RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.1.39-46

Occurrence of diarrheagenic *E. coli* pathotypes in broiler chicken birds reared in Kerala#

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Citation: Asha, K., Sethulekshmi, C., Renuka N., Vinod, V.K., Vergis, J., Sumod, K., Murugadas, V. and Prejit. 2024. Occurrence of diarrheagenic *E. coli* pathotypes in broiler chicken birds reared in Kerala. *J. Vet. Anim. Sci.* **55**(1):39-46
DOI: https://doi.org/10.51966/jvas.2024.55.1.39-46

Received: 16.09.2023 *Accepted: 16.10.2023* *Published: 31.03.2024*

Abstract

The diarrheal diseases caused by Escherichia coli (E. coli) are a very common occurrence in developing countries and the occurrence of pathogenic diarrheagenic E. coli (DEC) has been widely reported from different food animals from all over the world. The present study analysed 100 samples each of cloacal swabs and chicken slaughter waste from broiler chicken birds collected from two districts of Kerala. The samples were subjected to isolation and identification of E. coli using conventional method and biochemical characterisation and the isolates were confirmed using PCR amplification of uidA gene. The isolates were further subjected to detection of DEC pathotypes using PCR assay. The results of the analysis revealed that the overall occurrence of E. coli in broiler chicken samples from Wayanad and Ernakulam districts were 93.00 and 89.00 per cent, respectively. The most common DEC pathotype detected was EAEC followed by aEPEC. Only two isolates from Wayanad district carried genes suggestive of EHEC. None of the isolates from both the districts carried genes suggestive of ETEC.

Keywords: *Diarrheagenic E. coli, broiler chicken, slaughter waste, virulence genes*

The Indian poultry industry has shown a substantial growth in the production and consumption pattern in the recent years, and this is mainly attributed to the increasing public

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demand for the high-quality animal protein at a relatively lesser cost. The southern Indian state of Kerala has accounted to produce 178 thousand metric tons of chicken meat and accounts for 5.6 per cent of India's total share (Valsalan *et al*. 2023). The burden of food-borne infections has always been a pressing concern for public health and diarrheagenic *Escherichia coli* (*E. coli*) pathotypes is implicated to be one of the leading causes of such diseases (WHO, 2015). Due to the added consequences for food safety, the incidence and distribution of diarrheagenic *E. coli* pathotypes in broiler chicken birds have drawn growing attention.

Poultry farming plays a significant role in the Kerala's agricultural landscape. Thus, knowing the frequency of these pathogenic *E. coli* strains is critical not just for public health issues but also for farmers' livelihoods. This study aims to investigate the presence and diversity of diarrheagenic *E. coli* pathotypes in broiler chicken birds reared in Kerala, shedding light on the potential risks associated with poultry consumption and offering insights into strategies for mitigating their impact on both animal and human health. This study holds significant implications for the poultry industry, public health agencies, and consumers alike, as it contributes to the broader discourse on the safety of poultry products in the region.

Materials and methods

The study was designed to assess the occurrence of *E. coli* in broiler chicken samples collected from the two selected districts (Wayanad and Ernakulam) in Kerala. The study also determined the presence of diarrheagenic *E. coli* pathotypes in these samples by the molecular detection of selected virulence genes. A total of 200 samples consisting of 50 cloacal swab samples and 50 chicken slaughter waste (CSW) samples, from each district were collected for the analysis. The samples were collected in the Cary-Blair transport medium and transported under insulated chilled conditions. The samples were processed in the laboratory for isolation and molecular confirmation of *E. coli*. Buffered peptone water (BPW) at a rate of 1:10 dilution was used to enrich the samples and then streaked onto MacConkey agar and incubated at 37°C for 18 h for the isolation of *E. coli*. At the end of incubation, the typical pink-coloured colonies were presumptively identified as *E. coli*. Further, selective plating of *E. coli* was done on eosin methylene blue (EMB) agar and the colony characteristics were noted. Biochemical characterisation of bacterial isolates obtained from the cultural isolation was done as per the procedure described by Barrow and Feltham (2003).

Molecular confirmation of E. coli

Presumptive colonies of *E. coli* were confirmed using PCR targeting the *uid*A gene. The PCR mixture was prepared in a 0.2 mL PCR tube and vortexed thoroughly to mix the reagents properly. The PCR was performed in an automated thermal cycler (Bio-Rad, USA) with a preheated lid. The cycling conditions for the selected gene under study included an initial denaturation of DNA at 94°C for 5 min followed by 35 cycles each of 40 sec denaturation at 94°C, 40 sec annealing at 55°C and 50 sec extension at 72°C, followed by a final extension of 5 min at 72°C and hold at 4°C (Bej *et al*., 1991). The PCR products were separated and visualised by gel electrophoresis in 1.50 per cent agarose (Origin, India) containing ethidium bromide, in TAE buffer. The resolution of the amplified fragment in the gel was visualised and recorded by the gel documentation system (Bio-Rad, USA).

