Original Article

Isolation and Molecular Identification of the Enterotoxigenic Pathotype of *Escherchia coli* From Calf Diarrhea in Some Part of Iran

Mahmoud Khormali[1], Taghi Zahraei Salehi[1*], Bahar Nayeri Fasaei[1], Gholamreza Nikbakht Brujeni[1], Ramak Yahyaraeyat[1], Samad Lotfollah Zadeh[2]

1. Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
2. Department of Internal Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

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ABSTRACT

Background: Calf diarrhea is a common cause of pre-weaning morbidity and mortality in cattle within livestock operations.

Objectives: The primary aims of our study were to determine the prevalence and occurrence rate of genes that encode virulence factors (virotypes) in *Escherichia coli* strains isolated from calves with diarrhea.

Methods: Rectal swabs were collected from 156 calves exhibiting diarrhea, representing 12 distinct dairy farms located across five provinces in Iran. Through polymerase chain reaction (PCR) analysis, the *E. coli* isolates were evaluated for the presence of various virulence genes, including *f4*, *f5*, *f6*, *f41*, *f17*, *cfa/1*, *sta*, and *lt*.

Results: Approximately 78.84% of isolates were found to be positive for at least one of the virulence genes. The highest frequency, at 76.28%, was related to the *sta* virotype. Most isolates analyzed had a single gene, and no combination of fimbrial and enterotoxin genes was found to be predominant.

Conclusion: These findings underscore the importance of monitoring and understanding the epidemiology of enterotoxigenic *E. coli* pathotypes to develop effective strategies for managing calf diarrhea and mitigating associated economic losses in the Iranian cattle industry.

Keywords: Enterotoxigenic *E. coli*, Toxin, Fimbriae, Calf diarrhea, Iran

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*** Corresponding Author:**
Taghi Zahraei Salehi, Professor.
Address: *Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.*
Phone: *+98 (21) 61117051*
E-mail: *tsalehi@ut.ac.ir*

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a significant pathogen associated with diarrhea in young calves worldwide (Özcan et al., 2021; Jessop et al., 2024). Calves, particularly in their first weeks of life, are highly susceptible to ETEC infections, making it a serious concern for livestock farmers and veterinarians (Slanzon et al., 2022). This study aims to investigate the significance of ETEC in the occurrence of diarrhea in calves while focusing on the factors contributing to the pathogenesis of this microbial infection.

ETEC is responsible for inducing a large portion of neonatal and post-weaning diarrhea cases in calves. The severity of the disease can range from mild to severe, leading to significant economic losses within the livestock industry (Tarabees et al., 2021). These particular bacteria possess the capability to generate two distinct types of virulence factors. The first type, known as adhesins, aids in binding to specific receptors on enterocytes, thereby promoting intestinal colonization (Pakbin et al., 2021). Among the well-known adhesins are fimbriae, including F4 (also referred to as K88), F5 (K99), F6 (987P), F17, F41, and CFA (colonization factors) (Osek et al., 1999). The second type, referred to as enterotoxins, can be classified into two major classes: Heat-labile toxins (LT) and heat-stable toxins (STa, STb) (Wang et al., 2019). The STa toxin can be found in various bacterial strains, including *Vibrio cholerae* non-O1 and O1, *Vibrio mimicus*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, and *Citrobacter freundii* (Scheutz et al., 2014). Apart from the toxins, ETEC also produces other virulence factors that contribute to its pathogenicity. These factors include hemolysins, proteases, and iron acquisition systems, which contribute to tissue damage, immune evasion, and the host's survival. Along with other genes known to contribute to ETEC virulence and pathogenicity, colonization factors associated with human and animal diseases have been extensively studied. In human ETEC, at least 28 antigenically distinct CFs and 30 LT types have been identified and characterized (Shams et al., 2012). On the other hand, ETECs isolated from animals have shown 6 CFs (Salvadori et al., 2003; Zhang et al., 2007), indicating a lesser diversity compared to human ETECs in terms of CF virulence factors. Notably, 6 clinically significant CFs (F4, F5, F6, F41a, F17, and F18) have been recognized in ETEC-induced diseases in animals (Scheutz et al., 2024). The fimbrial adhesins F17, F5, and F41 are closely associated with ETEC infections in calves. Studies have shown that the virulence factors F5 and F41 are associated with diarrhea across various animal species, although the prevalence of these genes tends to decline in adult animals. These fimbriae are characterized by their unique properties, which include their amino acid composition and their capacity to agglutinate red blood cells (RBCs) (Ghavami et al., 2021). The combination of adhesion, enterotoxin production, and additional virulence factors makes ETEC a formidable pathogen in causing diarrhea in calves (Sora et al., 2021).

