Original Article

Frequency of *k99*, *stx1*, and *stx2* Virulence Factors in *Escherichia coli* isolated from Diarrheic and Clinically Healthy Suckling Calves in Sistan and Baluchistan Province, Iran

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Abstract

It is necessary to understand the frequency of virulence factor-encoding genes in the assessment of the carriage proportion. Moreover, it is required in the characterization of major unique antigens that are useful in the development of effective immunological-based preventive measures. The current study aimed to evaluate the frequency of three encoding-virulence genes associated with Enterotoxigenic (ET) and Shigatoxigenic *Escherichia coli* (*E. coli/EC)* pathotypes (*k99*, *stx1*, and *stx2*) in North of Sistan and Baluchistan Province, Iran. The frequency of *k99*, *stx1*, and *stx2* was determined via polymerase chain reaction among *E. coli* isolates collected from the feces of the clinically healthy suckling (n=50) and diarrheic calves (n=50). The *k99* gene was absent in all isolates, and the frequencies of the *E. coli* containing *stx1* and *stx2* or both *stx1* and *stx2* were estimated at 8%, 14%, and 4%, respectively, in the clinically healthy suckling calves (P>0.05), compared to 24%, 16%, and 6% in diarrheic animals (P<0.05). Among the three studied genes, there was a statistically significant difference between clinically healthy suckling and diarrheic calves in terms of the frequency of *E. coli* isolates containing *stx1*. On the other hand, the results of this study indicated that *k99* was not a major fimbrial antigen-encoding gene in the ETEC population in the region. It is assumed that in any health measure intended to control the pathogen, other genes involved with encoding fimbriae should also be considered. The noticeable high frequency of *E. coli* isolates bearing *stx1* and/or *stx2* virulence elements both in clinically healthy and diarrheic suckling calves in this study is a concern for public health. Accordingly, it is recommended that further epidemiological studies be conducted on the role of the *stx1* gene in the diarrhea of suckling calves in Sistan and Baluchistan Province, Iran.

Keywords: Calf, Diarrhea, *k99*, *stx1*, *stx2*

Fréquence des Facteurs de Virulence *k99*, *stx1* et *stx2* Chez *Escherichia coli* isolés Chez des Veaux de Lait Diarrhéiques et Cliniquement Sains Dans la Province du Sistan et du Baloutchistan, Iran

Résumé: Il est nécessaire de comprendre la fréquence des gènes codant le facteur de virulence dans l'évaluation de la proportion de transport. De plus, il est nécessaire dans la caractérisation des principaux antigènes uniques qui sont utiles dans le développement de mesures de prévention immunologiques efficaces. L'étude actuelle visait à évaluer la fréquence de trois gènes codant les virulences associés aux pathotypes Entérotoxigènes (ET) et Shigatoxigènes d'*Escherichia coli* (*E. coli / EC*) (*k99*, *stx1* et *stx2*) dans le nord de la province du Sistan et du Baloutchistan, en Iran. La fréquence de *k99*, *stx1* et *stx2* a été déterminée par réaction en chaîne par polymérase parmi les isolats d'*E. Coli* prélevés dans les matières fécales des veaux allaités cliniquement sains (n=50) et diarrhéiques (n=50). Le gène *k99* était absent dans tous les isolats, et les fréquences d'*E. Coli* contenant *stx1* et

stx2 ou les deux *stx1* et *stx2* ont été estimées à 8%, 14% et 4%, respectivement, chez les veaux allaités cliniquement sains (P>0.05), contre 24%, 16% et 6% chez les animaux diarrhéiques (P<0.05). Parmi les trois gènes étudiés, il y avait une différence statistiquement significative entre les veaux allaités cliniquement sains et les veaux diarrhéiques en termes de fréquence d'*E. Coli* isolats contenant *stx1*. D'un autre côté, les résultats de cette étude ont indiqué que *k99* n'était pas un gène majeur codant l'antigène fimbrial dans la population ETEC de la région. On suppose que dans toute mesure sanitaire destinée à contrôler le pathogène, d'autres gènes impliqués dans le codage des fimbriae doivent également être pris en compte. La fréquence élevée notable d'isolats d'*E. Coli* portant des éléments de virulence *stx1* et/ou *stx2* à la fois chez des veaux allaités cliniquement sains et diarrhéiques dans cette étude est préoccupante pour la santé publique. Par conséquent, il est recommandé que d'autres études épidémiologiques soient menées sur le rôle du gène *stx1* dans la diarrhée des veaux allaités dans la province du Sistan et du Baloutchistan, en Iran.

