International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; 9(6): 515-520

www.veterinarypaper.com
Received: 16-11-2024
Accepted: 19-12-2024

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Occurrence of ESBL: Producing *Enterobacter cloacae* in the broiler production chain in Wayanad, India

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DOI: https://doi.org/10.22271/veterinary.2024.v9.i6h.1923

Abstract
The emergence of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae has become an increasing concern in recent years, owing to its ability to evade the biocidal activities of antibiotics and its potential to cause severe infections in both humans and animals. One of the members of this family, *Enterobacter cloacae* is well known for its carriage of antimicrobial genes and also for its potential to cause nosocomial infections. This study investigated the prevalence of ESBL-producing *Enterobacter cloacae* (*E. cloacae*) in the broiler production chain, in the Wayanad district of Kerala, India. Out of the total 210 samples analysed, 94 samples tested positive for *E. cloacae* via culture method, out of which 89.36 per cent of isolates were confirmed as *E. cloacae* using PCR. The organism was detected in 12.72 per cent of the cloacal swab samples from live birds, 68.42 per cent of the broiler chicken meat samples, 48.21 per cent of the slaughter waste, and 26.19 per cent of the environmental samples. The phenotypic characterisation of the isolates using double disc synergy test revealed that 67.9 per cent were ESBL-producers and the PCR based screening showed that 73.80 per cent of the ESBL-producers harboured all the three selected ESBL genes *viz*., *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$. The findings highlighted public health concerns, particularly due to the high detection rate of this drug- resistant organism in the broiler production chain, posing risks to the consumers. The study underscored the necessity for continuous monitoring and improved biosecurity measures in broiler production to control the spread of this opportunistic organism, which can produce serious human infections.

Keywords: *Enterobacter cloacae*, ESBL, PCR, ECL gene

1. Introduction
The poultry industry is a cornerstone of global food production, evolving at an unprecedented pace to meet the surging demand for affordable protein while reshaping rural economies and modern agriculture. In India, this sector has transformed drastically in the recent years, propelling the country to the forefront as the third-largest producer of eggs and the fifth-largest producer of broiler meat globally (Kashyap *et al*., 2024) [1]. In addition to its crucial contributions to economic growth and food security, the industry plays a vital role in rural development by providing essential income and employment opportunities for small and marginal farmers.

Enterobacter cloacae (*E. cloacae*), a member of the family *Enterobacteriaceae* is a notorious pathogen, known for causing various nosocomial and community-acquired infections including pneumonia, bacteraemia, urinary tract infections, and intra-abdominal infections (Temsaah *et al*., 2024) [2]. This intestinal commensal is the predominant species associated with Enterobacter infections in humans, particularly targeting immunocompromised individuals and infants (Ioannou *et al*., 2022) [3]. This recognised nosocomial pathogen is also known for its capability to form biofilms and for the production of a range of cytotoxins such as enterotoxins, hemolysins, and pore-forming toxins responsible for its pathogenicity (Mezzatesta *et al*., 2012) [4]. The increasing threat posed by Enterobacter species, particularly their involvement in hospital-acquired infections and resistance to current antibiotic therapies, has led the World Health Organization (WHO, 2017) [5] to place them on its list of priority pathogens highlighting their significance in the present scenario.

Drug-resistant *Enterobacteriaceae* pose a critical global health challenge, particularly due to its resistance towards β-lactam antibiotics (Nossair *et al.*, 2022) [6]. This resistance is primarily mediated by plasmid-encoded β-lactamase genes, which encode for the extended-spectrum β-lactamases (ESBLs) capable of hydrolysing the β-lactam ring in antibiotics such as penicillins, cephalosporins, and monobactams, rendering them ineffective. Furthermore, they exhibit concerning resistance towards last resort options like carbapenems through the production of carbapenemases (Ku *et al.*, 2018) [7].

Although numerous studies have investigated the prevalence of such drug-resistant pathogens in the medical field, there is a notable scarcity of similar research in the veterinary domain. Thus, our study was designed to evaluate the occurrence of this drug-resistant opportunistic pathogen in the broiler production chain, a critical sector in poultry and one of the fastest-growing agro-based industries globally.

