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Phenotypic, Molecular Detection, and Antibiogram Patterns of *E. coli* isolates from Respiratory Infections of Commercial Layers in and around Tirupati

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ABSTRACT: A total of 78 pooled oral swabs, 82 pooled tracheal swabs, 86 pooled nasal swabs, and 25 pooled infraorbital sinus exudates were collected from the ailing birds, which were showing respiratory signs and were labeled farm-wise and specimen-wise. The PCR test was standardized to target the 16s rRNA gene, and the study found that 8 of 12 farms tested positive for *E. coli* infection. Among the 8 positive farms, 59 out of 78 (75.64% oral swabs), 72 out of 82 (87.80% tracheal swabs), 70 out of 86 (81.39% nasal swabs), and 18 out of 25 (72% infraorbital sinus exudates) were confirmed positive by PCR, with a predicted size of 585 bp for all positives. From the pooled samples, 32 samples (1 from oral swabs, 1 from tracheal swabs, 1 from nasal swabs, and 1 from infraorbital sinus exudate from each of the 8 positive farms) had been evaluated for antimicrobial susceptibility patterns using the Kirby-Bauer disc diffusion method. These 32 clinical *E. coli* isolates were isolated on selective EMB agar and MacConkey agar, which produced greenish metallic sheen and pink colonies, respectively. Gram staining confirmed these colonies, which had an IMViC pattern of ++--. The Kirby-Bauer disc diffusion method was used to test the antimicrobial patterns of pure clinical respiratory isolates of *E. coli*. All isolates were resistant to Ceftriaxone (CTR) and Cephalexin (CN), but sensitive to Enrofloxacin (EX), Tetracycline (TE), Ciprofloxacin (CIP), Co-trimaxazole (COT), and Colistin (CL). The gold standard test for confirmation of *E. coli* is isolation and identification of *E. coli* on EMB agar, which appear as greenish metallic sheen colonies, but the process is laborious and time-consuming. Similar results were obtained with molecular techniques like PCR, a confirmatory test with accurate results but less time-consuming. This study concluded that the prompt detection of respiratory diseases in poultry is accurate using molecular approaches, which will aid in the isolation of *E. coli*, antibiogram patterns, and the implementation of control measures that will guide the selection of suitable drugs for treatment.

Keywords: layers, *E. coli*, EMB agar, PCR, antibiogram.

INTRODUCTION

India is one of the world's leading producers of eggs and broiler meat, and demand is rising as the population grows, incomes rise, and dietary preferences evolve. Chickens account for over 95% of total egg production in India, with ducks and other poultry species making up the remainder. India ranks 3rd in egg output and 6th in chicken meat production globally. More output is required to meet the ICMR's recommended standards of 180 eggs and 10.8 kg of poultry meat per person per year. Many factors influence productivity; among them, infectious diseases are considered one of the most significant factors. The use of live vaccines, combined with the evolution of many pathogens, has resulted in a rise in disease incidence and resurgence, posing a threat to production. The most common respiratory pathogens, like *Mycoplasma, Infectious Bronchitis Virus, Infectious Laryngotracheitis Virus,* and *E. coli*, are the prevalent etiological agents that lead to financial and productivity losses. Avian colibacillosis is one of the major diseases caused by *E. coli* (Singh *et al.,* 2011). Infected birds exhibit symptoms like cough, respiratory discomfort, poor growth, and productivity (Pang *et al.,* 2002). *E. coli*, which belongs to the *Enterobacteriaceae* family and is considered the normal intestinal microflora of humans and birds, is typically present in the pharynx and trachea of birds. It is a gram-negative, non-acid-fast, non-sporulating, facultatively anaerobic, rod-shaped bacterium that ferments lactose, producing acid and gas. The majority of *E. coli* strains are non-pathogenic and are referred to as commensals. However, after acquiring certain virulence features, some of these bacteria have gained the ability to thrive in diverse organisms and are known as pathogenic *E. coli*, which cause clinical symptoms associated with intestinal and extraintestinal illnesses (Kaper *et al.,* 2005). *E. coli* can be classified into two types:

commensals and pathogenic. Pathogenic *E. coli* is divided into two subgroups: extraintestinal pathogenic *E. coli* (ExPEC) and diarrheagenic *E. coli* (DEC), both of which cause gastrointestinal illnesses. Avian pathogenic *Escherichia coli* (APEC), a kind of extraintestinal pathogenic *E. coli* (ExPEC), is the cause of avian colibacillosis, an infectious disease in birds. The most common infections in chickens caused by avian pathogenic *E. coli* (APEC) are septicaemia, enteritis, perihepatitis, pericarditis, airsacculitis, egg peritonitis, salpingitis, coligranuloma, omphalitis, cellulitis, osteomyelitis, and decreased egg yield, quality, and hatchability in layers (Dziva *et al.,* 2008). Layers are susceptible to APEC at any time during the growth and laying seasons, particularly during the late laying period and peak egg production, whereas broilers aged 4 to 6 weeks are more vulnerable (Dho-Moulin *et al.,* 1999). In the presence of stressors, APEC invades the gastrointestinal and respiratory tracts via abraded tracheal and intestinal epithelium, penetrates deeply into the mucosa and submucosa before entering the bloodstream, and spreads to internal organs, resulting in septicaemia (Dho-Moulin *et al.,* 1999; Dziva *et al.,* 2008; Rodrigo Guabiraba *et al.,* 2015). In most countries, poultry flocks are often raised under intensive conditions using a wide range of antimicrobials (Agunos *et al.,* 2012; Landoni and Albarellos 2015). These antimicrobials are typically given orally with the purpose of preventing and treating disease while also boosting development and production (Page and Gautier 2012). A considerable number of these antimicrobials are regarded as important and critical for human medicine (World Health Organization *et al.,* 2017). The indiscriminate use of antimicrobials in animals has accelerated the development of antibiotic-resistant bacteria, which can pose major public health threats (CDC: Atlanta, GA, USA *et al.,* 2019). Furthermore, *E. coli* has been identified as a natural reservoir of antibiotic resistance genes (ARGs), playing a significant role in the spread of antimicrobial resistance (AMR) (CDC: Atlanta, GA, USA *et al.,* 2019; Partridge *et al.,* 2018), and it is frequently used as an important biomarker for monitoring AMR (Brisola *et al.,* 2019; Poirel *et al.,* 2018). To avoid economic and production losses, it is essential to detect organisms and antibiogram patterns early, as well as propose area-specific medication for treatment. Regular isolation of organisms is must to know the conformation and antimicrobial pattern for selection of suitable drug. The current work sought to employ molecular approaches to detect *E. coli* in isolates from layers with respiratory infections and characterize their antimicrobial pattern, thereby assisting in the selection of appropriate treatments and control methods to prevent economic losses in the poultry industry.

MATERIALS AND METHODS

Sample collection: Samples were collected from 12 commercial layer farms located in and around Tirupati. A total of 78 pooled oral swabs, 82 pooled tracheal swabs, 86 pooled nasal swabs, and 25 pooled infraorbital sinus exudates were collected from the ailing birds, which were showing respiratory signs and were labeled farm-wise and specimen-wise.

Isolation and identification of *Escherichia coli*: After collection, the samples were inoculated into sterile test tubes containing nutrient broth and aerobically incubated at 37 °C in a bacteriological incubator for 24 hours. Following incubation, the inoculum was spread onto MacConkey agar and Eosin Methylene Blue (EMB) agar plates and incubated at 37 °C for 24 hours. Later, the inoculated plates were examined for pink-colored colonies on MCA and greenish metallic sheen colonies on EMB agar. To confirm, the colonies on EMB agar plates were subjected to gram staining and biochemical tests, *viz*., IMViC tests (indole production, methyl red, Voges Proskauer, citrate test), catalase test, urine production, and nitrate reduction tests, and the organisms showed the IMVC pattern of ++-- (Edwards *et al.,* 1972).

DNA Isolation: DNA isolation was done using the boiling and snap-chilling processes. Initially, *E. coli* isolates that had been cultured and biochemically confirmed were incubated in nutrient broth at 37 °C overnight. After 18 hours, 1.5 mL of the enriched broth culture was centrifuged for 10 minutes at 10,000 rpm and the pellet was resuspended in 100 µL of nuclease-free water. This mixture was heated in a boiling water bath at 100 °C for 10 minutes before being rapidly chilled in an ice box at -20 °C and centrifuged at 10,000 rpm for 5 minutes. The supernatant was stored at -20 °C and utilized as a DNA template in the PCR assay. DNA concentration was determined using Nanodrop (Thermo Scientific, USA).