Mots-clés: Veau, Diarrhée, *k99*, *stx1*, *stx2*

1. Introduction

Despite the importance of neonatal calf diarrhea (NCD) as the most significant cause of morbidity and mortality in neonatal calves, the numbers of calves' losses due to diarrhea are not declining (Younis et al., 2009). The effective control and prevention of NCD must be based on a proper understanding of those disease complexities because of its multifactorial nature (Cho and Yoon, 2014). The identification of the possible risk factors and delineation of the possible causative agent of diarrhea are important to allow targeted preventative measures, such as vaccination (Younis et al., 2009). Calf scours mainly caused by *Escherichia coli* (*E. coli*) and *Salmonella* in less than two months of age (Quinn et al., 2011). The frequency of causative agents of diarrhea varies by the geographical location of the farms, farm management practices, and herd size (Cho and Yoon, 2014).

Although most *E. coli* strains are inoffensive, pathogenic *E. coli* can make a range of diseases of zoonotic importance (Cho et al., 2010). Harmless *E. coli* may cause intestinal and extraintestinal diseases due to the translocation of the virulence genes by lateral gene transfer. Moreover, new pathotypes probably emerge resulting from the genetic recombination of some virulence genes (Cabal et al., 2016).

Enterotoxigenic *E. coli* (ETEC) and Shigatoxigenic *E. coli* (STEC) are two pathotypes that can produce intestinal disease in humans (Cabal et al., 2016). The

ETEC has been reported as the causative agent of the traveler's diarrhea in patients who traveled to developing countries (Cabal et al., 2016). It has been considered the most important economically and the most common cause of NCD among pathotypes of *E. coli* (Younis et al., 2009; Cho et al., 2010). This pathotype has many virulence factors. The expression of *K99* fimbriae (*K99* adhesion antigen) accounts for nearly all cases of ETEC (*E. coli k99+*) infection was found in newborn calves (Younis et al., 2009). The first and key step of ETEC pathogenicity is the capability to be colonized in the host body by adhesive agents, such as *K99* and *K88* (Quinn et al., 2011).

The STEC infection, a zoonotic pathogen (Cabal et al., 2016), can lead to hemorrhagic colitis, and in the most severe cases, a hemolytic-uremic syndrome in humans. Cattle have been concluded as the most important reservoir and carrier of STEC among ruminants (Jamshidi et al., 2015). Bovine STEC involved in economic losses may produce two types of Shigatoxins (i.e., *stx1* and *stx2* or both) inhibiting protein synthesis and killing the cultured Vero cells *in vitro* (Cobbold and Desmarchelier, 2001).

Outbreaks of STEC via food of cattle origin, especially undercooked meat and raw milk are documented indicating its importance to human health (Cobbold and Desmarchelier, 2001).

Diarrhea caused by *E. coli* in neonatal calves is treated via antibiotic therapy. It is logical to find alternative strategies to control NCD due to the increased incidence

of antibiotic resistance and the highly limited use of antibiotics in the feed industry (Alo et al., 2018). Therefore, the determination of the target antigen is critically necessary as the first step to produce the specific therapeutic antibodies against causing agents. On the other hand, there is limited knowledge in Iran about the burden of the virulence genes of *E. coli* in healthy individuals, which could help evaluate the results from clinically affected individuals (Cabal et al., 2016).