Understanding the pathogenesis factors involved in ETEC infections is crucial for developing effective preventive strategies and targeted interventions (Rueter and Bielaszewska, 2020).

In this research, we have simplified and expedited the detection of various CFs and toxins through the use of polymerase chain reaction (PCR) assays. This method combines the advantages of the PCR technique, known for its high sensitivity and specificity in detecting ETEC, with a set of previously established primers. Additionally, we have employed multiple primers for each gene, enhancing the accuracy and reliability of our detection process.

Materials and Methods

Collection of samples

Between April 2022 and September 2023, our study was conducted on 156 diarrheic neonatal calves aged between 1 and 10 days. These calves were raised on 12 farms located in 5 provinces of Iran, including Tehran, Alborz, Qazvin, Arak, and Khorasan Razavi. Notably, these farms were facing a widely reported problem of diarrhea in newborn calves, and there was no observed use of antibiotics for treating ETEC infection. A rectoanal mucosal swab specimen was obtained from each diarrheic calf, and subsequently placed into a tube containing Cary-Blair transport medium.

Isolation and identification procedures

The samples were transported to the laboratory on ice and subsequently streaked onto MacConkey and EMB (eosin methylene blue) agar plates. The plates were then incubated at 37 ˚C for 24 hours. After the incubation period, three colonies from each sample displaying the characteristic appearance of *E. coli* were carefully selected for further analysis. These colonies were of particular interest as *E. coli* is commonly associated with diarrheal cases in calves. The *E. coli* strain was identified through a series of biochemical tests, encompassing assessments for in-

dole production, citrate utilization, glucose and lactose fermentation, hydrogen sulfide production, and urease negativity. Following identification, the isolated bacteria were preserved in TSB with 20% glycerol at a temperature of -70 ˚C until further analysis.

DNA extraction and PCR reaction

Overnight cultures of bacteria were grown in 3 mL Luria-Bertani broth and subsequently centrifuged for 5 minutes at 3000×g. The resulting bacterial pellet was resuspended in 300 µL of distilled water and boiled for 10 minutes. To obtain the template DNA, the tubes were centrifuged at 10000×g for 10 minutes again, and the supernatant was collected.

The PCR mixture consisted of 12.5 µL of Taq 2x Master Mix Red (Ampliqon, Denmark), 1.5 mM $MgCl_2$, 1 µL (10 pM) of each primer, 2 µL of DNA template, and deionized water to reach a final volume of 25 µL. The amplification process involved an initial denaturation step at 94 ˚C for 5 minutes, followed by 30 cycles at 94 ˚C for 1 minute (denaturation), 56 ˚C (for *lt*, *f41*, *f4*, *f17*, and *f6*) and 62 (*sta*, *f5* and *cfa*/1) for 1 minute (annealing), and 72 ˚C for 1 minute and 30 seconds (extension). A final extension was performed at 72 ˚C for 10 minutes. The resulting PCR products were separated by 2% agarose gel electrophoresis and visualized using ethidium bromide staining on a UV transilluminator.

A PCR assay utilizing specific primers enabled the identification of six fimbrial genes (*f4, f5, f6, f17, f41,* and *cfa/1)*) in addition to two toxin genes (*lt* and *sta*) (Table 1).

