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Research Article

Determination of iron acquisition genes in *E. coli* from diarrheic calves in arid regions of India

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Abstract

Iron is an essential nutrient for nearly all living organisms. So, the motive of this study was to determine iron acquisition genes in *E. coli* from diarrheic calves in the Bikaner district of Rajasthan. Out of 35 isolates, 15 (42.86%) isolates were identified as iron acquisition genes- positive strains that had at least one of the three analyzed genes and the majority of the isolates carried the gene *iro*N (22.85%), followed by *chu*A (17.14%) and *fyu*A (17.14%). Five isolates carried two iron acquisition genes in a combination of (*chu*A and *iro*N, two isolates) and (*fyu*A and *iro*N, three isolates). The combination of *chu*A and *fyu*A genes was not to be found. In conclusion, this study provided information about the prevalence of different iron acquisition genes of *E. coli* originated from neonatal diarrheic calves in Bikaner.

Keywords: *Escherichia coli*, Iron acquisition genes, Neonatal calf diarrhea, PCR

Highlights

- Determine iron acquisition genes in *E. coli* by gene-specific PCR
- Prevalence of iron acquisition genes: *iro*N (22.85%), *chu*A (17.14%) and *fyu*A (17.14%)

INTRODUCTION

Neonatal calf diarrhea has a significant effect on the livestock population. It causes economic loss through mortality and reduces the growth rate. *Escherichia* (*E.*) *coli* is an important cause of diarrhea in farm animals (DebRoy *et al*., 2001; Nayak *et al.*, 2019; Kumar *et al*., 2022). *Escherichia coli* is a gram-negative, rod-shaped, flagellated, non-sporulating and facultative anaerobic bacterium of the family *Enterobacteriaceae*. *E. coli* pathotypes that cause diarrhea are referred to as diarrheagenic *E. coli* and were classified based on virulence potential, mechanism of pathogen entry, and interaction (Nataro and Kaper, 1998; Rathor *et al*., 2021).

Iron is an essential nutrient for nearly all living organisms. The requirement for iron is based on its role in cellular processes ranging from energy generation and DNA replication to oxygen transport and protection against oxidative stress. Iron requirement for the bacterial pathogen is a necessity inside the vertebrate host because of its replication in the host and establishment of infection in the host (Skaar, 2010; Gao *et. al*., 2012). Vertebrate immune systems have developed some modifications and restrictions to the availability of iron for microorganisms. However, some microorganism counters this restriction and makes availability of iron (Ganz, 2009; Light *et. al*., 2018).

Iron acquisition is a critical function required by bacteria to cause infections (Cassat and Skaar, 2013; Scott *et al.*, 2021). In bacteria mainly six pathogen-associated outer membrane iron receptors (*Chu*A, *Hma*, *Iha*, *Ire*A, *Iro*N and *Iut*A) and encoding ferric

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yersiniabactin uptake receptor (*fyuA*) were used in iron acquisition (Alteri *et. al*., 2009; Himpsl *et. al*., 2010, Kouse *et. al*., 2013).

Most studies about virulence factors are limited to ETEC and STEC associated genes. So, the motive of this study was to determine iron acquisition genes in *E. coli* from diarrheic calves in the Bikaner district of Rajasthan located in the arid climate regions of India.

MATERIALS AND METHODS

Collection of faecal samples: The study was conducted in the Bikaner region of Rajasthan located in the western part of India where the climate is arid. A total of 39 fecal samples were collected in winter season (December 2020 to February 2021) from calves below one month of age showing classical clinical signs of diarrhea at private dairy farms (19 sample from 3 farms) as well as animals of individual holdings (20 sample) in the (270 km²) Bikaner district of Rajasthan. The samples were collected directly from rectal aseptically in sterile swab and transported to a lab for further processing as soon as possible.

Isolation and identification of *E. coli*: The sample swabs were directly streaked over selective MacConkey agar followed by incubation at 37°C for 24 hrs. The isolation and identification of *E. coli* was carried out as per Quinn *et al*. (2005). The organisms were confirmed on the basis of cultural characteristics and biochemical test profiles. Further molecular confirmation was done using 16S rRNA ribotyping as per Abd El-Razik *et al*. (2010).

Determination of iron acquisition genes in *E. coli* isolates: The PCR method was used for amplification of iron acquisition genes in *E. coli* using specific primer sequences. The primer pairs used for the PCR are given in Table 1.