Moreover, it is essential to understand the frequency of virulence factor-encoding genes in assessing the proportion of carriage. It is also required in the characterization of major unique antigens that are useful in the development of effective immunological-based preventive measures. The ranges of the prevalence of *E. coli* isolates containing *k99*, *stx1*, *stx2*, and *stx1+2* were determined at 1-13%, 0.1-87%, 0.1-90%, and 0-35%, respectively (Lotfollahzadeh et al., 2004; Rezazadeh et al., 2004; Shams et al., 2010; Tahamtan et al., 2010; Dastmalchi and Ayremlou, 2012; Hashish et al., 2015; Jamshidi et al., 2015).

Since there is little information available regarding the occurrence and characteristics of ETEC and STEC isolated from calves in Sistan and Baluchistan, South East of Iran, the present study aimed to investigate the prevalence of their representative virulence genes of *k99*, *stx1*, and s*tx2* in *E. coli* isolates recovered from clinically healthy and diarrheic suckling calves.

2. Material and Methods

2.1. Sample Size and Study Design

The present study was the first comparative survey on the studied genes in Iran. The sample size, considered for the comparative research, was calculated using the formula described by Eng (2003). The numbers of subjects per group (healthy/diarrheic) were determined at 11,500 (*stx1*), 544 (*stx2*), and 437 (*k99*). However, each of which included 50 cases for both screening and comparing plan because it was not possible to collect and match the case-control design in the study area in line with the calculated sample size.

2.2. Specimen and Study Area

Individual fecal samples were collected during six months (September 2015 to February 2016) from the rectum of diarrheic suckling (n=50) and clinically healthy suckling calves (n=50) (age range: 1-30 days) at several farms located in Sistan and Baluchistan (30˚85' N 61˚76' E), South East of Iran. It should be noted that the sampled farms were the largest in the region. The sampled calves, which were of the cows, not the buffaloes, were traditionally maintained. Following that, the constant clinical signs observed in the examined calves were sudden onset of profuse yellow/white diarrhea leading to rapid and severe dehydration. None of the healthy individuals presented any gastrointestinal symptoms when the samples were collected. The median age of the studied calves was 13 days. The calves had eaten the colostrum at the same age and no one of which had been vaccinated against bacterial infections.

2.3. Bacteriological Examination

The samples were transported to the Laboratory of Microbiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran, with minimum delay in the icebox. They were then processed on the same day. One g of each fecal sample was inoculated in 5 ml of sterile Pepton water (Liofilchem, Italy) and incubated for 18-24 h at 37˚C. In the next stage, the samples were sub-cultured, and *E. coli* was identified biochemically. Briefly, a loop full of diluted specimens was inoculated on MacConkey agar (Liofilchem, Italy) and incubated at 37˚C for 18-24 h. Lactose fermenter (pink) colonies were streaked on Eosin Methylene Blue (EMB) agar (Liofilchem, Italy) plates and green colonies (presumptive *E. coli*) with metallic sheen growing on EMB confirmed as *E. coli* using Triple Sugar Iron Agar, IMViC test, Simmons' citrate agar, and SIM agar (Liofilchem, Italy) (Quinn et al., 2011).

2.4. Positive Control Strains

For polymerase chain reaction (PCR), a *k99* serologically-positive *E. coli* strain and an isolate of *E. coli* containing *stx1* and *stx2* genes were used as

positive controls. Double-distilled water was used as a negative control.

2.5. Polymerase Chain Reaction

The PCR was conducted for all isolates. The procedure was performed by the primers that amplify 180 bp fragment for *stx₁* (*stx₁F*: 5'-ATAAATCGCCATTCGTTGACTAC-3'*; stx₁R:* 5'-AGAACGCCCACTGAGATCATC-3') and 225 bp fragment for *stx₂* (*stx₂F*: 5'-GGCACTGTCTGAAACTGCTCC-3'*; stx₂R:* 5'-TCGCCAGTTATCTGACATTCTG-3') (Jamshidi et al., 2015). Meanwhile, in case of ETEC, oligonucleotide primers (*k99F*: 5'-TATTATCTTAGGTGGTATGG-3'; *k99R*: 5'-GGTATCCTTTAGCAGCAGTATTTC-3') that amplify a 314 bp fragment for the *k99* (*F5*) gene was used (Franck et al., 1998).