2. Materials and Methods

In the present study, a total of 210 samples were collected from the broiler production chain over a period of 10 months, from September 2023 to July 2024, across selected towns *viz*., Vythiri, Kalpetta, Sulthan Bathery, Panamaram, Meenangadi, and Meppadi in the Wayanad district, Kerala. The samples comprised 55 cloacal swab samples of live broiler chicken (irrespective of age), 57 meat samples, 56 slaughter waste samples and 42 environmental samples (Table 1). Specifically, 35 samples each were collected from Sulthan Bathery, Panamaram, Meppadi, and Vythiri towns while 34 and 36 samples were collected from Kalpetta and Meenangadi towns, respectively. All the samples were aseptically collected and transported to the laboratory under chilled condition and were processed within 24 h of collection.

Table 1: Details of samples collected in the study

Sl. No	Types of samples	Location of sample collection						
		Vythiri	Kalpetta	Meenangadi	Sulthan Bathery	Panamaram	Meppadi	Total
1	Chicken meat	10	9	9	9	10	10	57
2	Chicken slaughter waste	9	9	10	10	9	9	56
3	Chicken cloacal swab	9	9	10	9	9	9	55
4	Surface swab	1	1	1	1	1	1	6
5	Knife swab	1	1	1	1	1	1	6
6	Feed	1	1	1	1	1	1	6
7	Water	1	1	1	1	1	1	6
8	Litter material	1	1	1	1	1	1	6
9	Soil	1	1	1	1	1	1	6
10	Hand wash	1	1	1	1	1	1	6
	TOTAL: 210							

For the isolation and identification of *E. cloacae*, the collected samples were enriched in buffered peptone water (BPW) at a rate of 1:10 dilution, and streaked onto Endo agar followed by incubation at 37 °C for 24 h (Russo *et al*., 2022) [8] and the characteristic pink coloured colonies were selected (Fig 1). The recovered isolates were confirmed through biochemical tests like Gram staining, motility, catalase test, oxidase test and other secondary test reactions like methyl red, Voges-Proskauer, indole, citrate and urease (Barrow and Feltham, 2003; Hoffmann *et al*., 2005) [9, 10] and presumptive isolates were further genotypically confirmed using PCR targeting ECL gene (Ji *et al*., 2021) [11] (Fig 2).

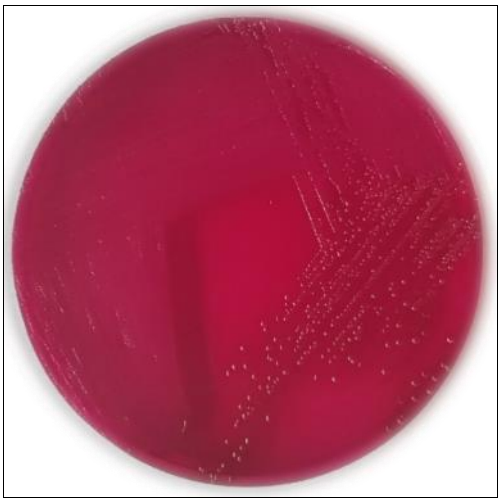

Fig 1: Colony characteristics of E. *cloacae*

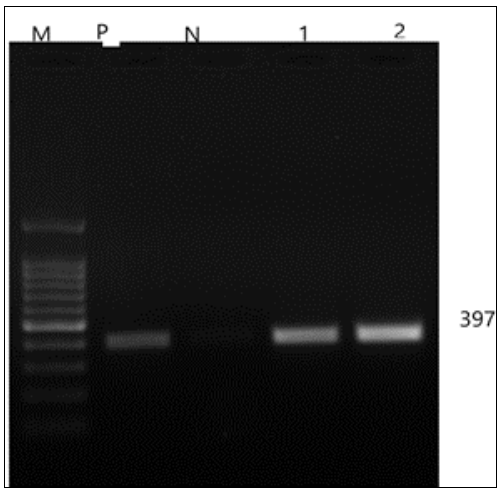

Fig 2: PCR standardisation of ECL gene

Lane M: 100 bp DNA Ladder, Lane P: Positive control
Lane N: Negative control, Lane 1-2: Samples

The confirmation of recovered isolates was done by the PCR based detection of the ECL gene using the primers: F: 5'-TGAAAACCTTATCCGCGA -3'; R: 5'-GGCAGGCTGGAAGATAAA-3' (Table 2). The reaction condition included an initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C for 15 s, 55 °C for 20 s, and 72 °C for 90 s), and a final extension at 72 °C for 5 min.