Identification of diarrheagenic E. coli (DEC) pathotypes

All the confirmed *E. coli* isolates were subjected to standardised PCR assays for identification of DEC pathotypes *viz*., entero-pathogenic *E. coli* (EPEC), entero-haemorrhagic *E. coli* (EHEC), entero-aggregative *E. coli* (EAEC) and entero-toxigenic *E. coli* (ETEC) (Vergis, 2013). The genes selected for the present study were *eae* (917 bp) and *bfp*A (385 bp) genes for EPEC, stx_1 (470 bp) and stx_2 (255 bp) genes for EHEC, *LT* (622 bp) and *ST* (171 bp) genes for ETEC and *ast*A (102 bp) gene for EAEC. In brief, the standardised PCR protocol for 20 µL reaction mixture included 10 µL of PCR master mix, 20 µM of a primer set containing forward and reverse primers (a final volume of 0.8 µL of each primer), 1.5 µL of DNA template and sterilised Milli-Q water to make

Table 1. Primer sequences used for the PCR for DEC pathotypes

	Gene name	Primer sequence	Product bp	Reference
DEC pathotypes	*eae*	F: 5'- GGAAGCCAAAGCGCACAAGATTA-3' R: 5'- GACCAGAAGAAGCATCCACCGAA-3'	917	(Vergis, 2013)
	*bfp*A	F: 5'- AATCTGCAATGGTGCTTGCGCTTG-3' R: 5'- CACCGTAGCCTTTCGCTGAAGTA-3'	385	
	*stx*1	F: 5'- TTACAGCGTGTTGCAGGGATCAGT-3' R: 5'- TTGTGCGTAATCCCACGGACTCTT-3'	470	
	*stx*2	F: 5'- GGCACTGTCTGAAACTGCTCC-3' R: 5'- TCGCCAGTTATCTGACATTCTG-3'	255	
	LT	F: 5'- GCGACAAATTATACCGTGCTGAC-3' R: 5'- TTGTGCTCAGATTCTGGGTCTCCT-3'	622	
	ST	F: 5'- TTTATTTCTGTATTGTCTTT-3' R: 5'- ATTACAACACAGTTCACAG-3'	171	
	*ast*A	F: 5'- TGCCATCAACACAGTATATCCG-3' R: 5'- ACGGCTTTGTAGTCCTTCCAT-3'	102	Muller *et al*., (2007)

up the reaction volume. The cycling conditions for PCR of *eae*, *bfp*A, stx_1, stx_2 and *LT* genes included an initial denaturation of DNA at 94°C, 5 min followed by 35 cycles each of 30 sec denaturation at 94°C, 1 min annealing at 59°C and 1 min 30 sec extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The cycling conditions for PCR of *ST* and *astA* genes included an initial denaturation of DNA at 95°C, 5 min followed by 40 cycles each of 45 sec denaturation at 95°C, 1 min, annealing at 60°C for 2 min extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. The PCR products were visualised by gel electrophoresis in 1.50 per cent agarose containing ethidium bromide, in TAE buffer. The *E. coli* standard cultures harbouring *LT* and *ST* genes procured from MTCC (MTCC 723 strain), *eae*, stx_1 and stx_2 genes procured from Sigma (EDL 933) and *E. coli* isolates carrying *bfp*A and *ast*A genes maintained in the repository of the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode were used as positive control for the standardisation of PCR in the study.

Results and discussion

The isolates which showed purple-black colonies with a greenish metallic sheen on EMB agar were presumptively identified as *E. coli* and were also subjected to biochemical tests for confirmation. The molecular-level confirmation of isolates identified as *E. coli* were done using a PCR technique targeting the *uid*A gene. Out of the 100 samples analysed from the Wayanad district, all the samples were positive for *E. coli* as per conventional method. However, the genotypic confirmation of *E. coli* using *uid*A gene amplification revealed that, out of the 50 samples each of cloacal swab and CSW, 94.00 and 92.00 per cent samples, respectively were harbouring *E. coli*. The results of the current study were in accordance with the study conducted by Al Azad *et al*. (2019) who also reported a 100 per cent isolation rate of *E. coli* from cloacal swabs of healthy broiler chicken. A similar study conducted by Alfifi *et al*. (2022) in Denmark also pointed out the presence of *E. coli* in broiler CSW like skin, inedible organs, blood etc. However, the rate of isolation was only 38.92 per cent which was substantially lower than that of the current study.