The DNA was extracted using the boiling method (Sadeghi Bonjar et al., 2017). The PCR reaction mixture with some modification in a 25 μL volume consisted of 5.5 μl DNA, 12.5 μl Master Mix with *Taq* DNA Polymerase (Amplicon, Denmark), 1 μl of each primer (10 μM), and 5 μl distilled water. The amplifications were performed by a programmable thermocycler (gradient Eppendorf's Master cycler® pro, Eppendorf, Hamburg, Germany).

Table 1 tabulates the conditions for the amplification of *k99*, *stx1*, and *stx2*. The amplified product was visualized by standard gel electrophoresis of 10 μl of the PCR reaction mixture on 1.5% agarose gel stained with ethidium bromide (Cinnagen, Iran). Visualized DNA fragments along with the relevant DNA marker (Fermentas, Germany) were pictured by gel documentation (Cambridge, UK).

2.6. Statistical Analysis

The data were analyzed using SPSS software (Version 16.0, USA). Moreover, the chi-square (X^2) test and Fisher's exact test were used to assess the significant differences between the diarrheic and healthy groups. Furthermore, the presence of *k99*, *stx1*, and *stx2* was investigated in *E. coli*. A p-value less than 0.05 was considered statistically significant.

Table 1. Polymerase chain reaction protocols used in this study

Gene	Polymerase chain reaction program						Reference
	Initial denaturation (°C/min)	Denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/min)	Final extension (°C/min)	No. cycles	
k99	94/5	94/30	50/45	72/1.5	70/10	25	
stx1	94/5	94/60	55/30	72/1	72/10	35	(Franck et al., 1998)
stx2	94/5	94/60	55/40	72/1.5	72/10	35	

3. Results

Positive control of *k99* showed the representative band (314 bp) via electrophoresis, while none of the *E. coli* isolates, both in clinically healthy (0%) and diarrheic (0%) suckling calves carried *the k99* gene (Figure 1 A).

In the present study, among *E. coli* isolated from clinically healthy suckling calves, the frequencies of the *E. coli* containing *stx1* and *stx2*, or both (*stx1+2*) were 8%, 14%, and 4%, respectively (P<0.05). Furthermore, in *E. coli* isolated from diarrheic suckling calves, the corresponding frequencies were estimated at 24%, 16%, and 6%, respectively (P<0.05).