Table 2: Primer details

Species	gene	PCR conditions	Base pair	Reference
E. cloacae	ECL	F: 5'-TGAAAACCTTATCCGCGA -3' R: 5'-GGCAGGCTGGAAGATAAA-3'	397	Ji *et al*., 2021[11]
ESBL genes	*bla*$_{CTX-M}$	F: 5'CGCTTTGCGATGTGCAG-3' R: 5'-ACCGCGATATCGTTGGT-3'	550	Bhoomika *et al.*, 2016[12]
	bla$_{SHV}$	F: 5'-GATGAACGCTTTCCCCATGATG-3' R: 5'-CGCTGTTATCGCTCATGGTAA-3'	214	
	bla$_{TEM}$	F: 5'- ATGAGTATTCAACATTTCCG-3' R: 5'-GTCACAGTTACCAATGCTTA-3'	847	

Table 3: PCR conditions

GENE	Initial Denaturation		Denaturation		Annealing		Extension		Cycles	Final Extension	
	Temp (°C)	Time (Min)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)		Temp (°C)	Time (min)
ECL gene	95	3	95	15	55	20	72	90	30	72	5
ESBL genes	94	5	94	60	56	60	72	60	35	72	5

The isolates were further characterised for ESBL-production as per the guidelines provided by the clinical laboratory standards institute (CLSI, 2023) [13]. The *E. coli* ATCC 25922 was used as the quality control strain. The commercial antibiotic discs namely, cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), ceftazidime/ clavulanic acid (CAC 30/10 µg), and cefotaxime/ clavulanic acid (CEC 30/10 µg) were used for the characterisation of ESBL production based on double - disc synergy test (DDST). An increase of ≥ 5 mm in zone diameter for either of the antimicrobial agent tested in combination with clavulanate versus the zone diameter when tested alone was considered positive for ESBL production.

The isolates were also genotypically characterised for ESBL-production by targeting three ESBL genes *viz*., *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$.. The isolates were subjected to a multiplex PCR assay with an annealing temperature of 56 ºC (details of which are given in Table 2 and 3) and the PCR products formed with specific product sizes were visualized using a gel documentation system. All data obtained were statistically analysed using SPSS version 24.0.

3. Results and Discussion

Out of the 210 samples analysed, the presence of *E. cloacae* was identified from 94 samples using culture and biochemical methods, out of which 89.36 per cent of the isolates were confirmed as *E. cloacae* using PCR. Sample-wise analysis revealed the presence of this organism in 68.42 per cent of the broiler chicken meat samples, 48.21 per cent of the slaughter waste samples, 26.19 per cent of the environmental samples and in 12.7 per cent of the cloacal swab samples collected from live birds. Among the environmental samples, half of the litter materials, 33.33 per cent of soil, water and surface swab samples, and 16.7 per cent of knife swab samples and hand washings of bird handlers revealed the presence of this organism. However, none of the analysed feed samples were positive.

Many foods, particularly those of animal origin, have been recognised as carriers for transmitting pathogens to humans, with poultry and its products being one of the major contributors (Kim *et al*., 2018; Hafez., 2023) [14, 15]. Members of the Enterobacteriaceae family are increasingly associated with foodborne outbreaks and public health issues due to their significant ability to acquire antimicrobial resistance and their biofilm-forming potential (Edris *et al.*, 2024) [16]. The current study indicated the prevalence of *E. cloacae* in the broiler production chain, with the highest occurrence observed in the meat samples analysed. The prevalence rate observed was notably higher than the findings of Al-Esawi *et al.* (2024) [17] who had observed a prevalence of 15 per cent. This increased prevalence could be attributed to faecal contamination, which may occur during slaughtering or handling, or as a result of cross-contamination with equipment or surfaces in the butcher shop. In addition to its disease-causing potential, the presence of *E. cloacae* in meat is notable due to its capacity to produce biogenic amines, such as putrescine, which may pose toxicological risks to consumers. Its detection serves as an indicator of microbial spoilage, thereby impacting meat quality and posing potential public health concerns (Jairath *et al.*, 2015) [18]. However, the prevalence of this organism in slaughter waste samples appears to be a normal finding, as all the analysed samples were intestines of broiler chicken, where it commonly resides as a commensal organism. The recovery of *E. cloacae* from the environmental samples further emphasised the possibility of environmental contamination - altering the environmental microbiome, thus contributing to broader public health risks through the food supply chain. A chi-square test ($\chi 2$) result of 41.14 with a p-value < 0.001 indicated that there is a significant difference in the presence of the organism of interest across the different sample types.

