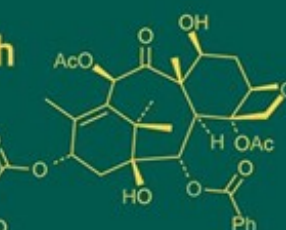
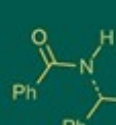


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
Indexing: Embase
IJABR 2026; 10(9): 590-599
www.biochemjournal.com
Received: 02-07-2026
Accepted: 18-08-2026
Published: 23-09-2026

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Etiopathology of cellulitis in broilers from Nagpur region of central India

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i9h.10129>

ABSTRACT

Avian cellulitis, bacterial disease of broilers caused by avian pathogenic *Escherichia coli* (APEC) contributes substantial economic losses to the broiler industry due to condemnations of carcass during post-mortem examination at slaughter house. The present study was conducted to study etiopathology of cellulitis cases and to characterize the *E. coli* isolates recovered from affected broilers across Nagpur region of central India. Total 9 broiler farms affected with cellulitis were screened and post-mortem examination was conducted on 28 broiler carcasses. The post mortem examination was carried at Department of Veterinary Pathology, Nagpur Veterinary College, Nagpur. Grossly the birds affected with cellulitis showed thickened and yellowish discoloured skin on inner aspect of thigh muscle, wings, breast, keel bone area, back and flank region. Fibrinopurulent exudate in subcutaneous tissue was observed. Microscopically, hyperkeratosis, thickened dermis, heavy infiltration of mononuclear cells and heterophils along with fibrinocaseous exudates and necrosis of the skin was observed. Samples from the affected portion of birds were cultured on Brain Heart Infusion (BHI) agar and Eosin Methylene Blue (EMB) agar media and overnight incubated at 37 °C. The result revealed that most of the samples showed growth of *E. coli* (93%), mixed with other bacterial isolates mainly *Staphylococcus*, *Enterococcus* and *Proteus* spp etc. Confirmation of the bacterial colonies was done by MALDI-TOF MS analysis. Antibiotic sensitivity test showed maximum sensitivity towards colistin sulphate. Later the virulence profiling of these *E. coli* isolates was done by PCR detection of *cvl* and *tsh* genes. Genotyping of the samples was done by ERIC-PCR; comparison of the cellulitis *E. coli* samples was done with the other non-cellulitis *E. coli* of poultry, dog, cow and buffalo. This study demonstrated the *E. coli* is major pathogen causing cellulitis in Nagpur region, the isolates are sensitive to colistin sulphate and these isolates are typed similar on ERIC PCR.

Keywords: Cellulitis, *Escherichia coli*, ERIC-PCR, MALDI-TOF MS analysis

Abbreviations: BHI= Brain Heart Infusion agar, EMB= Eosin Methylene Blue agar, MALDI-TOF MS= Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry, ERIC-PCR= Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction, AST= Antibiotic Sensitivity Test, PCR= Polymerase Chain Reaction, *cvl*= colicin V immunity protein, *tsh*= temperature-sensitive hemagglutinin.

Introduction

Cellulitis usually refers to the inflammation of subcutaneous tissue, almost occurring in all the anatomical areas, but most typically observed adjacent to the lower abdomen, breast, thigh and in the regions with loose subcutaneous connective tissue with presence of fibrinonecrotic plaques in broiler chickens (Mellata, 2013) [1]. Cellulitis is important from salubrious point of view, representing its public health issue, but is also relevant in terms of animal welfare (Bianco *et al.*, 2016) [2]. Over the last few decades, cellulitis has emerged as economic important disease of broilers and turkeys (Lighty *et al.*, 2015). Cellulitis may be a secondary finding in a number of conditions. *Escherichia coli* is the predominant microorganism isolated from cellulitis although other agents, such as *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Enterobacter agglomerans*, *Proteus vulgaris* and *Streptococcus dysgalactiae* have also been isolated from cellulitis lesion (Norton *et al.*, 2000; Gomis *et al.*, 2000) [4, 3].

