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Original article

Prevalence of neonatal calf diarrhea caused by *Escherichia coli* and investigation of virulence factors, serotypes, and antibiotic susceptibility

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Abstract

Neonatal calf diarrhea (NCD) is one of the most important concerns in cattle production. Escherichia coli is the most important bacterial agent of NCD. Although vaccination and antibiotic treatment are common in NCD, the high antigenic diversity of E. coli and the increase in antibiotic resistance cause difficulties in the control. The study aimed to investigate the rate of E. coli in calf diarrhea, isolate an agent of the NCD E. coli strain, determine antimicrobial resistance, and find out about some surface antigens. Fecal samples (n=115) were analyzed to isolate pathogenic E. coli strains with nine mixed infections; sixty-one strains isolate from fifty diarrhoeic calves. Among the isolates from diseased animals, 22 K99+STa+F41, 3 K99+STa, 3 strains F41, 2 strains Stx1, one strain K99, one strain eae, and one strain Stx2+eae were detected. 27 strains of F17- associated fimbriae have been identified. 17 strains F17a, 6 strains F111, 3 strains F17c, one strain carrying the F17a and F17c gene regions, whereas subfamily typing of one strain could not be performed. Serotypes were determined by molecular and serological methods: 32/61 (52.5%) isolates were O101 and 2/61 (3.3%) isolates were O9 serotypes. But 27 strain serotypes could not be detected. The antibiotic resistance profiles of the isolates were determined by the disc diffusion method. The resistance rates to antibiotics were trimethoprim-sulphamethoxazole 91.7%, ampicillin 86.7%, enrofloxacin 86.7%, gentamicin 45%, tobramycin 41.7%, cefotaxime 3.3%, and ceftazidime 1.7%. Due to increasing antibiotic resistance, prophylaxis is gaining importance. In further research, E. coli surface antigenic structures should be examined in detail, and it should form the basis for vaccine and hyperimmunization studies to be developed.

Keywords: antimicrobial susceptibility, calf diarrhea, Escherichia coli, serotype, virulence factor

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Introduction

Neonatal calf mortality is one of the most significant problems of dairy and beef production. Neonatal calf mortality was found 10.7% in Korea (Hur et al. 2013) and 7.8% in suckling calves in the United States (USDA 2008). The most important infectious causes of calf mortality are diarrhea and pneumonia. Calf diarrhea causes 15-56% calf mortality (USDA 2008, Gulliksen et al. 2009, Hur et al. 2013). Calf diarrhea either results in death, or calves recovering from the disease have lower body weight, and a decrease in milk yield (Donovan et al. 1998, Caffarena et al. 2021). Many infectious agents, such as bacteria, viruses, and protozoa, cause calf diarrhea (Cho and Yoon 2014).

Escherichia coli is the most important bacterial agent in neonatal calf diarrhea (NCD). Some *E. coli* pathotype strains, such as Enterotoxigenic *E. coli* (ETEC), Shiga Toxigenic *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), and Enterohemorrhagic *E. coli* (EHEC) cause NCD (DebRoy and Maddox 2001, Dubreuil et al. 2016, Pervez et al. 2018). ETEC strains such as K99 (F5), F41, the F17 family, and STa are one of the main causative agents of NCD, causing diarrhea by the effect of the fimbriae and the toxin (Dubreuil et al. 2016). Furthermore, ETEC strains pathogenic ability correlates with calf age. ETEC strains are more pathogenic in newborn animals younger than one week (Yuyama et al. 1993). Shiga Toxigenic *E. coli* (STEC) is present in cattle as a reservoir. At the same time, STEC causes calf diarrhea depending on the age, immune system, and gastrointestinal system conditions of calves (Bielaszewska et al. 2000, Algammal et al. 2020). Besides the presence of Shiga toxin, Enterohemorrhagic *E. coli* causes bloody diarrhea in calves due to its attaching and effacing effect, likewise Enteropathogenic *E. coli* (Thiry et al. 2017).

Antimicrobial resistance is spreading rapidly among *Escherichia coli* strains. Antibiotic resistance is increasing due to the overuse and misuse of antibiotics in cattle breeding. Although most of the antibiotics are consumed by livestock breeding (Van Boeckel et al. 2017), there is poor antibiotic diversity. The increase in antibiotic resistance in *E. coli* could cause difficulties in treatment and increase the mortality rate of NCDs.

