



Investigation of Enterotoxigenic *Escherichia coli* Species Which Take Role in Calf Diarrhea and Detection of their Antimicrobial Susceptibility



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Abstract

The scope of this study was determination of enterotoxigenic *Escherichia coli* identification by detection of phenotypic and genotypic methods and determination of antibiotic susceptibilities of isolated strains from animals which had clinical findings such as diarrhea in Aydin province. Between September-December 2015, 50 calves with diarrhea symptoms were found in cattle farms in and around Aydin province. A total of 50 stool swabs taken from diarrheal calves were brought to the laboratory of the Department of Microbiology and Microbiology at the Adnan Menderes University Veterinary Faculty under the cold chain. Identification of was carried out by phenotypic methods from swab samples. The isolates identified as *Escherichia coli* were genotypically confirmed by PCR and the presence of enterotoxigenic was also investigated by PCR. Antibigrams of strains identified in the last phase of the study were made by disk diffusion method. Isolation and identification of from 47 (94%) of 50 rectal swab samples examined. A total of 47 isolates (100%) examined after PCR using specific primers were found to be positive for uidA gene. Molecularly confirmed strains were identified as only one (2%) strain of enterotoxigenic when examined for the presence of ETEC-specific est1B gene. Antibigram tests revealed that isolates were sensitive to 89% for Gentamycin, 61% for Cefoperazone, 51% for Amoxicillin-Clavulanic Acid, 44% for Danofloxacin, 42% for Enrofloxacin and Penicillin G and Erythromycin 100%, Tetracycline and Trimethoprim-Sulfamethoxazole to 80% and Kanamycin to 76.5%.

As a result, 94% of and 2% of these isolates were identified by phenotypic and genotypic methods from rectal swab samples taken from diarrheal calves in our study. In conclusion of antibiogram tests of isolates, multiple antibiotic resistance development was observed.

Keywords: Enterotoxigenic *E. coli* ; Calf diarrhoea; Identification; PCR

Introduction

Bacteriologically-originated digestive system diseases widely seen all over the world are still an important health problem that affects both human health and animal health negatively. These diseases have an important cause of economic loss. Known as "Calf Diarrhea" or "Calf Septisemia" in the literature, colibacillosis is encountered in the calves during 2-10 days after birth. *Escherichia coli* is the causative agent of the disease that can be found in every enterprises. This bacteria is always abundant in the environment and in the intestines of healthy animals. The disease is more likely to occur in immunosuppressed animals due to inadequate care and nutritional conditions. The immunosuppressed calf intakes the bacteria through the respiratory, mouth, or umbilical cord. In sick calves, decrease in body temperature, yellow-whiteish and odorless watery diarrhea symptoms is seen in the tail parts of sick calves. However, there is a need for laboratory diagnosis in order not to interfere with diarrhea caused by other factors. For this purpose, if the calf is dead, it should be sent to the laboratory without being subjected to putrification. Some of sick calves recover within 5-10

days. Some of them suddenly become sick and die within 6 hours after clinical symptoms [1].

E. coli is known to be a Gram negative, rod-shaped, mostly moving, aerobic/facultative anaerobic growing microorganism found in the normal intestinal flora of mammals and poultry. While non-pathogenic strains usually do not cause infection, pathogenic strains can cause serious infections. Enterotoxigenic strains cause gastroenteritis and travel diarrhea in humans and urogenital system infections, colibacillosis and colisepticemia in animals [2,3]. It is known that wild birds such as seagulls, as well as domestic animals such as cattle and sheep play an important role in infecting pathogenic strains to humans [4,5]. The agent is able to infect humans with direct contact with faeces of various domestic and wild animals or consumption of contaminant foods (uncooked meat and unpasteurized milk products). Since people in contact with sick or transporting animals are at risk, it is very important to prescribe and treat pathogenic agents, especially in healthy animals [6-8].

