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Molecular characterization of Escherichia coli from colibacillosis affected neonatal calves, Wayanad, Kerala

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Abstract
Colibacillosis is an economically important disease, causing neonatal diarrhoea and septicaemia in calves. It has worldwide occurrence and has been reported from almost all states of India. The present study was conducted to identify different strains of *Escherichia coli* associated with colibacillosis in calves from Wayanad district by identification of virulence factors through PCR. Thirty calves under the age of 28 days with signs of diarrhoea, pyrexia and dehydration were selected for the study. Faecal samples were collected to sterile sample collection vials and laboratory culture was performed. Lactose fermenting colonies from MacConkeys agar were futher cultured in EMB agar which is the selective media for *E. coli*. Bacterial DNA was isolated from isolated *E. coli* colonies and subjected to PCR. Virulence factors of pathogenic *E. coli* were targeted using specific primers against *K99, F41, STa, intimin*, *Stx1* and *Stx2* genes. Seventeen samples were found positive for Intimin, *Stx1* and *Stx2* together or alone suggesting the presence of EHEC/EPEC.

Keywords: PCR, colibacillosis, calves, diarrhoea, neonates

Introduction
Escherichia coli, pathogenic bacteria which causes colibacillosis in neonatal calves. Common clinical signs associated with colibacillosis were diarrhoea, dehydration, metabolic acidosis and pyrexia. Sometimes complication like meningitis and arthritis could occur. Common clinical signs associated with colibacillosis were diarrhoea, dehydration, and pyrexia (Radostits *et al*. 2007) [6]. MacConkey (1905) [4] identified lactose fermenting activity of *Coli* (*B. colicommunis* (Escherich) like bacteria and these bacteria were negative for the Vogus-Proskauer test. Holt-Harris and Teague (1916) [3] developed a new culture medium based on methylene blue and eosin for *Bacillus typhosus.* This media was known as Eosin Methylene Blue (EMB), was effective for the isolation of *E. coli.* Franck *et al.* (1998) [2] developed a multiplex PCR for detecting enterotoxigenic *E. coli*, attaching and effacing *E. coli* and shiga toxin producing *E. coli* strains from calves. Targeted genes were Intimin, Stx1, Stx2, sta, K99, and F41. Pourtaghi *et al*. (2012) [5] conducted a study on virulence genes in *E. coli* isolated from calves with diarrhoea in Iran and found out that all ETEC carried both K99 and F41 fimbriae and possessed *Sta* enterotoxin gene. Vanitha, (2017) [7] found of EHEC positive *E. coli* strains from mastitis milk samples through the PCR technique from Thrissur district, Kerala. Amrutha (2018) [1] conducted a study on neonatal calf diarrhoea using PCR and the genes for Stx1, Stx2, and Intimin specific for enterohemorrhagic *E. coli* and enteroaggregative *E. coli* were amplified.

Materials and methods
The present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, between May 2018 and April 2019. A total of thirty neonatal calves affected with diarrhoea were selected from those presented at Teaching Veterinary Clinical Complex, CVAS, Pookode, dairy farms and rural households in Wayanad district.

Sample collection
Faecal samples were collected directly from rectum of diarrheic calves in a sterile collection bottle for isolation and identification of *Escherichia coli* and molecular diagnosis.

Bacterial culture
All samples were first inoculated in MacConkey's agar and incubated at 37oC for 24 hours.

From the culture, lactose fermenting colonies further inoculated in Eosin Methylene Blue media incubated at 37ºC for 24 hours.

Bacterial genomic DNA isolation for PCR

Bacterial genomic DNA isolation was carried out with HiPurA™ Bacterial Genomic DNA Purification Kit. Reconstitution of reagents and DNA isolation was done as per the manufacturer's instructions. The purity and concentration of the extracted genomic DNA was estimated using Thermo Scientific NanoDrop™ 2000.

Polymerase Chain Reaction (PCR)

Reagents

Taq DNA polymerase (Sigma Aldrich, USA), 10X PCR buffer (Sigma Aldrich, USA), Primers (Sigma Aldrich, USA), Deoxy nucleotides mix (dNTPs) (Sigma Aldrich, USA), 100 bp DNA ladder (Thermo-scientific)

Procedure

BIO RAD T100™ Thermal Cycler was used for PCR. The PCR was performed for the following virulence factors of *E. coli*. The details of the primers and protocols were given in the Tables 1, 2, 3, 4, 5 and 6.