Results

Our research team utilized PCR to detect the presence of six fimbrial genes (*f4*, *f5*, *f6*, *f17*, *f41*, and *cfa/1*), along with six toxin genes (*lt* and *sta*) (Figure 1). Among the 156 diarrheic calves examined using PCR analysis, positive results were obtained for the fimbrial and toxin genes. Detailed data on the number and frequency of fimbriae genes and toxins are presented in Table 2. However, it is important to note that no positive cases were discovered for fimbriae F6 (Table 2).

Table 1. Primers used to detect gene prevalence

Row	Primer Name	Sequence (5'-3')	Gene	Size (bp)	Annealing (˚C)	Ref.
1	LT-F	GCACACGGAGCTCCTCAGTC	*lt*	218	56	Azimi et al. (2021)
	LT-R	TCCTTCATCCTTTCAATGGCTTT				
2	STa-F	TCCGTGAAACAACATGACGG3'	*sta*	244	62	Sinha, et al. (2018)
	STa-R	ATAACATCCAGCACAGGCAG3				
3	F4-F	GGTGATTTCAATGGTTCGGTC	*f4*	704	56	Xu et al. (2020)
	F4-R	ATTGCTACGTTCAGCGGAGCG				
4	K99-F	TATTATCTTAGGTGGTATGG	*f5(K99)*	314	62	Franck et al. (1998)
	K99-R	GGTATCCTTTAGCAGCAGTATTTC				
5	F6-F	TCTGCTCTTAAAGCTACTGG	*f6*	333	56	Sinha et al. (2018)
	F6-R	AACTCCACCGTTTGTATCAG				
6	F17c-F	GCAGGAACCGCTCCCTTGGC	*f17*	416	56	Bertin et al. (1996)
	F17c-R	CAACTAACGGGATGTACAGTTTC				
7	F41-F	GCATCAGCGGCAGTATCT	*f41*	380	56	Franck et al. (1998)
	F41-R	GTCCCTAGCTCAGTATTATCACCT				
8	CFA/1 F	GCTTACTCTCCCGCATCAAA	*cfa/1*	170	62	Rasul et al. (2022)
	CFA/1 R	ACTTGTCCTCCCCATGACAC				

Table 2. Frequency presence of adhesion factor and enterotoxin genes in *E. coli* from calves in Iran (n=156)

Row	Gene	Number of Positive Results	No. (%)
1	*f4(K88)*	3	3(1.92)
2	*f5(K99)*	18	18(11.53)
3	*f6*	0	0(0)
4	*f17*	4	4(2.56)
5	*f41*	12	12(7.7)
6	*cfa/1*	11	11(7.05)
7	*lt*	21	21(10.9)
8	*sta*	119	119(76.28)

We examined 156 *E. coli* isolates obtained from 10-day-old calves with diarrhea. The results showed that a significant number of the isolates carried virulence traits. Specifically, 119 samples (76.28%) were positive for the *sta* gene, 21 samples (13.5%) for the *lt* gene, 18 samples (11.53%) for the *f5* gene, and 12 samples (7.7%) for the *f41* gene. Additionally, 11 samples (7.07%) contained the *cfa/1* gene, while a smaller group of 4 samples (2.56%) had the *f17* gene, and 3 samples (1.29%) carried the *f4* gene. These findings highlight the widespread presence of various virulence factors in *E. coli* strains linked to diarrheal illness in young calves.

Among these isolates, 64(41.02%) exclusively possessed the *sta* gene. Additionally, 13 isolates (8.33%) had both the *f5* and *sta* genes, while another 11 isolates (7.05%) carried both the *cfa/1* and *sta* genes. The distribution of isolates with multiple gene combinations included 8 isolates (5.12%) with the *f41* and *sta* genes, 4 isolates (2.56%) with the *f17* and *sta* genes, and 2 isolates (1.28%) with the *f4* and *sta* genes. One isolate (0.64%) was identified as having three genes: *f5*, *sta*, and *lt*. Two isolates (1.28%) had three genes: *f5*, *cfa/1*, and *sta*, while one isolate carried the genes *sta*, *f5*, and *lt* together. Notably, 13 isolates (8.33%) were characterized solely by the *sta* and *lt* genes, two isolates (0.64%) contained two genes, including lt and *f5* (1.28%), and another 2 isolates had two genes, *lt* and *f41* (1.28%).