Our results demonstrated that *E. coli* harboring *stx1*, *stx2*, and *stx1+2* in samples from diarrheic calves were more frequent than those of healthy ones. Moreover, a statistically significant difference was observed between the isolates recovered from clinically healthy and diarrheic suckling calves in terms of *E. coli* harboring *stx1* (P<0.05).
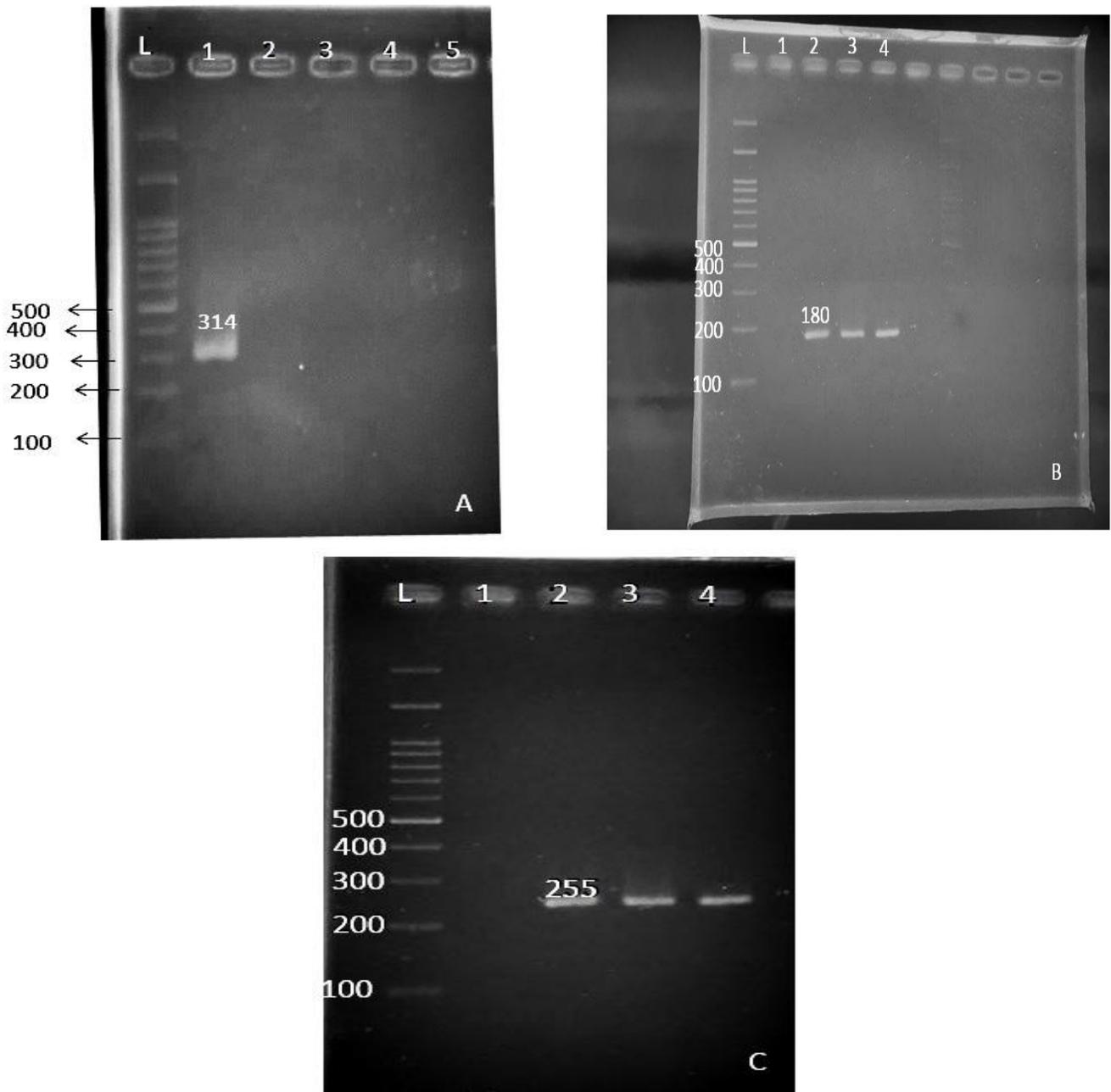

Figure 1. Gels represented the amplification of the selected *E. coli* isolates from healthy and diarrheic calves for *k99* (A) Lane 1: Positive control, Lane 2: Negative control, Lane 3-5: selected studied isolates; *stx1* (B) Lane 1: Negative control, Lane 2: Positive control, Lane 3 and 4: Selected investigated isolates; and *stx2* (C) Lane 1: Negative control, Lane 2: Positive control, Lane 3 and 4: Selected investigated isolates. L indicates GeneRulerTM 100 bp DNA ladder marker.

4. Discussion

According to the literature, for many years, there have been no investigations regarding healthy calves in terms of ETEC *k99* in Iran, and the most recent study was conducted in 2010. The frequencies of *k99* in *E. coli* isolates obtained from diarrhea calves (0%) in this study are lower than those reported in Iran and abroad in the studies conducted by Lotfollahzadeh et al. (2004) in Ghaemshahr-Babol (1.07%), Rezazadeh et al. (2004) in the suburbs of Tehran (1.2%), Shams et al. (2010) in Fars Province (4.3%), and Hashish et al. (2015) in Egypt (13.3%).

These investigations were performed just in diarrheic calves, and all performed a nearly different method (conventional microbiological techniques), except for Hashish et al. (2015) who conducted a PCR method.