To prevent NCD, practices such as active immunization of pregnant cows and passive immunization of calves are carried out (Dubreuil et al. 2016). The efficiency of these applications is necessary to reveal the diversity of surface antigenic structures and adhesion factors of region-specific *E. coli* strains. In this study, surface antigenic structures and adhesion structures, some virulence genes, and antibiotic resistance profiles of *E. coli* strains that cause diarrhea in calves were determined.

Materials and Methods

Sample collection

Study stool samples were taken from 1-10-days-old 115 calves with diarrhea in Kars, Turkey. Stool samples from calves diagnosed with diarrhea were taken directly from the rectum using a swab by the veterinarian and quickly delivered to the laboratory under the cold chain.

Bacterial strains isolation, PCR analysis and identification

The isolation of *E. coli* strains was applied by modifying the method of Coura et al. (2015). Briefly, the samples were dissolved in 3 ml of PBS. Then, 1 ml was inoculated into 9 ml of buffered peptone water and incubated for 18 hours at 37ºC in a shaking incubator. At the end of the pre-enrichment, DNA extraction was performed by the boiling method (Franck et al. 1998), and K99 (F5), F41, Stabile Toxin a (STa), intimin (eae), Shiga toxin 1, 2 (Stx1,2) gene regions, and F17 fimbriae and subfamilies were investigated by PCR, which is described below. Samples with at least one of the investigated gene regions were selected, passaged on Mac Conkey agar, and incubated at 37ºC for 18 hours. Ten colonies similar to *E. coli* strains were selected, passaged on EMB agar, and incubated at 37ºC for 18 hours. Metallic green colonies were selected and subcultured on nutrient agar incubated at 37ºC for 18 hours.

DNA isolation and PCR analysis

Fresh subcultured strains were homogenized in 100 µl of nuclease-free water, boiled at 100ºC for 10 minutes, then centrifuged at 8000 rpm for 3 minutes, and the supernatant was stored at -20ºC as a source of DNA (Franck et al. 1998). The samples were analyzed for STa, K99 (F5), F41, Stx1, Stx2, and, eae gene regions as previously reported by Franck et al. (1998) (Table 1). The PCR assay was carried out in a total volume of 50 µl of a mixture containing 5 µl of 10xPCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTP (Ampliqon), 0.5 µM of each primer sequence (Sentebiolab), 1.25 U Taq polymerase (Thermo Fisher), and 5 µl of bacterial DNA, and completed with nuclease-free water up to 50 µl. Samples were amplified under the following conditions: 1 minute for the initial denaturation at 94ºC, 25 cycles beginning with 30 seconds denaturation at 94ºC, annealing for 45 seconds at 50ºC, extension for 1,5 minutes at 70ºC. The extension time was ramped for an additional

Table 1. The primers used for determination of Shiga toxin 1(Stx1), Shiga toxin 2 (Stx2), intimin (eae), F41, K99 (F5), Stabile toxin a (STa) genes.

	Virulence Factor	Primer Name	Primer 3'-5'	PCR product (bp)	Referance
Multiplex PCR-1	Shiga Toxin 1	Stx-1	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT	555	(Paton et al. 1995)
	Shiga Toxin 2	Stx-2	GTGCCTGTTACTGGGTTTTTCTTC AGGGGTCGATATCTCTGTCC	118	(Paton et al. 1993)
	İntimin	eae	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA	425	(Jerse et al. 1990, Yu and Kaper 1992)
	F41	F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT	380	(Fidock et al. 1989)
	K99	K99	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC	314	(Roosendaal et al. 1984)
	Stabile Toxin a	STa	GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	190	(Sekizaki et al. 1985)

3 seconds per cycle, and 10 minutes at 70ºC for the final extension (Franck et al. 1998).