Four isolates (2.56%) had only the *lt* gene (Table 3). This genetic analysis underscores the diverse array of gene combinations present in the ETEC strains studied.

Discussion

In this study, we examined 6 different types of fimbriae and utilized 2 primers to identify the genes responsible for ST and LT toxins, respectively. This approach was compared to previous studies conducted in the field.

According to research conducted in numerous countries, the recorded incidence of ETEC infection varies widely, ranging from around 1% to as high as 50%. Shams et al.'s extensive study in Fars Province, conducted in 2008 and 2009, involved 268 samples, indicating that 5.3% of the isolates identified were ETEC strains (Shams et al., 2012). A similar study by Pourtaghi et al. (2015) in Alborz Province in 2015 found that 18.33% of the 60 rectal swab samples were positive. In 2021, Ghavami et al. in Hamadan reported a 7.5% prevalence rate for F5 from a total of 120 samples (Ghavami et al. 2021).

The prevalence of F5, F41 fimbriae, and STa toxin genes was 5.3%, 5.3%, and 4.02%, respectively. A similar result was reported by Younis et al. (2009); however, a higher prevalence was reported by Acha et al. (2004), who reported a prevalence rate of 40%. On the contrary, lower prevalence rates (0.57%, 2.3%, and 7.3%) were recorded (Zhang et al., 2007).

F5 is one of the three major fimbriae expressed by ETEC strains that colonize the intestines of neonatal piglets, and it is implicated as a major cause of neonatal diarrhea in calves (Salvadori et al., 2003). During the study, it was observed that the occurrence of f5 was 11.53%. However, it is interesting to note that the prevalence of F5 varied across different regions in Iran.

Table 3. Coincidence of adhesion factor and enterotoxin genes in *E. coli* from calves in Iran (n=156)

Major±Associated Virotype	Number of Calves with Indicated *E. coli* Virotype*	No. (%)
STa (only)	64	64(41.02)
STa + F5	13	13(8.33)
STa + CFA/1	11	11(7.05)
STa + F41	8	8(5.12)
STa + F17	4	4(2.56)
STa + F4	2	2(1.28)
STa + F4 + F41	1	1(0.64)
STa + F5 + CFA/1	2	2(1.28)
STa + F5 + LT	1	1(0.64)
STa + LT	13	13(8.33)
LT + F5	2	2(1.28)
LT + F41	2	2(1.28)
LT (only)	4	4(2.56)

*Strains that do not express any fimbrial genes for F6.

According to a study conducted in the Republic of Argentina and Korea, the *f17* gene showed a prevalence of 16% and 72.2%, respectively, among *E. coli* strains isolated from diarrheic calves (Ryu et al., 2020). In our study, we observed that the frequency of *f17* was 2.56%. Interestingly, before this research, no other studies had been reported on this matter in Iran.

The significance of fimbriae F41 in ETEC isolated from calf diarrhea, along with its frequency ranging from 11% to 16%, has been investigated (Cengiz & Adıgüzel, 2020). The findings of this study reveal a similar result, with a 5.6% frequency, which closely aligns with a corresponding study conducted in Iran (7.7%).

CFA/1 is a protein found on the surface of certain strains of ETEC, commonly associated with diarrhea in various animal species, including calves (von Mentzer & Svennerholm, 2023). There is no specific information available on the prevalence of CFA/1 in ETEC isolated from calves with diarrhea. In this study, 11 samples (7.05%) had the *cfa/1* gene.

The frequency of F4 and F6 in ETEC can vary depending on the specific strain and geographical location. ETEC strains can possess different combinations of fimbriae, and the presence of F4 is more commonly associated with ETEC strains that infect pigs and other animals. These genes have not been investigated in any of the studies conducted in Iran.

There have been reports of variations in the frequency of toxin types across different geographic areas (Shen et al., 2022; Umpiérrez et al., 2021). Shahrokhi et al. (2011), for example, found that the most common toxin type in Iran was ST-only, accounting for 60.3% of cases, followed by LT-only (31.3%) and LT/ST (8.4%). A similar dominance of ST-expressing ETEC has been observed in Egypt, Bangladesh, and Iran (Qadri et al., 2005; Darbandi et al., 2016). In this study, the frequency of samples with the *lt* gene is 10.9% and the frequency of the *sta* gene is 76.28%.