Polymerase Chain Reaction (PCR): All extracted DNA samples were submitted to PCR using particular primers to target the 16s rRNA gene for species-specific characterization. Table 1 lists the primers used in the investigation. The reaction used 12.5 µl of master mix, 4 µl of DNA template, 1 µl of forward and reverse primer, and 6.5 µl of nuclease-free water for a total of 25 µl. The PCR reaction was carried out in a thermal cycler with a heated cover under standardized cycling conditions, as indicated in Table 2. The amplified PCR products obtained after the reaction were run through 1.5% agarose gel electrophoresis in a horizontal electrophoresis apparatus at 5 V/cm and ethidium bromide at 0.5 µg/ml. The Alpha Innotech gel documentation system was used to examine the gel under UV transillumination in order to observe the bands.

Table 1: Primers used for detection of *E. coli* (Nagendra Reddy Thopireddy, 2023).

Primers	Primer Sequence (5'to 3')	Amplicon size
16s rRNA gene-F	5'-GACCTCGGTTTAGTTCACAGA-3'	585 bp
16s rRNA gene-R	5'-CACACGTGACGCTGACCA-3'	

Table 2: Cyclic conditions used for amplification of 16s *r*RNA gene of *E. coli*

Sr. No.	Step	Temperature (°C)	Time	No of cycles
1.	Initial Denaturation	95°C	5 min	1
2.	Denaturation	94°C	30 sec	35
3.	Annealing	62°C	30 sec	
4.	Extension	72°C	30 sec	
5.	Final extension	72°C	2 min	1

Antimicrobial Sensitivity Test: The Kirby-Bauer disc diffusion method was used to assess antibiotic susceptibility patterns in 32 *E. coli* isolates, and the results were interpreted as susceptible (S), intermediate (I), or resistant (R) in accordance with CSLI (2021). *E. coli* overnight cultures were infused into sterile PBS and matched to the 0.5 MacFarland turbidity standard. A sterile swab dipped in the bacterial suspension was equally distributed throughout the Mueller-Hinton agar plate. Antimicrobial discs were placed on the agar, and the plates were incubated at 37 °C for 24 hours.

RESULTS

Out of 271 samples, 219 were positive for *E. coli,* with an overall prevalence rate of 80.81%. On testing, 8 farms out of 12 were found positive for the *E. coli* infection. Among the 8 positive farms, 59 out of 78 (75.64% oral swabs), 72 out of 82 (87.80% tracheal swabs), 70 out of 86 (81.39% nasal swabs), and 18 out of 25 (72% infraorbital sinus exudates), totaling 219, were confirmed positive by PCR, which produced a predicted size of 585 bp in all positives. Random selection of the isolated *E. coli* was performed, and 32 [4 (1 from oral swabs, 1 from tracheal swabs, 1 from nasal swabs, and 1 from infraorbital sinus exudate) from each positive farm] isolates were chosen and then subjected to antimicrobial sensitivity tests. The highest resistance was observed for Cephalexin (100%) and Ceftriaxone (100%). The highest sensitivity was observed for Cotrimaxazole (65.62%), followed by Tetracycline (28.12%), Enrofloxacin (25%), Ciprofloxacin (21.87%), and Colistin (15.62%) (Table 3).

Table 3: Antibiotic susceptibility patterns of *E. coli* isolates from respiratory infections of commercial layers.

Sr. No.	Antimicrobial agents	Sensitive	Intermediate	Resistant
1.	Ceftriaxone (CTR), 10µg	0 (0%)	0 (0%)	32 (100%)
2.	Cephalexin (CN), 30µg	0 (0%)	0 (0%)	32 (100%)
3.	Tetracycline (TE), 30µg	9 (28.12%)	5 (15.62%)	18 (56.25%)
4.	Enrofloxacin (EX), 5µg	8 (25%)	7 (21.85%)	17 (53.12%)
5.	Cotrimaxazole, (COT), 25µg	21 (65.62%)	1 (3.12%)	10 (31.25%)
6.	Ciprofloxacin (CIP), 5µg	7 (21.87%)	6 (18.75%)	19 (59.37%)
7.	Colistin (CL), 10µg	5 (15.62%)	4 (12.5%)	23 (71.85%)

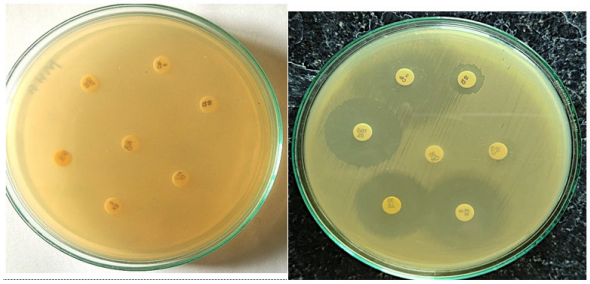

Fig. 1. Antibiotic sensitivity patterns of *E. coli* isolates.