Although *E. coli* is a microorganism that constitutes the healthy intestinal flora, infection can occur with an increased number of bacteria. In addition, oral intake of pathogens with faeces and contaminated feed and water is among the important reasons for the formation of the infection. In general, the intestinal tract has two primary mechanisms that control the number of *E. coli*. The first factor is known as bacterial interference between other bacteria in the flora and, while the other factor is known as inadequate passive immunity. In the newborn calves, the intestinal tract is sterile. However, microorganisms in the vaginal and perianal flora of the mother can colonize the intestinal tract very quickly through the mouth and rectum. Coliform group agents, lactobacilli and obligatory anaerobic bacteria form this colonization within a few hours after birth. Gram positive, anaerobic lactobacilli are nonpathogenic and usually found in the small intestine, they decrease the pH due to the acid they produce and therefore cannot grow. On the other hand, colostrum and milk to be fed to the calf contain specific and nonspecific antibodies synthesized in the mother against. Thus, the gastrointestinal bacterial flora is kept in equilibrium with the immunoglobulin taken by colostrum and milk. It is reported that non-hygienic conditions, stress factors, climate changes, irregularities in nutrition increase of burden in the intestinal canal [6].

The scope of this study was to identify enterotoxigenic *E. coli* agent which is one of the most important agent of calf diarrhoea by phenotypic and genotypic methods from calves with diarrhoea. It is aimed to determine the most effective antimicrobial drugs as a result of antibiotic susceptibility tests and to keep the treatment costs which play an important role in calf breeding at a minimum level also.

Material and Methods

Specimen collection

This research was carried out between September-December 2015 by visiting cattle enterprises in and around Aydın province. The animal material of the study was composed of 50 calves presenting the symptoms of diarrhoea. Samples were collected by swabs animals' rectum and delivered to the Routine Diagnostic Laboratory of the Department of Microbiology, Department of Veterinary Medicine, Adnan Menderes University in cold chain. These samples were investigated for the presence of enterotoxigenic *E. coli*. Adnan Menderes University Animal Experiments Local Ethics Committee (ADÜ-HADYEK) dated 14.08.2015 and numbered 64583101/2015/092 have not found any objection to the investigation.

Isolation

It was aimed to obtain pure *E. coli* from the laboratory samples. For this, swab samples were enriched in MTSB and incubated for 24 hours at 42 °C. Blood agar, EMB, MacConkey agar passages were performed to investigate the phenotypic characteristics of the strains that were observed at the end of 24 hours. After 24 hours of incubation, Gram staining method was applied to colonies. Identification of strains were determined by carbohydrate fermentation, indole, methyl red, oxidase, H₂S, Voges Proskauer and citrate reac-

tions [9,10].

Standard strains

ATCC® 25922 and ATCC® 35469 were obtained from the American Type Culture Collection (ATCC).

Primers

Primers for a putative toxin gene of (*uidA*-F: 5' ATGCCAGTCCAGCGTTTTT GC 3', *uidA*-R: 5' AAAGTGTGGGTCAATAATCAGGAAGTG 3') (1487 bp) and a plasmid encoding heat stable toxin (*est1b*-F: 5' TGTCTTTTTCACCTTTTCGCTC 3', *est1b*-R: 5' CGGTACAAGCAGGAT-TACAACAC3') (171bp) of enterotoxigenic [11] were ordered from MacroGen® (Republic of Korea).

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 25µl, containing a final concentration 1×Taq enzyme buffer solution, 100mM KCl, 25mM magnesium chloride (MgCl₂), 10mM dNTPs, 0.2µM of each primer and 5U Taq DNA polymerase (Genet Bio® Exprime Taq DNA Polymerase, Republic of Korea) for identification of *uidA* and *est1b* gene-specific products in enterotoxigenic *E. coli* isolates [11].

The PCR products were loaded on a 2% Tris-EDTA buffer agarose electrophoresis gel stained with 3µl 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.

Antibiotic susceptibility test

The disc diffusion method was applied using Mueller-Hinton Agar (Merck Millipore®, Germany) to determine the antibiotic susceptibilities of enterotoxigenic isolates. The antimicrobial agents used in the antibiotic susceptibility testing were Penicillin G, Tetracycline, Amoxicillin, Trimethoprim-sulfamethoxazole, Erythromycin, amoxicillin-clavulanic acid, Gentamicin, Enrofloxacin, Kanamycin, Danofloxacin.