Two distinct multiplexes of PCR were used to investigate the existence of F17 fimbriae and its subfamily, as described by Bertin et al. (1996) (Table 1). The PCR assay was conducted using mix consisting of 5 µl of 10xPCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTP (Ampliqon), 0.6 µM of each primer sequence (Sentebiolab), 1.25 U of Taq polymerase (Thermo Fisher), 5 µl of bacterial DNA, and nuclease-free water was added up to 50 µl. For the amplification process, the following procedures were applied: 5 minutes for the initial denaturation at 94ºC, 25 cycles of denaturation at 94ºC for 2 minutes, annealing at 55ºC (mPCR1) and 59ºC (mPCR2) for 45 seconds, extension at 72ºC for 1 minute, and 5 minutes at 72ºC for the final extension (Bertin et al. 1996).

At the end of the PCR, the result showed a single type of virulence gene profile, and a randomly selected strain was kept for further studies. In mixed infections, a strain was selected for each profile. *E. coli* was identified with positive results for catalase, indole, and methyl red, and negative results for oxidase, hydrogen sulphate, Voges-Proskauer, citrate, and urease. Identified *E. coli* strains were stored at -20ºC in TSB with 20% glycerin.

Serotyping of *E. coli* isolates

The serotypes of virulent *E. coli* strains were determined using molecular and serological methods. Serotypes O5, O8, O9, O15, O20, O26, O55, O86, O101, and O118, which are the most common in calf diarrhea, were selected and investigated. The PCR assay was carried out in a total volume of 50 µl of a mixture containing 5 µl of 10xPCR buffer, 1 mM MgCl2, 1 mM dNTP (Ampliqon), 0.5 µM each primer sequence (Sentebiolab), 2 U Taq polymerase (Thermo Fisher) and 5 µl of bacterial DNA, and completed with nuclease-free water up to 50µl. For the amplification process, the following procedures were applied: 5 minutes for the initial denaturation at 94ºC, 25 cycles of denaturation at 94ºC for 30 seconds, annealing at 58ºC 30 seconds, extension at 72ºC for 2 minutes, and 10 minutes at 72ºC for the final extension (Iguchi et al. 2015). The strains serotyped by PCR were serologically confirmed with monovalent antisera (SSI diagnostica).

Antimicrobial susceptibility test

Antibiotic susceptibility tests for *E. coli* strains were performed by disk diffusion test according to CLSI M100-ED31-2021 standards. Ten different antimicrobial agents belonging to four different antimicrobial families were tested [(ampicillin (10 µg), cefotaxime (30 µg), ceftazidim (30 µg), ertapenem (10 µg), and meropenem (10 µg), trimethoprim-sulphamethoxazole (1.25/23.75 µg), gentamicin (10 µg), kanamycin (30 lg), enrofloxacin (5 µg)]. ATCC 25922 strain was used as a quality control strain.

Results

Isolation and pathotyping

In the present study, *E. coli* carrying at least one virulence gene was isolated in 50/115 (43.5%) calves with diarrhea. PCR was performed on 10 colonies taken from each of the samples in which the presence of virulence gene region was detected as a result of pooling PCR. As a result of PCR performed on 506 isolated col-

Table 2. Distribution of Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Shiga Toxigenic *E. coli* (STEC), and Enterohemorrhagic *E. coli* (EHEC) strains pathotyped according to virulence gene presence.

Pathotype	Virulence gene		Strain number	%
Enterotoxigenic *E. coli* (ETEC)	K99 Fimbria, Stabil toxin A, F41 fimbria		22	36,1
	K99 Fimbria, Stabil toxin A,		3	4,9
	F17 fimbria	F17a	17	27,9
		F17d (F111)	6	9,8
		F17c	3	4,9
		F17a+F17c	1	1,6
		F17 (non-determined)	1	1,6
	F41 fimbria		3	4,9
	K99 Fimbria		1	1,6
Enteropathogenic *E. coli* (EPEC)	Intimin		1	1,6
Shiga Toxigenic *E. coli* (STEC)	Shiga toxin 1		2	3,4
Enterohemorrhagic *E. coli* (EHEC)	Intimin, Shiga toxin 2		1	1,6
Total			61	100

onies, 299 *E. coli* strains had at least one virulent gene. In 41 calves, one type of virulent *E. coli* strain was isolated. Two different virulence gene profiles were detected in seven calves, and three different virulence gene profiles were detected in two calves. One strain was chosen based on each virulent profile. Consequently, a total of 61 different *E. coli* strains were randomly selected for further study (Table 2).