Table 4: Sample-wise occurrence of *E. cloacae* in different samples

Type of sample	Total sample taken	Positive cases	
		No.	Per cent
Meat	57	39	68.42^{a}
Slaughter waste	56	27	48.21^{b}
Cloacal swab	55	7	12.73^{c}
Environmental samples	42	11	26.19^{c}
χ^2 Value (P-value)		41.14** (<0.001)	

** Significant at 0.01 level. Percentage having different letter as superscript differ significantly

In the town-wise analysis, samples collected from Meenangadi showed the highest prevalence at 72.22 per cent, this high prevalence rate could be due to inadequate hygienic practices, including the absence of proper waterlines and reliance on stored water for cleaning and insufficient sanitation measures for the equipments used for slaughtering. Additionally, factors such as high stocking density and poor ventilation may contribute to the increased incidence of *E. cloacae* in farms within these areas, while samples from Vythiri had the lowest prevalence at 11.43 per cent, may be due to the better abattoir practises and slaughtering methods followed. The chi-square ($\chi 2$) test result of 36.16 with a p-value of 0.004 indicated a significant difference in the occurrence of *E. cloacae* across these locations. This discrepancy could be attributable to the microclimate, differences in hygienic practices or due to broiler health and immune status. Sample-wise occurrence of *E. cloacae* from each location is detailed in table 6.

Table 5: Location-wise occurrence of *E. cloacae*

Location	Total sample taken	Positive cases	
		No	Per cent
Kalpetta	34	17	50.00[b]
Meenangadi	36	26	72.22[a]
Meppadi	35	18	51.43[ab]
Panamaram	35	11	31.43[b]
Sulthan Bathery	35	8	22.86[cd]
Vythiri	35	4	11.43[d]
χ^2 Value (P-value)		36.16** (0.004)	

** Significant at 0.01 level. Percentage having different letter as superscript differ significantly

Table 6: Sample-wise occurrence of *E. cloacae* from each location

Type of sample	Kalpetta	Meenangadi	Meppadi	Panamaram	Sulthan Bathery	Vythiri	Total
Meat	8 (88.9)	9 (100)	9 (90)	9 (90)	3 (33.3)	1 (10)	39 (68.4)
Slaughter waste	9 (100)	7 (70)	6 (66.7)	2 (22.2)	3 (30)	0	27 (48.2)
Cloacal swab	0	4 (40)	1 (11.1)	0	1 (11.1)	1 (11.1)	7 (12.7)
Surface swab	0	1 (100)	1 (100)	0	0	0	2 (33.3)
Knife swab	0	1 (100)	0	0	0	0	1 (16.7)
Litter	0	1 (100)	0	0	1 (100)	1 (100)	3 (50)
Soil	0	1 (100)	0	0	0	1 (100)	2 (33.3)
Water	0	1 (100)	1 (100)	0	0	0	2 (33.3)
Hand wash	0	1 (100)	0	0	0	0	1 (16.7)
Feed	0	0	0	0	0	0	0
Total	17 (50)	26 (72.2)	18 (51.4)	11 (31.4)	8 (22.9)	4 (11.4)	84

Values in the brackets are percentages out of total samples in each type in each location