DISCUSSION

E. coli belongs to the *Enterobacteriaceae* family. *E. coli* can be classified into two types: commensals and pathogenic. Pathogenic *E. coli* is divided into two subgroups: extra intestinal pathogenic *E. coli* (ExPEC) and diarrheagenic *E. coli* (DEC). ExPEC is further divided into six subpathotypes: uropathogenic *E. coli* (UPEC), sepsis/newborn meningitis-associated *E. coli* (NMEC), avian pathogenic *E. coli* (APEC) (Kunert Filho *et al.,* 2015), sepsis-associated pathogenic *E. coli* (SePEC) (Mokady *et al.,* 2005), mammary pathogenic *E. coli* (MPEC) (Shpigel *et al.,* 2008), and endometrial pathogenic *E. coli* (EnPEC). DEC is divided into eight subpathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) (Huang *et al.,* 2006), adherent invasive *E. coli* (AIEC), and Shiga-toxin-producing enteroaggregative *E. coli* (STEAEC).

APEC can cause both systemic and localized forms of colibacillosis, acting as a primary or secondary agent. The most prevalent localized infections caused by APEC as the primary pathogen are omphalitis, yolk sac infections, and reproductive tract infections (Landman *et al.,* 2013). *E. coli*, as a secondary pathogen, contributes to respiratory colibacillosis and causes colisepticemia. Colisepticemia develops secondary to viral infections such as *Newcastle disease (NDV), avian influenza (AIV), infectious bronchitis (IBV), Mycoplasma gallisepticum (MG)*, immunosuppressive diseases (infectious bursal disease (IBD)), and stressors (overcrowding, excessive dust, and ammonia levels) (Dho-Moulin *et al.,* 1999; Ghunaim *et al.,* 2014).

In all 12 poultry farms, clinical signs including ruffled feathers, nasal discharges, facial swelling, labored breathing, huddling, depression, sinus swelling, a decrease in egg production and poor egg quality, reluctance to stand or move, decreased food and water consumption, and complete or extreme prostration. Similar observations were documented by Ramasamy *et al*. (2008); Gowthman *et al*. (2013); De Carli *et al*. (2014); Veeraselvam *et al*. (2019); Thopireddy*et al*.(2023). The current study identified *E. coli* as the predominant concurrent infectious agent linked with respiratory disease complex in eight farms (80.81%), corroborating the findings of previous researchers (Gowthaman *et al*., 2013; Chowdhury *et al*., 2018; Kaore *et al*., 2018).

Post-mortem examination of affected birds revealed polyserositis characterized by white to yellow exudates

on the liver surface (fibrinous perihepatitis), heart (fibrinous pericarditis), (Fig. 2), and intestines (peritonitis), along with blood vessel congestion and lung consolidation indicative of severe *E. coli* infection (Yadav *et al.,* 2018; Surjagade *et al.,* 2020; Halder *et al.,* 2021). The study also identified an acute form of the disease marked by septicemia leading to death and a subacute form characterized by pericarditis, airsacculitis, and perihepatitis, as reported by Dhama *et al*. (2013); Dadheech *et al*. (2016) ; Thopireddy (2023).

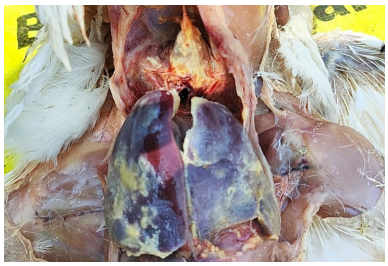

Fig. 2. PM examination of the affected bird showing pericarditis and perihepatitis.