Serotyping of *E. coli* strain

The serotype was determined by molecular and serological methods, revealing that 32/61 (52.5%) isolates were serotype O101 and 2/61 (3.3%) were serotype O9 serotypes. The distribution of serologically and molecularly detected O serotypes according to their virulence characteristics is shown in Table 3.

Antimicrobial susceptibility test

According to the results obtained, resistance rates to antibiotics were: trimethoprim-sulphamethoxazole 91.7% (55/60), ampicillin 86.7% (52/60), enrofloxacin 86.7% (52/60), gentamicin 45% (27/60), 41.7% (25/60) tobramycin, 3.3% (2/60) cefotaxime and 1.7% (1/60) ceftazidime. The results were summarized in Table 4. One of the strains was identified as ESBL by the double disc synergy test.

Discussion

Neonatal calf diarrhea (NCD), which is one of the most important problems in cattle breeding, causes serious economic losses and adversely affects animal welfare (Smith 2012). The high rates of morbidity for diarrheal agents in general, the high costs of treatment, and the immense labor force spent on treatment cause difficulties in combating disease. As a result of inadequate or untreated treatment, calves face problems such as dehydration, electrolyte imbalances, and metabolic acidosis (Foster and Smith 2009). In calves that survived the disease, lower body weight, a delay in the first calving age, and a decrease in milk yield were observed compared to those who did not get diarrhea at later ages (Caffarena et al. 2021).

Many infectious and noninfectious factors play a role in NCD. *Escherichia coli* is one of the most important infectious agents (Foster and Smith 2009). *E. coli* is also defined as one of the bacteria that develop resistance to antibacterials most frequently in the world (Boucher et al. 2009). Due to increasing antibiotic resistance, the importance of prophylaxis is increasing day by day. Vaccination and hyperimmune serum applications are widely used for prophylaxis. However, *E. coli* has surface antigenic diversity. For prophylactic applications to be effective, regional *E. coli* diversity should be revealed, and products specific to regional differences should be developed. The surface antigenic diversity of *E. coli* strains causing NCD has been found to be very little in the literature.

High antimicrobial resistance (AMR) rates against the most commonly used antibiotics in veterinary medicine were observed, such as trimethoprim-sulfamethoxazole, ampicillin, enrofloxacin, gentamicin, and tobramycin. Our results regarding antibiotic resistance should be considered worrying. Antimicrobial resis-

Table 3. Virulence and serotype diversity profiles of the 61 E. coli isolate recovered from diarrheic calves.

Serotype	Virulence gene	Strain number	%
O101	K99+Sta+F41	12	19,7
	F17	18	29,5
	K99	1	1,6
	Stx1	1	1,6
O9	K99+Sta	1	1,6
	F17	1	1,6
Non-determined	K99+Sta+F41	10	16,4
	K99+Sta	2	3,3
	F17	9	14,8
	F41	3	4,9
	eae	1	1,6
	Stx1	1	1,6
	Stx2+*eae*	1	1,6
Total		61	100

Table 4. Antibiotic resistance/sensitivity profiles of *E. coli* strains as tested via disc diffusion test.

Antimicrobial Family	Antimicrobial (n=60)	Susceptible		Intermediate		Resistant	
		n	**%**	**n**	**%**	**n**	**%**
Beta-lactam	Ampicillin	5	8,3	3	5,0	52	86,7
	Cefotaxime	58	96,7	0	0,0	2	3,3
	Ceftazidime	58	96,7	1	1,7	1	1,7
	Ertapenem	60	100,0	0	0,0	0	0,0
	Meropenem	60	100,0	0	0,0	0	0,0
Aminoglycoside	Gentamicin	26	43,3	7	11,7	27	45,0
	Tobramycin	28	46,7	7	11,7	25	41,7
Sulfonamide	Trimethoprim-sulphamethoxazole	4	6,7	1	1,7	55	91,7
Fluoroquinolone	Enrofloxacin	6	10,0	2	3,3	52	86,7

tance rates were similar to those reported by Guler et al. (2008), Mohammed et al. (2019), and Prieto et al. (2022).