Of the 100 samples (50 nos. each of cloacal swab and CSW) collected from the Ernakulam district, 96.00 and 94.00 per cent samples, respectively, yielded *E. coli* based on conventional method. The genotypic confirmation of isolates using PCR method showed that 88 and 90 per cent of samples collected from the Ernakulam district were harbouring *E. coli*. The presence of *E. coli* in the intestine of broiler birds is a common occurrence however, the unsanitary conditions prevailing

Table 2: Details of *E. coli* isolated from different sources from the two districts

Sl no.	Sources	Total no. of samples	No. of *E coli* isolates from Wayanad district		Total no. of samples	No. of *E coli* isolates from Ernakulam district	
			Isolated by Culture method	PCR Confirmed		Isolated by Culture method	PCR confirmed
1	Cloacal swabs of broiler chicken	50	50	47 (94.00)	50	48	44 (88.00)
2	CSW	50	50	46 (92.00)	50	47	45 (90.00)
	TOTAL	100	100	93 (93.00)	100	95	89 (89.00)

in the farm environment, use of contaminated water in the farm and the overcrowding of birds in the chicken slaughter stall cages might have contributed to the very high prevalence rate of *E coli* in the samples. The findings of the current study were in agreement with the result of a similar study conducted by Mahmud *et al*. (2018) who reported that 83.08 per cent of cloacal swab samples from healthy broiler chicken in Bangladesh, were positive for *E. coli*. However, a lower isolation rate of 77.80 per cent was reported by Afsal *et al*. (2021) who had done a similar investigation in Kerala and isolated *E. coli* from cloacal swab samples of broiler chicken. The occurrence of *E. coli* in CSW samples from Ernakulam district was found to be slightly lower than the findings reported by Bagheri *et al.* (2014) who isolated *E. coli* from skin and other parts of chicken carcasses collected from Iran and reported that cent per cent of skin and other external surface samples of chicken carcasses carried the organism under study. A slightly higher finding was also reported by Khalafalla *et al*. (2015) who observed that cent per cent skin samples of fresh broiler chicken carried *E. coli*.

The statistical analysis of the data using the z-test for two independent proportions revealed that there was no significant difference ($p>0.05$) between the occurrences of *E. coli* in cloacal swab as well as CSW samples from the two selected districts. The occurrence of *E. coli* in the cloacal swab samples from Ernakulam district was lower than what was observed in samples from the Wayanad district. This could be attributed to the better management practices followed in the Ernakulam district farms. The

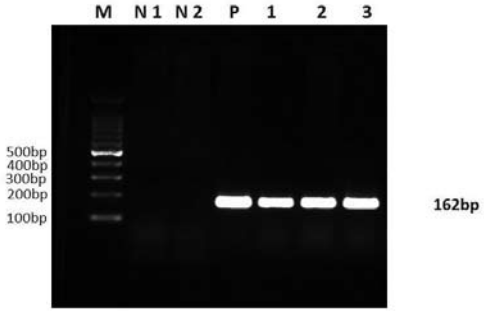

Fig.1. Standardisation of *uidA* gene amplification
Lane M-Marker, Lane N1, N2: Negative samples;
Lane P: Positive Control; Lane 1, 2, 3: Positive samples

presence of *E. coli* in cloacal swab samples is a normal occurrence as the organism is enteric in origin however, the significance increases if the *E. coli* is a virulent pathotype or is harbouring AMR genes.

Diarrhoeagenic E. coli (DEC) pathotypes in the samples

The PCR-based screening of isolates for detection of DEC pathotypes revealed that among the isolates from the Wayanad district, EPEC, aEPEC, EHEC and EAEC were detected. A total of five isolates recovered from cloacal swab samples and three isolates recovered from CSW samples, were found to be atypical EPEC (aEPEC) and three isolates recovered from CSW samples were found to harbour genes indicative of EPEC type. The typical EPEC is defined as *E. coli* isolates harbouring both *eae* and *bfp*A genes whereas