The various frequency of *E. coli k99*[+] could be related to different techniques applied for screening along with the geographical variety. Traditional microbiological methods were used by previous investigations. Many accessible diagnostic tests, hemagglutination inhibition, serum agglutination, indirect fluorescent antibody tests, and enzyme-linked immunosorbent assay have been introduced; however, PCR has been a specific and convenient method for large-scale screening of ETEC *k99* for its sensitivity, simplicity, and rapidity (Younis et al., 2009; Cho and Yoon, 2014).

Many factors might contribute to the negative outcome of the PCR used in the present study. The detection limit of the PCR is related to the number of colonies forming units, and it clarifies why the target gene was not detectable (i.e., a few numbers of colony-forming units). In addition, the presence of antibiotic residues might explain the falsely negative bacteriological results because the withdrawal time may not be regarded in the studied herds by the farmers (Fricker, 1987). Since the history of prescription of antibiotics was not accessible, the farmer's claim was considered in this connection to clarify whether or not the antibiotic was prescribed. Moreover, the lack of *k99* may result from some functional changes of the receptors or the presence of immature receptors in the intestine of the suckling calves that prevented ETEC *k99* to be colonized in the intestines (Quinn et al., 2011). It leads to a decline in the population of the ETEC *K99* in the intestinal environment. Studies have shown that ETEC strains are more inclined to colonize in jejunum and ileum (Radostitis and Blood, 2017). Therefore, the sampling site could be suggested as a factor that alters the frequency of the isolation of *E. coli* harboring *k99*. In this connection, according to some studies (Lotfollahzadeh et al., 2004; Rezazadeh et al., 2004), the feces were collected directly from the rectum. The highest prevalence of ETEC *k99* was reported in December and March (Younis et al., 2009). Although there was an attempt to perform sampling in these months, no frequency of *k99* was observed for both isolates recovered from clinically healthy and diarrheic suckling calves, which indicated the need for the year-wise distribution of ETEC *k99* isolation for both isolates recovered from clinically healthy and diarrheic suckling calves in the study area.

Studies showed that rotavirus infection, vaccination of the animals with combined vaccine against rotavirus, coronavirus and ETEC *k99*, colostrum feeding practice, animal age, Vitamin E, and selenium supplementation of pregnant cows alter the prevalence of ETEC *k99* infection in healthy and/or diarrheic calves (Younis et al., 2009). In this connection, it was to match control-case samples equitably based on the history and clinical signs.

Bacterial host properties can interfere with the outcome. This lack of frequency also indicated that *k99* is not the major fimbrial antigen-encoding gene and other genes are also probably involved in binding this bacterium to the target cell and should be taken into consideration for the ETEC of suckling calves of the study area for detection or prevention purpose.

Primers for *stx1* and *stx2* produced 180 and 255 bp PCR products, respectively, such as the control strain (Figures 1B and Figure 1C).

Recently, there have been few reports in Iran and

other countries that evaluate the frequency of *stx1, stx2,* or *stx1+2* in calves with or without diarrhea, individually.

A higher proportion of *stx1* (45.2%, Tahamtan et al., 2010; 15%, Jamshidi et al., 2015; 12.5%, and Dastmalchi and Ayremlou, 2012), *stx2* (88.3%, Tahamtan et al., 2010 and 19%, Jamshidi et al., 2015), and *stx1+2* (34.9%, Tahamtan et al., 2010; 8%, Jamshidi et al., 2015; and 12.5%, Dastmalchi and Ayremlou, 2012) along with a lower proportion of *stx2* (12.5%, Dastmalchi and Ayremlou, 2012) have been reported among *E. coli* from healthy calves, compared to those in the present study.