Results

Phenotypic identification and biochemical characterization

After gram-staining, the gram-negative bacilli were subjected to biochemical identification. Lactose-positive pink colonies on MacConkey agar, which had a metallic-green sheen on the EMB agar, were preliminary identified as *Escherichia sp.* Subsequently, mannitol, glucose, indole, methyl red positive; oxidase, H₂S, Voges Proskauer and citrate negative colonies were identified *E. coli*. As a result, isolation and identification of *E. coli* from 47 (94%) of 50 rectal swab samples examined.

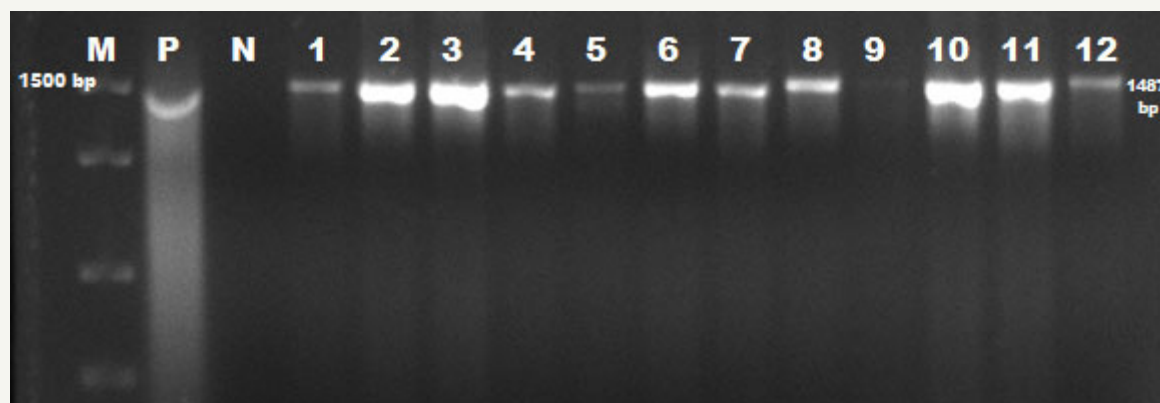


Figure 1: *E. coli* uidA specific PCR results.

M: 1500bp DNA Ladder; P: Positive Control (*E. coli* ATCC 25922); N: Negative Control; 1-12: uiaA gene Positive Samples

Genotypic identification

Forty-seven isolates identified as by biochemical identification profile exhibited expression of the uidA gene and were confirmed

as *E. coli*. Only 1 enterotoxigenic *E. coli* was identified from strains examined after PCR performed using eST1B specific primers. Electrophoresis images obtained from the PCR are shown in Figure 1 & 2.

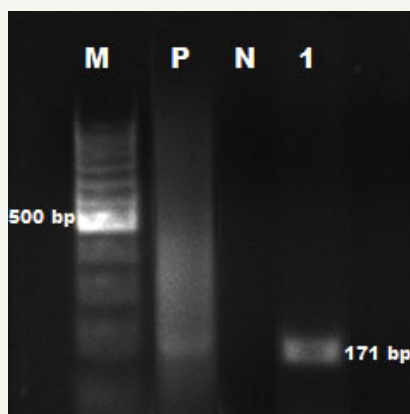


Figure 2: ETEC est1B specific PCR results.

M: 100bp DNA ladder; P: Positive Control (*E. coli* ATCC 25922); N: Negative Control; 1: ETEC *est1B* gene Positive Samples

Antibiotic susceptibility results

The antibiotic resistance rates determined against isolates are presented in Table 1. As a result of the antibiogram tests, the isolates of were susceptible to Gentamycin (89%), Sefoperazone (61%), Amoxycillin-Clavulanic acid (51%), Danofloxacin (44.6%), Enrofloxacin (42.5%) and resistant to Penicillin G (100%), Erythromycin (97.8%) Tetracycline (80%), Trimethoprim-Sulfomethoxazole (80%) and Kanamycin (76.5%).