Table 1: Details of the primer sets used for detection of virulence factors of *E. coli*

S. No.	Virulence Factor	Primers (5'- 3')	PCR product (bp)	References
1	Stx1	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT	555	Franck *et al.*, 1998 [2]
2	Intimin	ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA	425	Franck *et al.*, 1998 [2]
3	F41	GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCAC CT	380	Franck *et al.*, 1998 [2]
4	K99	TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATT TC	314	Franck *et al.*, 1998 [2]
5	Sta	GCTAATGTTGGCAATTTTTATTT CTGTA AGGATTACAACAAAGTTCACAG CAGTAA	190	Franck *et al.*, 1998 [2]
6	Stx2	GTGCCTGTTACTGGGTTTTTCTTC AGGGGTCGATATCTCTGTCC	118	Franck *et al.*, 1998 [2]

Table 2: PCR reaction mixture for detection of *E. coli* virulent factors

S. No	Name of the reagent	Quantity (µL)
1	2 X PCR Master mix	12.5
2	Forward Primer	1
3	Reverse Primer	1
4	Nuclease free water	7.5
5	Template DNA	3
Total		25

Table 3: PCR protocol for detection of genes for *F41, K99, and Sta* (Franck *et al*., 1998) [2]

S. No.	PCR Programme	Temperature	Time	Cycles
1	Initial denaturation	94	5min	1 cycle
2	Denaturation	94	30 sec	25 cycles
3	Annealing	50	40 sec	
4	Extension	70	1.30 min	
5	Final extension	70	10 min	1 cycle

Table 4: PCR protocol for detection of genes for *Stx1* (Franck *et al*., 1998) [2]

S. No.	PCR Programme	Temperature	Time	Cycles
1	Initial denaturation	95	5min	1 cycle
2	Denaturation	95	30 sec	35 cycles
3	Annealing	56	40 sec	
4	Extension	70	1.30 min	
5	Final extension	70	10 min	1 cycle

Table 5: PCR protocol for detection of genes for *Stx2* (Franck *et al*., 1998) [2]

S. No.	PCR Programme	Temperature	Time	Cycles
1	Initial denaturation	95	5min	1 cycle
2	Denaturation	95	30 sec	40 cycles
3	Annealing	46	1.30 sec	
4	Extension	70	1.30 min	
5	Final extension	70	10 min	1 cycle

Table 6: PCR protocol for detection of genes for *Intimin* (Franck *et al*., 1998) [2]

S. No.	PCR Programme	Temperature	Time	Cycles
1	Initial denaturation	95	5min	1 cycle
2	Denaturation	95	30 sec	35 cycles
3	Annealing	52	40 sec	
4	Extension	70	1.30 min	
5	Final extension	70	10 min	1 Cycle

Results and discussion

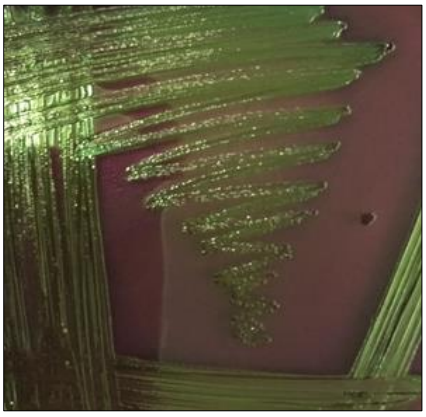
Bacterial isolation

Lactose fermenting colonies from MacConkey's agar and purple colonies with black centre and green metallic sheen from EMB agar confirmed the presence of *Esherichia coli* (Table 7).

Table 7: Results of bacterial isolation.

Culture medium	Faecal sample (30)	Milk samples (1)	Water (1)	Inference
MacConkey's agar	Lactose fermenting colonies	Lactose fermenting colonies	Lactose fermenting colonies	Positive for *E. coli*
Eosin Methylene Blue Agar	Purple with black centre and green metallic sheen	Purple with black centre and green metallic sheen	Purple with black centre and green metallic sheen	Positive for *E. coli*