Out of the recovered isolates obtained from the cloacal swab samples, it was observed that 85.71 per cent isolates were ESBL producers which was lower than the findings of Dandachi *et al.* (2018) [19], while 66.71 and 63.00 per cent of the isolates recovered from the meat and slaughter waste samples respectively were identified as ESBL producers, higher than the findings of Tekiner and Özpınar (2016) [20]. Analysis of the environmental samples revealed diverse ESBL production patterns among the *E. cloacae* isolates. Isolates obtained from litter samples, soil and water samples, knife swabs, surface swabs, and hand washings of bird handlers were phenotypically confirmed as ESBL producers. The results are concerning from a public health perspective as high detection rate observed from the cloacal swab suggests significant colonisation within the birds, serving as a reservoir for antimicrobial resistance (AMR) (Akkari *et al.*, 2024) [21], similarly, the detection of ESBL producers in meat poses direct risks to humans through handling or consumption, while their presence in slaughter waste, often used as fish feed, facilitates the indirect transmission of AMR to humans through the food chain. Further their occurrence in the environmental samples, underscores their potential for transmission to both human and animal subsystems.

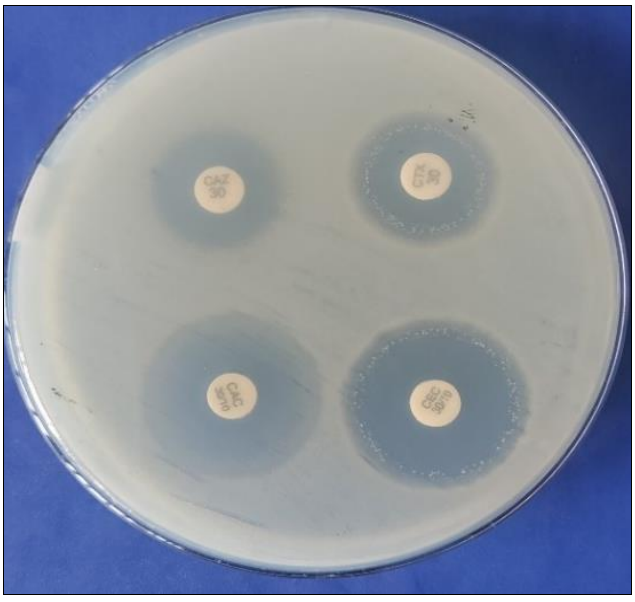

Fig 3: Phenotypic characterisation of isolates for ESBL production

Genotypic characterisation of ESBL production revealed that the *bla*$_{SHV}$ gene was ubiquitous across all broiler samples, contrary to the findings of Tekiner and Özpınar. (2016) [20], and Abdallah *et al.* (2015) [22]. Among the isolates, 73.80 per cent of the isolates harboured all three selected ESBL genes. Additionally, 91.95 per cent of the isolates carried two of the selected genes, either *bla*$_{SHV}$ and *bla*$_{CTX-M}$ or *bla*$_{SHV}$ and *bla*$_{TEM}$. Notably, all isolates carried at least one of the three selected ESBL genes. The elevated prevalence of these intestinal commensals is alarming; however, their antibiotic resistance profiling presents an even greater challenge with significant public health implications. This situation highlighted the potential for antimicrobial resistance genes to spread from poultry to humans through the food chain. Since most ESBL genes are plasmid-borne, they can spread rapidly among bacteria through various mechanisms like horizontal gene transfer, conjugation etc, introducing these resistance genes into the naive bacterial populations (Benz *et al*., 2021) [23]. This process significantly enhances their potential for widespread dissemination, exacerbating the challenges posed by antibiotic-resistant infections.