The antibiotic resistance rates in studies on commensal *E. coli* isolates are significantly lower than the resistance rates found in this study. Hang et al. (2019) found resistance to sulfamethoxazole (49.3%), ampicillin (48.6%), trimethoprime (43.8%), and gentamicin (9.8%) in their study on commensal *E. coli* strains. In countries of the European Union, indicator *E. coli* isolates isolated from cattle less than one year old were found to be resistant to sulfamethoxazole in 34.6%, ampicillin in 30.2%, trimethoprim in 24.9%, and gentamicin in 4.8%. In this study, the resistance rates of trimethoprim-sulphamethoxazole, ampicillin, and gentamicin were found to be high. Antibiotic resistance rates in virulent strains of *E. coli* are high. Antibiotic resistance increases due to exposure to antibiotics, misuse, or inappropriate antibiotic selection. As the concentration in the environment increases, it creates a selective pressure and increases the rate of resistant bacteria (Andersson and Hughes 2011).

In the present study, pathogenic *E. coli* was isolated from 50 (43.5%) of 115 diarrheal calves, and with mixed infections, 61 *E. coli* strains were isolated. Among isolated pathogen strains, the most common virulence factor is the F17 family (24%). F17 was the only virulence factor found in 21 calves (18.2%). The prevalence rate was similar to Umpiérrez et al. (2016). The K99, F41, STa, Stx1, Stx2, and eae gene regions were examined, and 28.7% (33/115) of *E. coli* strains were isolated. This rate is close to 24.81% (33/133)

found in the study carried out by Cengiz and Adiguzel (2020). Considering the prevalence of *E. coli* strains with the K99 gene, were 22.6% (26/115). Bendali et al. (1999) and Shahrani et al. (2014) determined similar rates of 20.3% and 28.41%, respectively.

In the present study, shiga toxins 1 and 2 were determined in 2.6% (3/115) of calves with diarrhea. In various studies, the presence of Shiga toxigenic *E. coli* in calf faces was found to be 2.7-9.73% (Wani et al. 2003, Guler et al. 2008). As a reason for the low presence of STEC, it was considered to limit the age of the calf selected for sampling to 1-10 days.

Apart from these isolates, three strains carrying only the F41 gene were isolated. To (1984) showed in their in vitro study that strains with the F41 virulence factor may be the cause of diarrhea. This atypical causative agent of NCD, which was isolated in an outbreak of NCD on a farm during the study, resulted in high mortality rates.

The O antigen is important for vaccine studies and epidemiological data for monitoring the disease. In the PCR and antiserum-based study performed to determine some important O serotypes, 32 of the *E. coli* strains isolated were defined as O101 and 2 strains as O9. However, none of the remaining 27 strains could be serotyped. Studies on the O serotype of *E. coli* strains that cause calf diarrhea are scarce. However, commercially recommended NCD-causing *E. coli* strain O-serotypes were found to be ineffective in detecting serotype diversity due to regional differences.

The present research has also shown that *E. coli* strains that cause NCD have increased AMR rates. The importance of prophylactic measures is increasing. Product selection is required that could affect the antigenic structural diversity of *E. coli* strains due to regional differences. The questions raised by this study are: serotype determination of non-serotypeable *E. coli* strains, determining differences between strains to be isolated from different regions, and development of appropriate vaccines and hyperimmune serum against strains that are country-wide.

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References

Algammal AM, El-Kholy AW, Riad EM, Mohamed HE, Elhaig MM, Yousef SaA, Hozzein WN , Ghobashy MOI **(2020)** Genes Encoding the Virulence and the Antimicrobial Resistance in Enterotoxigenic and Shiga-Toxigenic *E. coli* Isolated from Diarrheic Calves. Toxins 12**:** 383.

Andersson DI , Hughes D **(2011)** Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol Rev 35**:** 901-911.

Bendali F, Bichet H, Schelcher F , Sanaa M **(1999)** Pattern of diarrhoea in newborn beef calves in south-west France. Vet Res 30**:** 61-74.