PCR was performed in a volume of 25 µL containing 12.5 µL of green mater mix, 1 µL (20 pmol) of each primer, 3 µL of template DNA and nuclease-free water up to 25 µL. The optimum cyclic conditions for virulence gene amplification in *E. coli* isolates are given in Table 2.

PCR was performed in a Veriti 96-well thermal cycler (Applied Bio System, USA) with a heated lid following the steps and cycle conditions provided in the table. The PCR product was analyzed by agarose gel electrophoresis to examine the amplicon sizes (Table 1) of the iron acquisition genes.

Table 1. Details of the primers used for the iron acquisition genes of *E. coli* isolates

Sl. No	Target gene	Primer	Sequence (5′----3′)	Amplicon size (bp)	Reference
1	*chu*A	*chuA*-F	ATGGTACCGGACGAACCAAC	288	Clermont *et al.* (2013)
		chuA-R	TGCCGCCAGTACCAAAGACA		
2	*iro*N	*iroN*-F	AAGTCAAAGCAGGGGTTGCCCG	665	Chapman *et al.* (2006)
		iron-R	GACGCCGACATTAAGACGCAG		
3	*fyu*A	*fyuA*-F	TGATTAACCCCGCGACGGGAA	880	
		fyuA-R	CGCAGTAGGCACGATGTTGTA		

Table 2. Steps and cyclic conditions of PCR for iron acquisition genes in *E. coli* isolates

Primer	Initial denaturation (°C/min)	Denaturation (°C/sec)	Annealing (°C/sec)	Extension (°C/sec)	Final extension (°C/min)	No of Cycles
*chu*A	95/5	95/60	60/60	72/60	72/10	30
*iro*N	95/3	95/60	60/60	72/60	72/07	30
*fyu*A	95/4	94/60	50/60	72/60	72/07	30

RESULTS

In the present study, out of 39 fecal samples collected from diarrheic calves, 35 samples were found positive for *E. coli* by conventional and 16S rRNA ribotyping methods. All the 35 *E. coli* isolates from diarrheic calves produced pink colonies on MacConkey agar showing lactose fermentation and when further streaked on EMB agar, the isolates produced a characteristic green metallic sheen. All the 35 isolates were gram-negative, medium-sized rods, motile, catalase test positive and oxidase test negative. The results of biochemical tests were interpreted according to the manufacturer's instructions and all the 35 isolates followed the typical IMViC pattern of indole and MR test positive and VP and Citrate test negative (++--). After primary identification further molecular confirmation was done through 16S rRNA ribotyping and all the 35 isolates produced an amplicon of 662 bp confirming them to be *E. coli*.

In our study, 35 confirmed *E. coli* isolates were subjected to PCR using specific primers for selected genes *viz. chu*A, *iro*N, *and fyu*A and the desired amplicons of the 288 bp, 665 bp and 880 bp sizes for the *chu*A, *iro*N, *and fyu*A genes, respectively, were observed (Fig. 1). Out of 35 isolates, 15 (42.86%) isolates were identified as iron acquisition genes-positive strains that had at least one of the three analyzed genes and the majority of the isolates carried the gene *iro*N (22.85%), followed by *chu*A (17.14%) and *fyu*A (17.14%) genes (Table 3). Five isolates carried two iron acquisition genes in a combination of (*chu*A & *iro*N, two isolates) and (*fyu*A & *iro*N, three isolates). The combination of *chu*A & *fyu*A genes was not to be found.

DISCUSSION

An iron uptake system known as siderophores is encoded by many pathogens, including *E. coli*. Siderophores produced by these strains are said to be responsible for the enhanced pathogenicity of invasive strains of *E. coli* (Williams, 1979, Tivendale *et al*., 2010). Bacteria need access to iron to regulate their metabolic activities and virulence properties (Skaar, 2010; Sarowska *et al*., 2019), but as only a small amount of iron in nature is available to bacteria, alternative strategies must be developed to fulfill its requirements (Ganz, 2009; Kohler and Dobrindt, 2011). The gene for siderophores is located either on a chromosome or on a plasmid, where many antimicrobial resistance genes are also located (Johnson *et al*., 2002). In our present study, the prevalence of two such siderophore genes, *i.e.*,

