fergusonii*. The PCR tests performed in our study represent a simple method that could reduce the identification period of *E. fergusonii* from six days to three days.

E. fergusonii utilises adonitol for growth and thus generates light yellow-coloured colonies on adonitol-supplemented SCA agar. On the other hand, *E. coli* typically does not use adonitol or citrate as

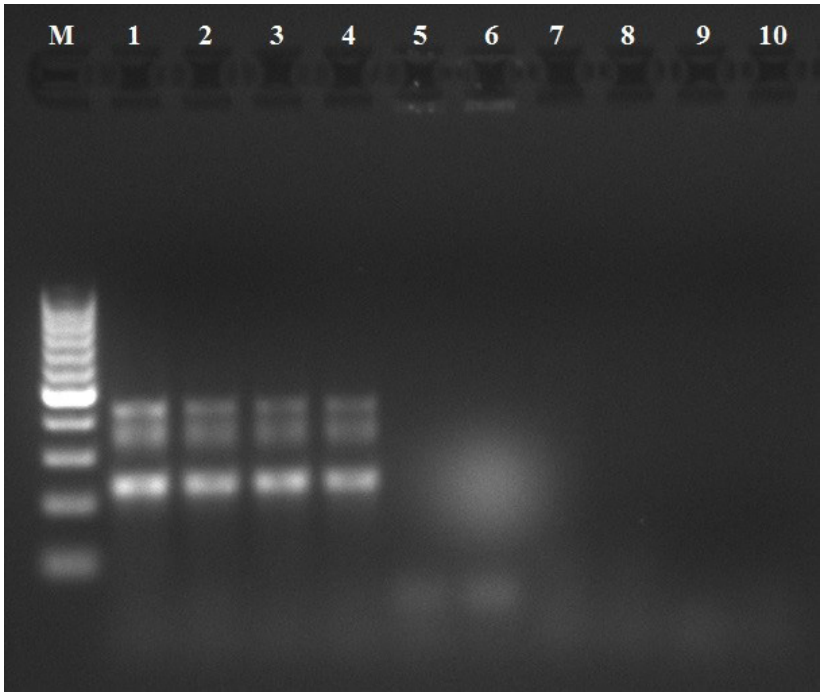

Figure 3. The specificity of PCR analysis

M = 100 bp DNA ladder, 1 = *E. fergusonii* ATCC® 35469, 2–4 = *Escherichia fergusonii* isolates of this study, 5 = *Escherichia coli* ATCC® 25922, 6 = *Escherichia coli* O157:H7 RHFS 232, 7 = *Salmonella enterica* serovar Enteritidis ATCC® 13076, 8 = *Salmonella enterica* serovar Typhimurium ATCC® 14028, 9 = *Staphylococcus aureus* ATCC® 25923, 10 = negative control (blank)

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a primer carbon source and cannot grow intensively on this medium (Foster et al. 2010). However, in this study, *E. coli* strains were identified that could use adonitol differently. *E. fergusonii* does not utilise sorbitol as a carbon source in SMAC medium, generating white or beige colonies. The growth of *E. coli* in SMAC leads to pink colonies, but lactose-negative *E. coli* variants can be misidentified as *E. fergusonii* as they generate colourless colonies (Simmons et al. 2014). In this study, 22 of 35 adonitol-positive and lactose-negative presumptive *E. fergusonii* isolates were identified as *E. coli* in PCR results. It has been argued that different modes of genetic regulation have emerged in the different *Escherichia* species and that *E. fergusonii* has evolved more rapidly compared to *E. coli* (Walk et al. 2009). The results of this study indicate that *E. coli* strains have a wide range of resistance to penicillin G, erythromycin, oxytetracycline, trimethoprim-sulfamethoxazole, gentamycin and florfenicol. The *E. fergusonii* strains have also developed resistance against penicillin G and erythromycin; however; in contrast to *E. coli* stains, *E. fergusonii* strains were found to be susceptible to amoxicillin-clavulanic acid, florfenicol, gentamycin and to be intermediately susceptible to trimethoprim-sulfamethoxazole. This study is the first to report the detection of multiple antibiotic-resistant *E. fergusonii* in Turkey. The data obtained in this study support the idea that using biochemical methods alone may not be sufficient for reliable identification results. In our study, the *uidA* gene was detected in 13 *E. fergusonii* strains. In this case, it is obvious that *E. fergusonii* carries gene sequences that are homologous to those of *E. coli*. Our study highlights the emergence of multiple resistant strains of *E. fergusonii* and *E. coli* in different farms of Denizli province in Turkey.

In conclusion, accurate identification is important to facilitate the ecological investigation of these opportunistic pathogenic *E. fergusonii* strains since they have potential to emerge as antibiotic-resistant gut bacteria. The PCR detection methods described in this study allowed clear differentiation between *E. fergusonii* and genetically related species. These identification methods are adaptable for safe and rapid use in the laboratory environment and specifically can be applied to inspection and diagnosis of recurrent diarrhoea as well as food safety and environmental assessment studies.

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