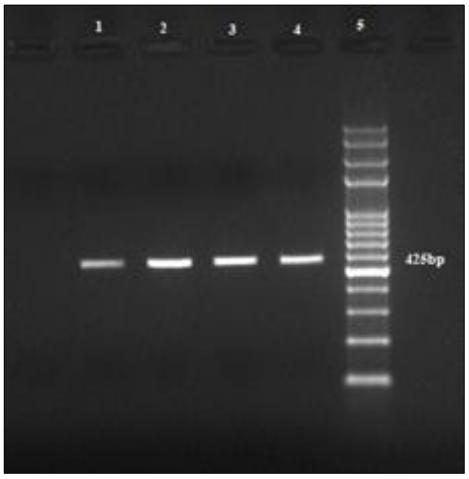
Lactose fermenting colony of *E. coli* in Macconkey Agar



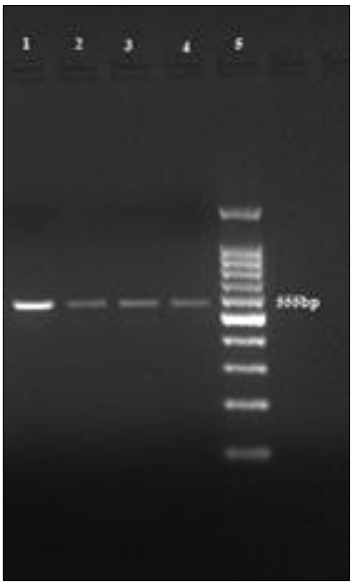
E. coli in EMB Agar

Polymerase chain reaction (pcr)

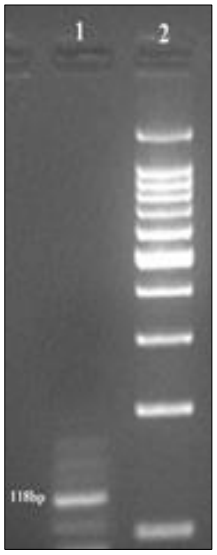
PCR was performed to detect different pathogenic strains of *E. coli.* Sta, K99 and F41 were used to detect Enterotoxigenic *E. coli*, Stx1, Stx2 and Intimin were used to detect enterohemorrhagic *E. coli* or Enteropathogenic *E. coli.* A total of thirty-two samples were subjected to PCR. For that DNA isolation was carried out and DNA isolates with more than 30 ng/µl were selected for amplification (table 8).



Agarose gel electrophoresis of PCR products for *Intimin* gene (425bp)



Agarose gel electrophoresis of PCR products for *Stx 1* gene (555bp).



Agarose gel electrophoresis of PCR products for *Stx 2* gene (118bp).

Table 8: PCR results

Samples	Virulence factors		Pathotype	Total
	Detected	Total number		
Faecal sample	Stx 2	1	EHEC	14
	Stx 1 and Stx 2	1		
	Intimin and *Stx1*	9		
	Stx1 and Stx2	1		
	Intimin, Stx1 and Stx2	2		
	Intimin	3	EPEC/EHEC	3

In the present study Stx1, Stx2 and intimin genes were detected and these genes confirmed presence of EHEC and EPEC in neonatal diarrhoea of calves from Wayanad. Stx1 and Stx2 genes are responsible for shigatoxin production which can cause apoptosis and vascular lesions in various cells including intestinal epithelium and Intimin causes attaching and effacing lesions in intestinal epithelium (Croxen *et al.*, 2013).

The current results are in agreement with Amrutha (2018) [1] who detected Stx1, Stx2 and intimin genes from neonatal diarrhoeic calves in Thrissur district and confirmed EHEC and EPEC as pathotypes.

Conclusion

Colibacillosis is an economically important disease, causing neonatal diarrhoea and septicaemia in calves. It has worldwide occurrence and has been reported from almost all states of India. The current study was conducted to identify

pathogenic *E.coli* in calves of Wayanad district. Thirty calves under the age of 28 days with signs of diarrhoea, pyrexia and dehydration were selected for the study. Faecal samples were collected to sterile sample collection vials and laboratory culture was performed. Lactose fermenting colonies from MacConkeys agar were futher cultured in EMB agar which is the selective media for E. coli. Bacterial DNA was isolated from isolated E. coli colonies and subjected to PCR. Virulence factors of pathogenic *E. coli* were targeted using specific primers against *K99*, *F41*, *STa*, *intimin*, *Stx1* and *Stx2* genes. EHEC and EPEC are the main causative agents of colibacillosis in neonatal calves in Wayanad district. Good managemental practices like colostrum feeding, proper disinfection, early diagnosis and timely treatment for neonatal diarrhoea is very important to control neonatal colibacillosis in calves.

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