Isolation and identification of *E. coli.* After 24 hours of incubation at 37°C in aerobic conditions, all *E. coli* positive isolates on MacConkey agar exhibited lactose-fermenting pink colonies (Fig. 3), whereas *E. coli* colonies on EMB agar exhibited greenish metallic sheen (Fig. 4), consistent with findings by Matin *et al.* (2017); Veeraselvam *et al*. (2019); Surjagade *et al*.(2020); Thopireddy *et al*. (2023). The *E. coli* isolates appeared as pink-colored gram-negative bacilli, occurring singly or in pairs, (Fig. 5), aligning with observations by Amer *et al.* (2015); Boro *et al*.(2019). All *E. coli* isolates displayed an IMViC pattern of ++-- (Edwards *et al*. 1972), were urease negative, catalase positive, and oxidase negative, paralleling results reported by Amin *et al*. (2017); Ibrahim *et al*. (2019); Nagendra Reddy *et al.* (2023).

Fig. 3. MCA showing pink colored colonies of *E. coli*.

Fig. 4. EMB agar showing greenish metallic colonies of *E. coli.*

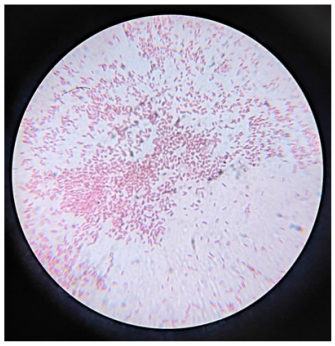

Fig. 5. Gram staining showing pink coccobacillary rods of *E. coli.*

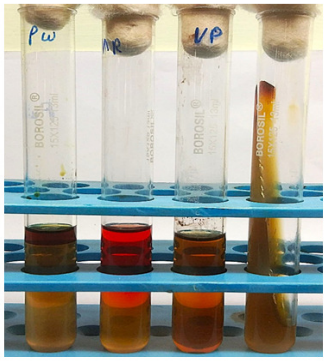

Fig. 6. IMViC tests pattern for *E. coli* (1. Indole test-positive, 2. Methyl red-positive, 3. Vp test-negative, 4. Citrate test-negative).

All suspected samples (271 samples) from 12 farms were initially tested for *E. coli* by targeting 16sr RNA gene and the study found that 8 farms with a total of 219 samples, of which 59 (74.64% from oral swabs), 72 (87.80% from tracheal swabs), 70 (81.39% from nasal swabs), and 18 (72% from in fraorbital sinus exudates) tested positive. These results are higher than those reported by Nayak *et al*. (2017), who found a positivity rate of 14.2% in both tracheal and nasal swabs, and align with Banu *et al*. (2017), who reported a positivity rate of 57% in tracheal swabs and 57.1% in infraorbital sinus exudates. The current study observed an *E. coli* isolation rate of 80.8%, similar findings have been reported by other researchers, 80% by Tonu *et al*. (2011), 82.83% by Ievy *et al*. (2020) and 100% by Ibrahim *et al*. (2019). The high isolation rate of *E. coli* observed in our study, as well as in others, could be due to stringent adherence to microbiological isolation techniques, resulting in improved bacterial recovery. Such a high rate of isolation, positions *E. coli* as an ideal candidate for assessing antimicrobial resistance (AMR) prevalence and patterns across various production systems, including layer poultry farms (Mudenda *et al.,* 2023).

An investigation in Andhra Pradesh identified *E. coli* on 12 farms, with a PCR positivity rate of 63.15% and a SYBR Green Real Time PCR positivity rate of 71.2%. On these farms, PCR detected *E. coli* in 92 nasal swabs (40.35%), 106 tracheal swabs (51.75%), 64 tracheal tissues (42.10%), and 72 lung tissues (47.36%). Furthermore, 118 nasal swabs (49.56%), 128 tracheal swabs (56.14%), 68 tracheal tissues (44.73%), and 77

lung tissues (50.65%) tested positive for SYBR Green Real Time PCR by Nagendra Reddy *et al.* (2023).

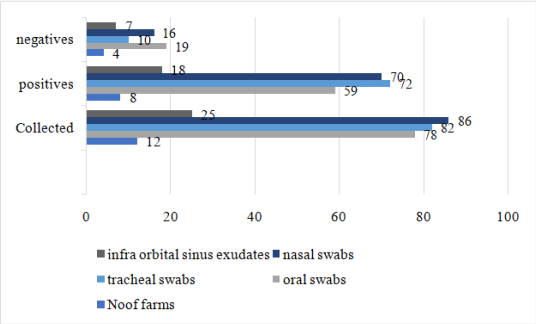

Fig. 7. Data table representing sample size.