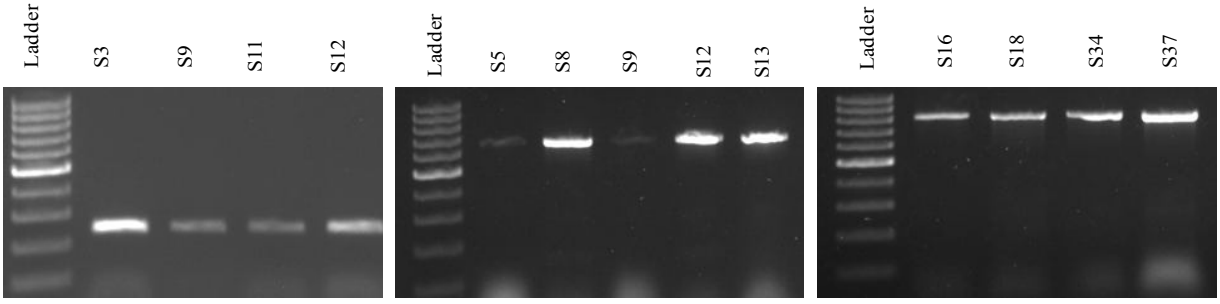

Fig. 1. Amplification of iron acquisition genes in *E. coli* isolates. The genes were amplified *chu*A (288 bp), *iro*N (665 bp) and *fyu*A (880 bp), repectively

Table 3. Prevalence of iron acquisition genes in *E. coli* isolates

Sl. No.	Virulence gene	Isolate ID	Total no. of *E. coli* isolates	Prevalence of virulence gene (%)
1	*chu*A	S3, S9, S11, S12, S14, S39	6	17.14%
2	*iro*N	S5, S8, S9, S12, S13, S18, S24, S34	8	22.85%
3	*fyu*A	S13, S16, S18, S22, S34, S37	6	17.14%

*fyu*A (encoding ferric yersiniabactin uptake receptor) was 17.14%, and *iron* (iron uptake) was 22.85% in *E. coli* isolates. The iron acquisition systems of strains can maximize the chance of these strains for successful persistence and pathogenesis in the host. deVerdier *et al.* (2012) reported a higher prevalence of the 35% *fyuA* gene in *E. coli* isolates from dairy calves in Sweden. In our results, 17.14% of isolates carried *chu*A gene and *chu*A, a gene required for heme transport in enterohemorrhagic *E. coli* (Clermont *et al.*, 2013). A total of 133 clinically isolated *E. coli* strains, which included urinary tract infections and fecal isolates, were carried several virulence factors involved with iron acquisition (*chu*A, *fyu*A, and *irp*2) were significantly associated with high pathogenicity (Hashimoto *et al.*, 2021).

Pathogenic bacteria that require iron during pathogenesis confirm expression and surface expos of the iron acquisition system in the host. Pathogenic bacteria mostly iron is acquired by many routes such as; receptor-mediated recognition of transferrin, hemoglobin, hemopexin, lactoferrin and complex of hemo–haptoglobin. In addition, secreted siderophores can be capable to remove the iron from transferrin, ferritin, or lactoferrin wherewith cognate receptors of the bacterial surface recognize the siderophore–iron complexes. These siderophore–iron complexes provide the iron receptor to the bacterial interaction in the viable vaccine candidates and protect from the infection of bacteria (Skaar, 2010). These types of candidate vaccines are developed on the iron utilization system with a combination of siderophore receptor (*iro*N) (Russo *et. al.*, 2003; Garber *et. al.*, 2020) and iron acquisition proteins (Alteri *et. al.*, 2009).

In conclusion, this study provided information about the prevalence of different iron acquisition genes of *E. coli* in Bikaner. The prevalence of iron acquisition genes was reported moderate to high and majority with *iro*N gene. Iron is an important source to establish infection or viability of bacteria so we need concern about iron acquisition genes with the main virulence factors.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: SK: Designed the study, wrote the protocol, conducted the experiments and wrote the first draft of the manuscript; S, KD, RD, SK: Managed the analyses of the study and contributed to conducting experiments; TB: Supervised all the authors for conducting this research, guided the methodology and analysis of the study and reviewed the original draft. All authors read and approved the final manuscript.

Ethics approval: This study was conducted following approval by the research committee and Institutional Animal Ethics Committee (Reg. No.-2044/GO/Re/SL/18/CPCSEA), Rajasthan University of Veterinary and Animal Sciences, under permission number CVAS/IAEC/2021-22/108.

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