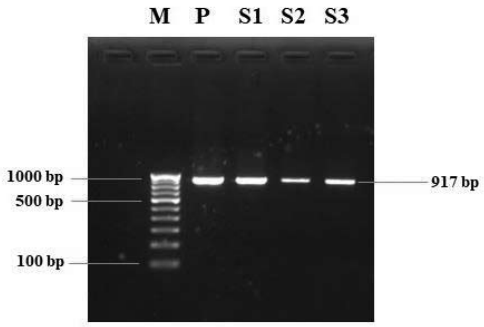

Fig. 2. Standardisation of *eae* gene amplification
Lane M: Marker
Lane S1, S2, S3: Sample
Lane P: Positive control

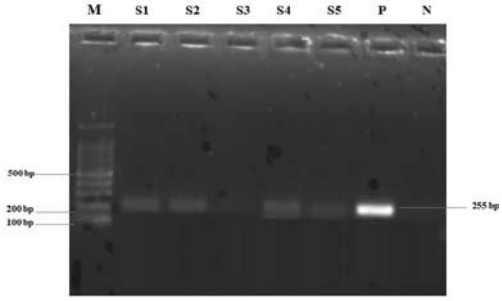

Fig. 3. Standardisation of *stx*$_2$ gene amplification
Lane M: Marker
Lane P: Positive control
Lane N: Negative control
Lans S1-S5: Sample

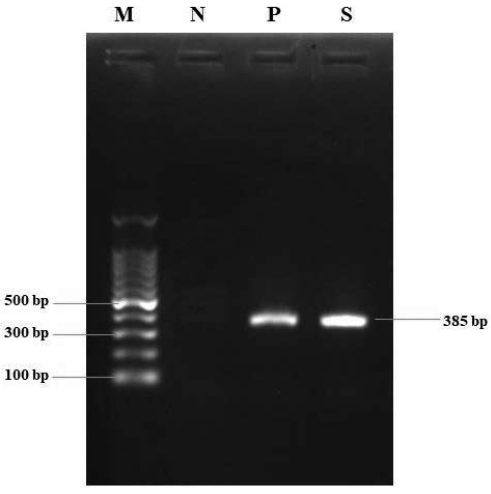

Fig. 4. Standardisation of *bfp* A gene amplification
Lane M: Marker
Lane N: Negative control
Lane P: Positive control
Lane S: Sample

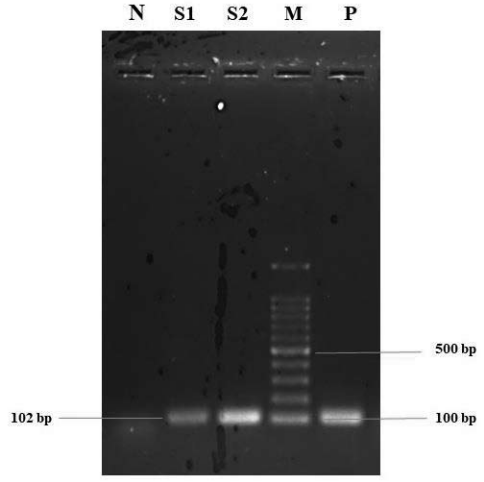

Fig.5. Standardisation of *ast* A gene amplification
Lane M: Marker
Lane P: Positive control
Lane S1, S2: Sample
Lane N: Negative

atypical EPEC carries only *eae* and not *bfp*A gene (Amir *et al*., 2021). The EPEC was the first recognised *E. coli* pathotype and is known to cause infantile diarrhoea mostly in developing countries. But nowadays it is reported that the aEPEC isolates are recovered from majority of diarrhoea cases in developing as well as developed countries (Mare *et al*., 2021) and it was also reported that the aEPEC has a more natural tendency to cause chronic infection compared to other DEC pathotypes (Ekici and Dumen, 2019). Out of the total 93 isolates recovered from the Wayanad district, 17 isolates were found to harbour *ast*A gene suggestive of EAEC and two isolates were found to carry *stx*$_2$ gene indicative of EHEC type. In the research work by Wang *et al*. (2017), it was observed that cent per cent poultry samples were positive for EAEC and only 25.0 per cent samples were positive for EPEC. In another study, Bhave *et al.* (2018) reported that EPEC and EAEC were detected most commonly among the *E. coli* isolates recovered from healthy chicken faecal samples. In most of the studies, it was observed that ETEC was detected in small number of samples, while EAEC and EPEC were the DEC pathotypes most commonly detected. This trend was found true in the current study also.