Bertin Y, Martin C, Oswald E , Girardeau J-P **(1996)** Rapid and specific detection of F17-related pilin and adhesin genes in diarrheic and septicemic *Escherichia coli* strains by multiplex PCR. J Clinic Microbiol 34**:** 2921-2928.

Bielaszewska M, Schmidt H, Liesegang A, Prager R, Rabsch W, TschäPe H, Cízek A, Janda J, Bláhová K , Karch H **(2000)** Cattle can be a reservoir of sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157: H− strains and a source of human diseases. J Clin Microbiol 38**:** 3470-3473.

Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B , Bartlett J **(2009)** Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48**:** 1-12.

Caffarena RD, Casaux ML, Schild CO, Fraga M, Castells M, Colina R, Maya L, Corbellini LG, Riet-Correa F , Giannitti F **(2021)** Causes of neonatal calf diarrhea and mortality in pasture-based dairy herds in Uruguay: a farm-matched case-control study. Braz J Microbiol 52**:** 977-988.

Cengiz S , Adiguzel MC **(2020)** Determination of virulence factors and antimicrobial resistance of *E. coli* isolated from calf diarrhea, part of eastern Turkey. Ankara Univ Vet Fak Derg 67**:** 365-371.

Cho YI , Yoon KJ **(2014)** An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. J Vet Sci 15**:** 1-17.

Coura FM, Freitas MD, Ribeiro J, De Leme RA, De Souza C, Alfieri AA, Facury Filho EJ, De Carvalho AU, Silva MX, Lage AP , Heinemann MB **(2015)** Longitudinal study of *Salmonella* spp., diarrheagenic *Escherichia coli*, Rotavirus, and Coronavirus isolated from healthy and diarrheic calves in a Brazilian dairy herd. Trop Anim Health Prod 47**:** 3-11.

Debroy C , Maddox C **(2001)** Identification of virulence attributes of gastrointestinal *Escherichia coli* isolates of veterinary significanc. Anim Health Res Rev 1**:** 12.

Donovan GA, Dohoo R, Montgomery DM , Bennett FL **(1998)** Calf and disease factors affecting growth in female Holstein calves in Florida, USA. Prev Vet Med 33**:** 10.

Dubreuil JD, Isaacson RE , Schifferli DM **(2016)** Animal Enterotoxigenic *Escherichia coli*. EcoSal Plus 7**:** 47.

Fidock DA, Mcnicholas PA , Lehrbach PR **(1989)** Nucleotide sequence of the F41 fimbriae subunit gene in *Escherichia coli* B41. Nucleic Acids Res 17**:** 2849.

Foster DM , Smith GW **(2009)** Pathophysiology of diarrhea in calves. Vet Clin North Am Food Anim Pract 25**:** 13-36, xi.

Franck SM, Bosworth BT , Moon HW **(1998)** Multiplex PCR for enterotoxigenic, attaching and effacing, and Shiga toxin-producing *Escherichia coli* strains from calves. J Clini Microbiol 36**:** 1795-1797.

Guler L, Gunduz K , Ok U **(2008)** Virulence factors and anti-

microbial susceptibility of *Escherichia coli* isolated from calves in Turkey. Zoonoses Public Health 55**:** 249-257.

Gulliksen SM, Lie KI, Loken T , Osteras O **(2009)** Calf mortality in Norwegian dairy herds. J Dairy Sci 92**:** 2782-2795.

Hang BPT, Wredle E, Börjesson S, Sjaunja KS, Dicksved J , Duse A **(2019)** High level of multidrug-resistant *Escherichia coli* in young dairy calves in southern Vietnam. Trop Anim Health Prod 51**:** 1405-1411.

Hur T-Y, Jung Y-H, Choe C-Y, Cho Y-I, Kang S-J, Lee H-J, Ki K-S, Baek K-S , Suh G-H **(2013)** The dairy calf mortality: the causes of calf death during ten years at a large dairy farm in Korea. Korean J Vet Res 53**:** 103-108.

Iguchi A, Iyoda S, Seto K, Morita-Ishihara T, Scheutz F , Ohnishi M **(2015)** *Escherichia coli* O-genotyping PCR: a comprehensive and practical platform for molecular O serogrouping. J Clini Microbiol 53**:** 2427-2432.