On the other hand, among *E. coli* from diarrheic calves, a higher proportion of *stx1* (86.7%, Hashish et al., 2015) and *stx2* (26.7%, Hashish et al., 2015; 90%, Behzadian Nezhad et al., 2011) along with a lower proportion of *stx1* (1%, Behzadian Nezhad et al., 2011; and 0.1%, Dastmalchi and Ayremlou, 2012), *stx2* (0.1%, Dastmalchi and Ayremlou, 2012), and *stx1+2* (0%, Dastmalchi and Ayremlou, 2012; 0%, Hashish et al., 2015; and 0%, Behzadian Nezhad et al., 2011) have been reported in the studies, compared to those in the present study.

Regardless of the health status of the calves, the fluctuation of the frequency of isolates containing *stx1, stx2,* and/or *stx1+2* caused by many factors. These factors included varieties in geographical differences that resulted in the various distribution of bacteriophages and harboring motile genetic elements; environmental conditions, such as temperature, humidity, sunshine, and sanitary condition; and the host physiology, such as dissimilarity in the excretion of fecal and/or genetic composition that could considerably affect the prevalence of *E. coli* and subsequently the tested genes (Behzadian Nezhad et al., 2011).

Since the bacteriophages transfer the motile genetic element (i.e., *stx*-coding gene) among bacteria via transduction procedure (Quinn et al., 2011), the transduction rate and the amount of the bacteriophages may elaborate the findings of the present study. Heat stress resulted mainly from environmental conditions or environment-host physiology interaction may affect the composition of the colostrum and sensitivity of the calves of the dams for a particular pathogen. The environmental condition, in which the calf is born, affects the absorption of immunoglobulin (Ig). The Ig, particularly IgA, the dominant Ig in the colostrum and/or mucosal surface of the intestine, neutralizes the virulence factors expressed on the surface of the bacterium that may lead to a decrease in the specific pathotype of bacteria via selecting pressure. Therefore, the correlation of the presence of the *stx1*, *stx2*, and *stx1+2* positive *E. coli* with the content of the IgA in the colostrum of the dams and/or intestine of the calves could address the different observations among studies. In other words, the interactions between host defense, pathogens, and environment elaborate the inconsistency of the frequency of the virulence factors among the *E. coli* isolates studied in the different geographical regions.

In line with our findings, the dominance of *stx2*, compared to *stx1* and *stx1+2*, have been reported among *E. coli* isolates from healthy cattle by Tahamtan et al., (2010) and Jamshidi et al. (2015). Moreover, the findings of a study conducted by Hashish et al. (2015) are consistent with our results indicating the higher frequency of *stx1*, compared to the *stx2* and *stx1+2* in diarrheic cattle. However, the results of a study performed by Behzadian Nezhad et al. (2011) are not in line with our findings in diarrheic cattle that depict the dominance of *stx2*, compared to the *stx1* and *stx1+2*. Since the transcription of *stx1* is iron-related, *in vivo* and the intestine environment is not iron-rich (Behzadian Nezhad et al., 2011), and the level of the transcription of *stx1* may shift in favor of high transcription of *stx2*. Consequently, virulent *stx1* positive isolates due to the iron-poor environment (intestine) were declined via selection pressure leading

to the higher detection of *stx2* positive *E. coli*.

Dastmalchi and Ayremlou (2012) reported in Urmia that among healthy and/or diarrheic cattle, the isolates harboring *stx1* and *stx2* were not dominant. This finding is not consistent with the results of the present study that can be due to various sources of information in methodology (STEC in the aforementioned study vs. *E. coli* in the present study).

To the best of our knowledge, this is the first report for the comparison of the frequency of *k99, stx1,* and *stx2* genes in *E. coli* isolated from healthy and diarrheic calves in the South East of Iran. According to the literature, there was only one study in Iran that was approximately similar to the present study in terms of the used method; however, *k99* was investigated in the present study (Dastmalchi and Ayremlou, 2012).