Discussion

Enterotoxigenic *E. coli* strains can cause severe diarrhoea especially in young animals with enterotoxins [2]. Enterotoxigenic characteristics of strains isolated from enteric infections are determined by enzyme immunoassay (EIA), passive hemagglutination (PA), various latex agglutination tests, intestinal ligature testing in different animals, infant mouse test (IFT), Vero, HeLa and Y1 cell cultures, rabbit vascular permeability test, DNA probing technique,

radioimmunoassay (RIA), immunoblotting and different PCR techniques [12-14].

It has been reported that studies on the determination of enterotoxin types in strains isolated from different animals have been found in percentages between 1-66.7% of stabile toxin (ST) and 1.8-13.2% of labile toxin (LT) [8,14,15]. Carroll et al. (1990) reported that all of 16 ETECs from bovine origin (ST1a) identified by DNA hybridization technique were found ST-positive BY competitive EIA and LT negative by latex agglutination test. In a similar study, Blanco et al. [15] reported that STa and LT were detected in one of the 197 strains isolated from diarrheal cows (STa) and LT in 2 (1.8%) in 3 isolates (2.7%) from healthy isolates. Rajkova et al. [8] reported that 2 of the 54 strains originated from cattle (3.7%) were found to be ST gene positive and 1 (1.8%) strain was positive by PCR for LT gene. Mainil et al. [16] detected STb genes by PCR in 7 of 870 ETEC strains of cattle origin (0.8%). In another study, Shin et al. [17] reported that 7 of the 666 bovine isolates (1.1%) were

found to be positive for the STb gene by DNA hybridization probe technique. In a study from Brazil, STA was detected in 8 (3.9%) and LT-II in 17 (8.3%) of 250 strains isolated from diarrheal calves [18].

In a similar study in the same country, Rigobelo et al. [19] showed that ST (25.4%) and LT (13.2%) genes were highly positive in calves with diarrhoea.

Table 1: The antibiotic resistance rates determined against *E. coli* isolates.

Antimicrobial Agent	n of Resistant Strains	%	n of Susceptible Strains	%
Amoxicillin-clavulanic acid	9	19	24	51
Cefoperazone	2	4	29	61
Gentamycin	4	8.5	42	89
Tetracyclin	38	80	9	19
Trimethoprim-sulfamethoxazole	38	80	1	2
Penicillin G	47	100	-	-
Erythromycin	46	97.8	-	-
Kanamycin	36	76.5	4	8.5
Danofloxacin	21	44.6	21	44.6
Enrofloxacin	20	42.5	20	42.5

In a study conducted in Turkey, Güler et al. [14] found that 12 of 75 isolates from diarrheic calves of different farms were detected as enterotoxigenic and 16 of 45 isolates from healthy calves were negative by multiplex PCR. In a similar study, 390 strains isolated from healthy calves in Osek and Winiarczyk [20] were found to be negative by PCR in terms of LT and ST genes. Mills and Tietze (1984) identified stable toxins by ELISA in 6 (2.4%) of 251 strains originating from calves. In another study Taku et al. [21] STA was detected in 31 (14.9%) calves with IFA, and STb was detected from 45 (21.6%) of 208 strains of calf origin by loop test. Wolk et al. [22] reported that 35 (58.3%) of 60 calf isolates were positive for ST by ELISA and 4 (66.7%) of 6 strains of lamb origin were positive for ST and all strains tested were negative for LT by the same method. In a study conducted in lambs, ST gene was determined by PCR in 2 (1.4%) of 146 strains [23].

The high prevalence of the geographic area and the incidence of species in calves were determined by Snodgrass et al. [24]. It was reported that season and geography play an effective role in the passive transfer of colostrum immunoglobulins in the calves [24]. It was reported that seasonal calf mortality was particularly important in immunoglobulin absorption in neonatal calves Fink [25]. One of the possible explanations for the high frequency of species during winter was the changes in barometric pressure along with climatic variables such as temperature, rain, and thunderstorms effective on the autonomic nervous system. Alternatively, another study suggests that high species in winter may be explained by the fact that the serum IgG1 concentration in winter is low and increases during spring and summer [26]. In our study, the transition of isolated strains from November to March appears to be seasonal.