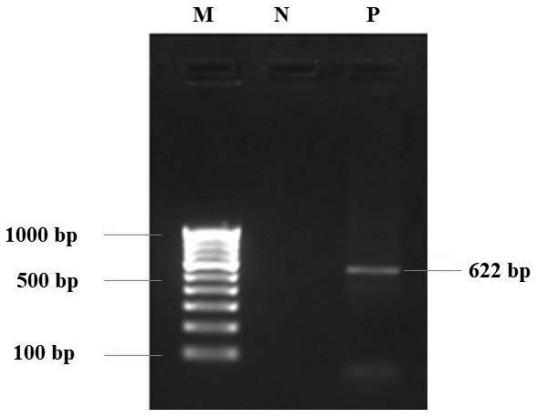

Fig. 6. Standardisation of *LT* gene amplification
Lane M: Marker
Lane N: Negative control
Lane P: Positive control

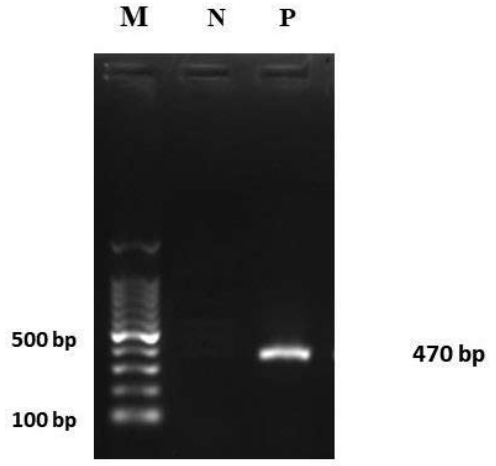

Fig. 7. Standardisation of *stx*$_1$ gene amplification
Lane M: Marker
Lane N: Negative control
Lane P: Positive control

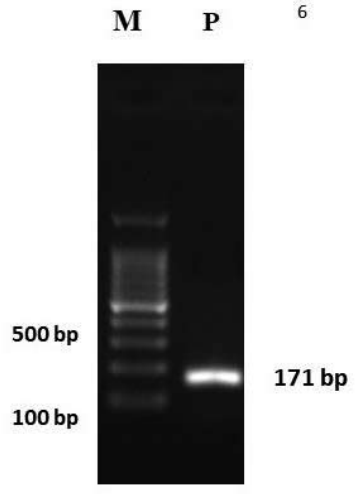

Fig. 8. Standardisation of *ST* gene amplification
Lane M: Marker
Lane P: Positive control

Out of the 44 cloacal swab isolates obtained from the Ernakulam district, three isolates were found to harbour *eae* and *bfp*A genes together, indicative of EPEC, four isolates were carrying only *eae* gene and not *bfp*A gene and were considered as aEPEC and 36 isolates were found to belong to EAEC type, as they carried *ast*A gene. All the other isolates were found negative for any of the pathotype-specific genes selected under this investigation. From the isolates recovered from CSW samples of Ernakulam district, the genes suggestive of EAEC type were detected from 18 isolates and none of the isolates were found to harbour

Table 3: Details of DEC pathotypes detected from different sources from the two districts

Sl No.	Type of sample	Sample No.	No. of PCR confirmed *E. coli* isolates	Pathotypes detected						
				EPEC		EHEC		ETEC		EAEC
	WAYANAD			*eae*	*bfp*A	*stx*$_1$	*stx*$_2$	*LT*	*ST*	*ast*A
1	Cloacal swabs of broiler chicken	50	47	5	-	-	-	-	-	7
2	CSW	50	46	6	3	-	2	-	-	10
	ERNAKULAM									
3	Cloacal swabs of broiler chicken	50	44	7	3	-	-	-	-	18
4	CSW	50	45	-	-	-	-	-	-	18

genes specific for EPEC, EHEC and ETEC types. The EAEC type is known to cause acute and persistent watery diarrhoea especially in children and is widespread in developing countries. Hence the zoonotic significance of this pathotype is more as the chances of the spread of such pathogens to humans through unscientifically processed chicken meat is very high especially in Indian scenario (Nataro and Kaper, 1998).

Conclusion

The presence of diarrheagenic *E. coli* in food animals is raising concern among the global public health agencies as these pathogens can cause chronic infections in human and animal populations. Also, if the DEC pathotypes acquire drug resistance, they might cause infections which are difficult to treat and incur huge economic loss. The high level of EAEC among the *E. coli* isolates recovered from broiler chicken samples from both the districts indicated higher degree of contamination of poultry products in the state and hence indicated increased chances of entry of such pathogens into human food chain. The present scenario could be improved by increasing the awareness among poultry handlers and slaughterers and by strictly implementing a hygienic production protocol in the broiler chicken industry.

Acknowledgements

The authors are thankful to the Kerala Veterinary and Animal Sciences University for providing the facilities needed for carrying out the research.

Conflict of interest

The authors declare that they have no conflict of interest.

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