Dastmalchi and Ayremlou (2012) determined the dissemination of the STEC in feces of 2-6-month-old healthy and diarrheic calves in Urmia, Iran. The isolates of fecal samples were tested for *stx1*, *stx2*, and other virulence marker genes. It is worth mentioning that the isolates containing *stx1, stx2,* and *stx1+2* were more prevalent in healthy calves (12.5%, 12.5%, and 12.5%), compared to the diarrheic ones (0.1%, 0.1%, and 0%), which was not consistent with the results of this study. The variety of the abundance between healthy and diarrheic ones reported by Dastmalchi and Ayremlou (2012), compared to the present study, irrespective of the variety in the sources of information in methodology (STEC in their study vs. *E. coli* in the present study), may result from the variety in the animal age, farm management/conditions, and/or geographical location (Behzadian Nezhad et al., 2011), which require more investigation and examination.

Some studies have revealed that strains possessing only *stx2* are potentially more virulent than those harboring *stx1* or even strains carrying both *stx1 and stx2* (Tahamtan et al., 2010). Furthermore, *stx2* harboring bacteria has more ability to survive in the intestine (Behzadian Nezhad et al., 2011) which consequently increases the possibility of lateral gene transfer leading to incorporating virulence genes or

genetic recombination to emerge new virulent strain causing diarrhea (Cabal et al., 2016). These explanations may elaborate the reason for the function of the *stx2* positive isolates in diarrheic calves despite their fewer frequency, compared to the healthy calves in a study conducted by Dastmalchi and Ayremlou (2012).

Notably, the iron content of the intestine between the two groups (healthy and diarrheic) should be taken into account to elaborate the compared data (Behzadian Nezhad et al., 2011). Further investigation to test this hypothesis is suggested locally, regionally, and nationally.

The findings in this study are important for public health and preventive veterinary medicine since the isolates recovered from both healthy and diarrheic suckling calves in the study area contained *stx1*, *stx2*, or *stx1+2,* and the main virulence factors/markers for zoonotic bacteria (i.e., STEC). Moreover, despite the low frequency of the isolates containing *stx1+2*, compared to the isolates harboring *stx1* and *stx2* in this study, clinically healthy cattle could be considered the primary reservoir of the STEC strains which may help transfer the pathotype to human or other animals in the study area due to the statistically equal distribution of isolates containing *stx1+2* between clinically healthy and diarrheic suckling calves of the study area.

Moreover, the *stx1* can act as a virulence factor among *E. coli* isolates of diarrheic suckling calves of the study area indicating that it could be regarded as the main factors for the pathogenicity of *E. coli* in the study area and could be used for the diagnosis of a virulent strain of *E. coli* isolated from calves.

Our analysis may be biased towards two *E. coli* pathotypes; therefore, it is recommended to consider all pathotypes when comparing *E. coli* from stools. The present case-control study aimed to investigate and analyze three genes represented in two *E. coli* pathotypes, isolated from suckling calves with and without diarrhea and accompanied with fairly matched case-control samples. The critical decision to evaluate only STEC and ETEC was not the best since there was

no information in the area and selected representative genes. Our study, as a preliminary survey, compared the frequency of three genes of *E. coli* among the isolates from suckling calves with and without diarrhea to propose a probable candidate gene to control the diarrhea measures.

5. Conclusion

The isolates recovered from both clinically healthy and diarrheic suckling calves in the study area contained the remarkable virulence factors of *E. coli*, *stx1* (dominant in diarrheic calves), *stx2*, or *stx1+2*, alarming for public health. Furthermore, *k99* was not regarded as the major fimbrial antigen-encoding gene, and other genes (i.e., encoding fimbriae) should also be taken into consideration for ETEC when formulating a strategy for health purposes in the study area. Future epidemiological studies on the *stx1* gene, other than *k99* and *stx2,* are suggested for calf diarrhea in the study area.

Authors' Contribution

All authors have approved the final article. S. S. designed the study. N. K. and S. S. performed the experiment and data analysis, respectively. A. R. designed the study, interpreted the data, and drafted the article. S. S. and A. R. revised the manuscript critically for the important scientific content.

Ethics

This study was carried out according to the legal requirements of the relevant local authority.

Conflict of Interest

The authors declare that they have no conflict of interest.

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     Keykhaei et al / Archives of Razi Institute, Vol. 76, No. 2 (2021) 283-292

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