Molecular characterization of *E. coli.* This work used PCR to quickly identify 16s rRNA gene of *E. coli*. The technique used DNA isolated from *E. coli* isolates and PCR primers specific to the 16s rRNA gene, which resulted in a 585 bp amplicon confirming *E. coli* presence. 16s rRNA was identified in 219 of 271 samples using PCR, corroborating the findings of Tonu *et al*. (2011); Islam *et al*. (2014); Matin *et al*. (2017).

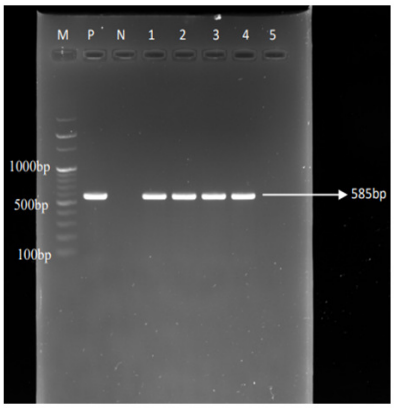

Fig. 8. Agarose gel displaying the 585 bp*E. coli* 16s rRNA gene product amplified by PCR.

Antibiotic sensitivity of *E. coli.* *E. coli* includes a large number of antibiotic resistance genes, including beta-lactam resistance genes. The presence of beta-lactam antibiotic resistance genes in *E. coli* causes the production of beta-lactamase enzymes, which hydrolyze β-lactam antibiotics, a frequent resistance mechanism in the *Enterobacteriaceae* family (Pitout*et al.,* 1998). ESBLs are enzymes that efficiently hydrolyze third and fourth-generation cephalosporins and monobactams (e.g., aztreonam), but are inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam (Fernandes *et al.,* 2014). Furthermore, ESBL-producing *E. coli* frequently exhibits resistance to multiple classes of antimicrobials, primarily fluoroquinolones, sulfonamides, aminoglycosides, chloramphenicol, trimethoprim, and tetracyclines (Bonnet *et al.,* 2004). The major genes involved in ESBL production are the TEM (blaTEM), SHV (blaSHV), and CTX-M (blaCTX-M) genes. CTX-M-type ESBL-producing *E. coli* is the most common worldwide (Pfeifer *et al.,* 2010).

Our investigation found that *E. coli* was resistant to Tetracycline (56.25%) and Cotrimaxazole (31.25%), which is consistent with research on laying hens in Zambia by Mudenda *et al.* (2023). In contrast, a study from commercial farms in Zambia's Chisamba district by Mtonga *et al.* (2021) reported a 100% resistance of *E. coli* to Tetracycline. Additionally, a high resistance rate to Colistin (71.85%) was detected, echoing the results of Hess *et al.,* (2022). Our research indicated that the resistance of *E. coli* to Cephalexin was the highest (100%), with resistance to Ciprofloxacin and Enrofloxacin at 59.37% and 53.12%, respectively, corroborating Khanal *et al.* (2017) findings. Similarly, our study discovered *E. coli* to be highly resistant to Ceftriaxone (100%), which was also found in a study by Islam *et al.* (2023), that revealed *Escherichia coli* isolates demonstrating resistance to several cephalosporin antibiotics, ranging from 2.3% to 100% (95% CI ranged from 0.4% to 100%) for Ceftriaxone.

CONCLUSIONS

E. coli is the most frequent bacterial infection in poultry, causing significant economic losses globally. Controlling *E. coli* improves both animal and human health. Present study found that *E. coli* isolates from layers in and around Tirupati are resistant to at least one of the antimicrobial agents tested. Given the serious antimicrobial resistance (AMR) conditions, there's a pressing need to curtail antibiotic use, especially in layer farms. The emergence of significant antibiotic resistance and the high risk of transmitting resistant bacteria and genes to humans necessitate the development of new antibacterials for exclusive use in animals, without cross-resistance to existing antibiotics. This could be a viable solution for the future. It's crucial to enhance awareness and conduct scientific research through monitoring and surveillance programs on AMR to mitigate the adverse impacts of antimicrobial-resistant *E. coli* in the poultry industry.

Author's contribution. S. Surendranath Reddy carried out the entire investigation, including sample collection and processing. With assistance from, Dr. T. Nagendra Reddy, S.S. Reddy wrote the first draft of the manuscript and corrected it. Dr. T. Nagendra Reddy contributed to the study's design, coordination, and scientific discussion in addition to writing the final text. The final manuscript was read and approved by all authors.

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