Herrera Luna et al. (2009) found that 17% of healthy and diarrheic calves were infected with in all of Austria. In addition, 15.2% of the strains carried the shiga toxin genes including the stx1, stx2, ehly and eae genes. There is another research which reported a low incidence of VTEC phenotype and O157: H7 serotypes of strains of diarrheal calves in Najaf, Iraq [27]. In one study, no cNF1, cNF2, stx2, stBand genes were found when the K99 fimbriae, stA enterotoxin, stx1 and eae genes were identified as 8, 8, 1 and 1 isolates, respectively [28]. Another study reported that the prevalence of other agents including Salmonella and Campylobacter species was detected 2% and 11% of the animals, respectively, while that of infected calves was in the ratio of 76%. In the same study, it was reported that 22/55 (40%) of diarrheal calves and 14/88 (16%) of strains from healthy calves carried K99 adhesin (P=0.001) [29]. In our study all isolates were identified as by uidA gene specific identification. Thus, it is seen that isolates from calf diarrhea are predominant bacterial species.

When the literature data are taken into consideration, it is reported that the rates of ST and LT positivity in strains among domestic animals are variable. In addition, studies in healthy animals have reported that these toxin types are found at low rates [8,15,17] and are generally negative [7,20]. In strains isolated from diarrheic animals, higher positivity is reported in various studies [18,19,30]. In this study where strains isolated from diarrheic animals were used, only 1 strain was detected as enterotoxigenic in 47 isolated from diarrheal specimens. Thus, strains are thought to originate from other serotypes.

Multidrug resistance in strains has been reported in previous studies [19,31,32]. Rigobelo et al. [19] showed that strains were the most resistant to Cephalothin (46.1%), followed by Tetracycline

(45.7%), Trimetoprim-sulfadiazine (43.3%) and Ampicillin (% 41) were found to be resistant. Previous studies have shown that isolates have multiple resistances to beta-lactam antibiotics containing broad-range Aminoglycosides, Cephalosporins, Tetracycline, Sulfanomides and Fluoroquinolones [28]. De Verdier et al. [33], strains were found to be resistant to Sulfanomide, Streptomycin, Ampicillin and Tetracycline, while 61% of all strains were reported to be resistant to more than one antibiotic. Similar research has been conducted in Sweden by De Verdier et al. [33], in the United States [34,35]. The prognosis for calf diarrhea due to septicemia varies depending on how early the treatment is started and on the calf. In addition, although it is not a surprise that the selection of inappropriate antibiotics may increase the rate of resistance to antibiotics, more than 80% resistance to certain antibiotics has been detected in our research. Penicillin G and Erythromycin (100%), Tetracycline and Trimetoprim-Sulfomethoxazole 80%, and Kanamycin 76.5% resistance rates were detected in our study strains.

Conclusion

A total of 47 strains were identified as a result of isolation and identification studies of rectal swab samples collected from 50 diarrheal calves. Standard biochemical tests have shown that the isolates are molecularly confirmed by uidA, which is the control gene, and that it is fast and reliable in the identification of . Field strains that were confirmed as in the molecular context were subjected again to PCR testing for the presence of the ETEC est1b gene, resulting in only one enterotoxigenic identified. In addition, strains were found to have multiple antibiotic resistance and strains isolated from calf strains were found to have antibiotic resistance. It has been concluded that the high sensitivity to Gentamycin is due to the fact that the antibiotic is not used on the calves for its nephrotoxic effect. Amoxycillin-clavulanic acid combined preparations and ceftiofur (3rd generation) have been found as effective antibiotics against field strains.

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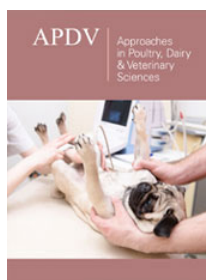
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