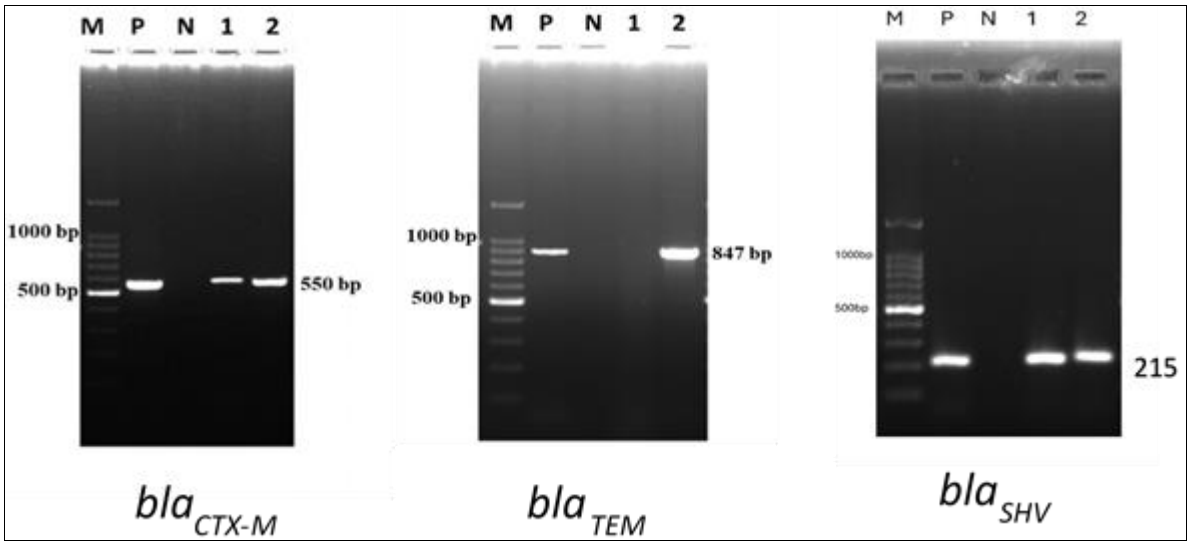

Fig 4: Genotypic characterisation of isolates for ESBL -production

Lane M: 100 bp DNA Ladder, Lane P: Positive control Lane, N: Negative control, Lane 1-2: Samples

Table 7: Characterisation of isolates of *E. cloacae* for ESBL production

Type of sample	PCR positive isolates	ESBL Producers	ESBL Genes		
			bla$_{SHV}$	*bla*$_{TEM}$	*bla*$_{CTX-M}$
Meat	39	26 (66.7)	39 (100)	34 (87.2)	39 (100)
Slaughter waste	27	17 (63)	27 (100)	20 (74.1)	25 (92.6)
Cloacal swab	7	6 (85.7)	7 (100)	6 (85.7)	6 (85.7)
Surface swab	2	2 (100)	2 (100)	0	0
Knife swab	1	1 (100)	1 (100)	0	0
Litter	3	2 (66.7)	3 (100)	2 (66.7)	3 (100)
Soil	2	1 (50)	0	1 (50)	2 (100)
Water	2	1 (50)	2 (100)	1 (50)	1 (50)
Hand wash	1	1 (100)	1 (100)	1 (100)	1 (100)
Feed	0	0	0	0	0
Total	84	57 (67.9)	82 (97.6)	65 (77.4)	77 (91.7)

Values in the brackets are percentages out of total PCR positive cases in each type

4. Conclusions

The study found that drug-resistant *E. cloacae* was widely distributed across various sample types collected and analysed from the broiler production chain. In light of the growing concern over antibiotic resistance, the presence of these pathogens in animal food sources presents a significant threat to both food safety and public health. The spread of ESBL-producing bacteria has become increasingly complex, with blurred lines between healthcare environments, communities, animals, and the environment. This underscores the urgent need for a One Health approach to tackle the interconnected challenges of antimicrobial resistance (AMR). It also highlights the critical need for enhanced biosecurity measures and stricter monitoring protocols throughout the production process. These findings have serious implications, emphasizing the necessity for immediate action to reduce the risk of pathogen transmission in broiler production and ensure the safety of poultry products for human consumption.

5. Conflict of Interest

The authors declare that there are no actual or potential conflicts of interest that could have influenced this work inappropriately.

6. Acknowledgements

The authors sincerely acknowledge the College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala, for offering the necessary research facilities that enabled the successful completion of this study.

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How to Cite This Article
Arya VS, Renukuttan A, Asha K, Vinod VK, Jess V, Rajagopal K, Aswathi PB and Hariharan R. Occurrence of ESBL: Producing *Enterobacter cloacae* in the broiler production chain in Wayanad, India. International Journal of Veterinary Sciences and Animal Husbandry. 2024;9(6):515-520.