Jerse AE, Yu J, Tall BD , Kaper JB **(1990)** A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Nat Acad Sci 87**:** 7839-7843.

Mohammed S, Marouf S, Erfana AM, El JK, Hessain AM, Dawoud TM, Kabli SA , Moussa IM **(2019)** Risk factors associated with *E. coli* causing neonatal calf diarrhea. Saudi J Bio Sci 26**:** 1084-1088.

Paton AW, Beutin L, Paton JC **(1995)** Heterogeneity of the amino-acid sequences of *Escherichia coli* Shiga-like toxin type-I operons. Gene 153**:** 71-74.

Paton AW, Paton JC, Manning PA **(1993)** Polymerase chain reaction amplification, cloning and sequencing of variant *Escherichia coli* Shiga-like toxin type II operons. Microb Pathog 15**:** 77-82.

Pervez A, Anjum FR, Bukhari AA, Anam S, S. R , Arshad MI **(2018)** Isolation and virulence genes characterization of diarrheagenic *Escherichia coli* from calves. Pak Vet J 38**:** 5.

Prieto A, Lopez-Novo C, Diaz P, Diaz-Cao JM, Lopez-Lorenzo G, Anton C, Remesar S, Garcia-Dios D, Lopez C, Panadero R, Diez-Banos P, Morrondo P , Fernandez G **(2022)** Antimicrobial susceptibility of enterotoxigenic *Escherichia coli* from diarrhoeic neonatal calves in Spain. Animals (Basel) 12**:** 12.

Roosendaal B, Gaastra W , De Graaf FK **(1984)** The nucleotide sequence of the gene encoding the K99 subunit of enterotoxigenic *Escherichia coli*. FEMS Microbiol Lett 22**:** 253-258.

Sekizaki T, Akashi H , Terakado N **(1985)** Nucleotide sequences of the genes for *Escherichia coli* heat-stable enterotoxin I of bovine, avian, and porcine origins. Am J Vet Res 46**:** 909-912.

Shahrani M, Dehkordi FS , Momtaz H **(2014)** Characterization of *Escherichia coli* virulence genes, pathotypes and antibiotic resistance properties in diarrheic calves in Iran. Biol Res 47**:** 13.

Smith DR **(2012)** Field disease diagnostic investigation of neonatal calf diarrhea. Vet Clin North Am Food Anim Pract 28**:** 465-481.

Thiry D, Saulmont M, Takaki S, De Rauw K, Duprez J-N, Iguchi A, Piérard D , Mainil JG **(2017)** Enteropathogenic *Escherichia coli* O80: H2 in young calves with diarrhea, Belgium. Emerg Infect Dis 23**:** 3.

To S **(1984)** F41 antigen among porcine enterotoxigenic *Escherichia coli* strains lacking K88, K99, and 987P pili. Infect Immun 43**:** 549-554.

Umpiérrez A, Acquistapace S, Fernández S, Oliver M, Acuña P, Reolón E , Zunino P **(2016)** Prevalence of *Escherichia coli* adhesion-related genes in neonatal calf diarrhea in Uruguay. J Infect Dev Ctries 10**:** 472-477.

Usda **(2008)** Dairy 2007, Part II: Changes in the U.S. Dairy Cattle Industry, 1991–2007. Fort Collins, CO: 57-60.

Van Boeckel TP, Glennon EE, Chen D, Gilbert M, Robinson TP, Grenfell BT, Levin SA, Bonhoeffer S , Laxminarayan R **(2017)** Reducing antimicrobial use in food animals. Science 357**:** 1350-1352.

Wani S, Bhat M, Samanta I, Nishikawa Y , Buchh A **(2003)** Isolation and characterization of Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) from calves and lambs with diarrhoea in India. Lett Appl Microbiol 37**:** 121-126.

Yu J , Kaper JB **(1992)** Cloning and characterization of the eae gene of enterohaemorrhagic *Escherichia coli* O157: H7. Mol Microbiol 6**:** 411-417.

Yuyama Y, Yoshimatsu K, Ono E, Saito M , Naiki M **(1993)** Postnatal change of pig intestinal ganglioside bound by *Escherichia coli* with K 99 fimbriae. J Biochem 113: 488-492.