The isolation and molecular characterization of ETEC pathotypes from calf diarrhea cases in various regions of Iran provides valuable insights into the epidemiology and virulence profiles of these diarrheagenic *E. coli* strains.

The high prevalence (78.84%) of *E. coli* isolates harboring at least one virulence gene, particularly the predominance of the *sta* virotype (76.28%), underscores the significant role of ETEC as a major etiological agent of diarrhea in young calves in the studied areas (Shahrani

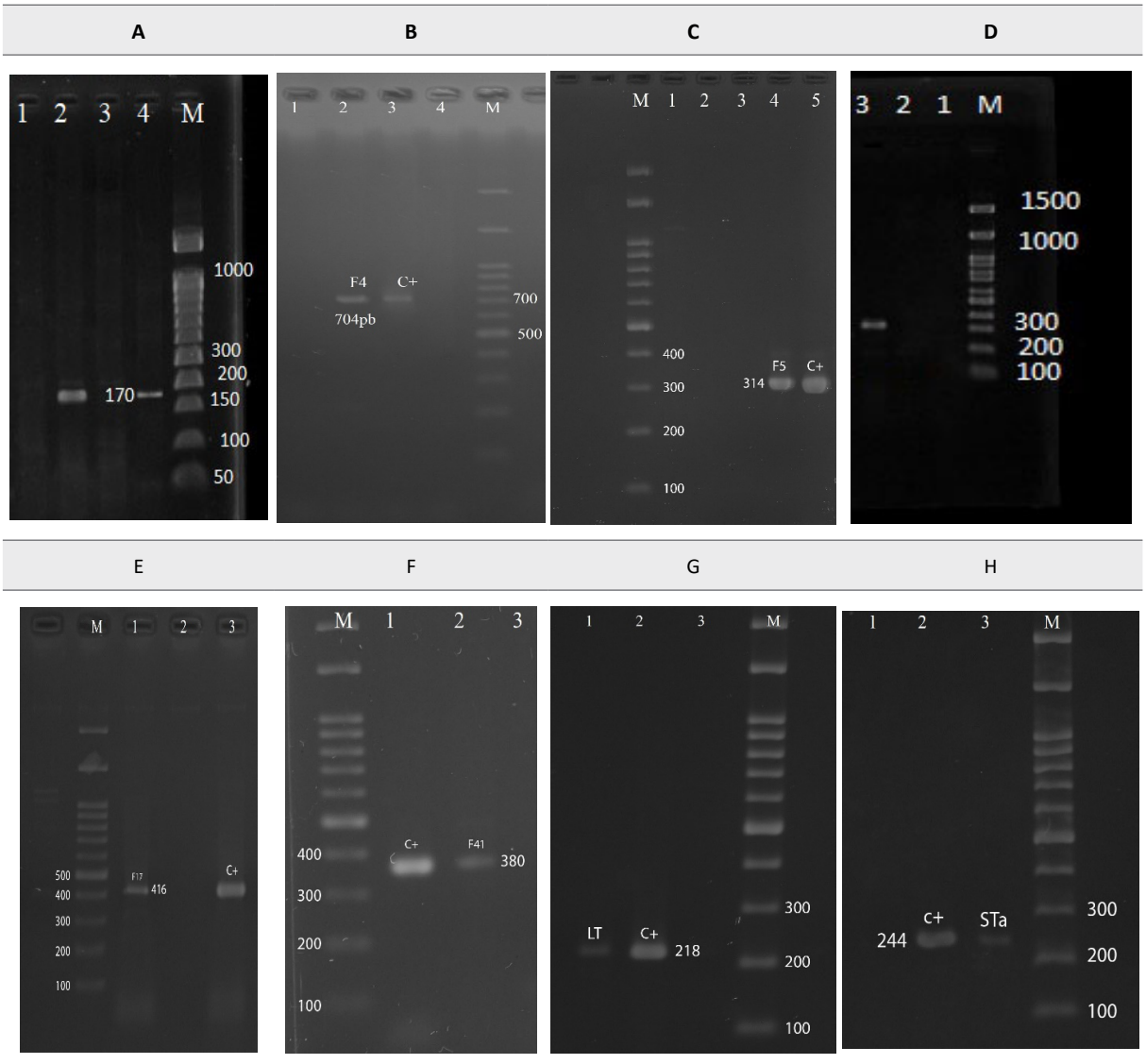

Figure 1. PCR results for the fimbria and toxin genes

A) the CFA/1 primers that amplify a 170-bp fragment: Lanes: 1, negative control; 2, positive control; 3, negative sample; 4, positive sample; B) the F4 primers that amplify a 704-bp fragment: Lanes: 1, negative sample; 2, positive sample; 3, positive control; 4, negative control; C) the F5 primers that amplify a 314-bp fragment: Lanes: 1, negative control; 2, negative sample; 3, negative sample; 4, positive sample; 5, positive control; D) the F6 primers that amplify a 333-bp fragment: Lanes: 1, positive control; 2, negative control; 3, negative control; E) the F17 primers that amplify a 416-bp fragment: Lanes: 1, sample positive; 2, sample negative; 3, positive control; 4, negative control; F) the F41 primers that amplify a 380-bp fragment: Lanes: 1, positive control; 2, positive sample; 3, negative control; G) the LT primers that amplify a 218-bp fragment: Lanes: 1, positive sample; 2, positive control; 3, negative control: H) the ST primers that amplify a 244-bp fragment: Lanes: 1, negative sample; 2, positive control; 3, positive sample; 4, negative control

et al., 2014). This finding is consistent with previous reports from Iran and other countries, which have also highlighted the importance of ETEC in causing calf diarrhea (Awad et al., 2020).

The detection of a diverse array of virulence genes, including fimbrial adhesins (*f4*, *f5*, *f41*, *f17*) and enterotoxins (*sta*, *lt*), suggests the ability of these ETEC strains to colonize the intestinal epithelium effectively and produce toxins that disrupt fluid homeostasis, leading to the development of diarrhea (Pourtaghi et al., 2013; Gebregiorgis & Tessema, 2016). The lack of a predominant combination of fimbrial and enterotoxin genes indicates the genetic heterogeneity of the ETEC isolates, which may contribute to the varied clinical presentations observed in calf diarrhea cases.

The high prevalence of ETEC pathotypes in the studied regions underscores the need for continued surveillance and monitoring of these diarrheagenic *E. coli* strains. Effective prevention and control strategies, such as improved biosecurity measures, targeted vaccination programs, and prudent use of antimicrobials, could help mitigate the impact of ETEC-associated calf diarrhea and reduce the associated economic losses in the cattle industry (Gebregiorgis & Tessema, 2016).

Further research is warranted to elucidate the molecular epidemiology, transmission dynamics, and potential zoonotic implications of the ETEC pathotypes identified in this study. Integrating these findings with data on antimicrobial resistance patterns and the distribution of other diarrheagenic *E. coli* pathotypes would provide a comprehensive understanding of the *E. coli*-mediated calf diarrhea burden in Iran.

Conclusion

According to the findings of this research, it seems that the presence of fimbriae may not be a necessary condition for causing diarrhea in calves. Other fimbriae, such as CFA/1, F5, and F41, are also important. The findings suggest that *E. coli*, excluding the virotypes STa and F5, is not a significant cause of diarrhea in calves. This finding may explain why F5 and F41 showed a low prevalence. The low frequency of F5 isolates can be due to farms frequently inoculating calves using a vaccine that targets this antigen. These findings highlight the importance of monitoring and understanding the epidemiology of ETEC pathotypes to develop effective strategies for managing calf diarrhea and reducing associated economic losses in the Iranian cattle industry.

Ethical Considerations

Compliance with ethical guidelines

The entire procedure has been conducted according to the instructor's guide and the University of Tehran's ethical standards for animals.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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