During, finisher stage of growth in broilers the development of disease is accompanied by interrupting the integrity of the skin, such as a traumatic wound or other cutaneous abrasion enabling a variety of bacteria to enter the wound and colonize the subcutaneous tissue (Quel *et al.*, 2013) [5]. Nutritional deficiency, contamination of water resources and Litter-moisture appears critical to cellulitis development (Hilmy *et al.*, 2002, Mellata *et al.*, 2013) [1].

With the advancement of molecular biological techniques, Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) methods could resolve the genotypes of the large number of antigen

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serotypes, thereby enabling identification of an appropriate immunogenic strain (Guay, 2009; Flores *et al.*, 2009; Indrawattana *et al.*, 2011) [7, 6, 8]. Further, would be useful in determining the sequence distribution and copy numbers of chromosomes for ERICs for showing the interspecies specificity (Lang *et al.*, 2013) [9].

Considering the importance of avian cellulitis in the context of poultry meat production, present study was aimed at performing microbiological isolation, detecting antimicrobial resistance genes in microorganisms and to establish an ERIC-PCR method for genotyping of *E. coli* strains in poultry cellulitis with other non-cellulitis *E. coli* cases of poultry, buffalo, cow, dog in and around Nagpur region of Central India,

Material and methods

Selection of birds with cellulitis: Total 9 broiler rearing farms around Nagpur were visited as they had problem of lingering mortality in the finisher stage of broiler production from November 2017 to June 2018. During visit the 28 broiler carcasses, which grossly showed the caudal abdominal discoloration and thickening of skin after feather removal, were collected prior to evisceration and examined for cellulitis, 28 birds from 9 flocks were sampled across Nagpur region and detailed post mortem examination was conducted at Department of Veterinary Pathology, Nagpur, India. Cellulitis lesions were characterized grossly by yellowish fibrin deposit underneath this discolored or thickened skin. Swabs from cellulitis lesions and any other gross lesions observed in the body cavity were propagated in nutrient broth and incubated overnight at 37°C followed by aseptically culture on BHI Agar and selective media that is Eosin Methylene Blue (EMB) Agar (Himedia, Mumbai) specially used for *E. coli* isolation. All the inoculated media were incubated at 37°C and inspected for growth for 24 hours. Then the Gram's staining was performed on the smear prepared from isolated colony. Gram negative isolates were subjected for molecular and phenotypic characterization along with Antibiotic Sensitivity Test (AST).

Histopathology

After complete examination of the carcass the skin sections from the affected areas (including the site where bacteria were lodged) like heart and liver were preserved in 10% buffered formalin, were processed, later embedded in paraffin and further sections were cut using a microtome. The sections were stained by the haematoxylin and eosin (HE) method and were examined under light microscopy.

Antibiotic susceptibility: The recovered *E. coli* isolates (26) were subjected to antibiotic sensitivity testing using disc diffusion method (Barbieri *et al.* 2013). The antibiotic discs were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. The isolates were tested against commonly used antibiotics Enrofloxacin (10 mcg), Ciprofloxacin (5 mcg), Chlorotetracycline (30 mcg), Furazolidone (100 mcg), Levafloxacin (5 mcg), Doxycycline (30 mcg), Oxytetracycline (30 mcg), Neomycin (30 mcg), Chloramphenicol (30 mcg), Bacitracin (10 units), Cephalaxin (30 mcg) and Colistin sulphate (10 mcg) and interpretations of zone diameters were done by the Quality Control Limits for Antibiotics based on results obtained by Muller Hinton Agar (Himedia, Mumbai).

MALDI-TOF MS analysis for the evaluation of the mass: Single bacterial colony of each isolates were picked up from primary culture and re-streaked on fresh Brain Heart Infusion (BHI) agar and incubated at 37°C for 24hrs to obtain pure colony of culture of the isolates. The organism was characterized by biochemical reactions using Biomerieux Vitek® 2 System and further identified by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS, Biomerieux, Marcy l'Etoile France) as per the method recommended by Barberis *et al.* (2014) [10].

DNA extraction and PCR: Genomic DNA isolation was performed by using Himedia DNA isolation Kit. PCR was performed by using Gradient Master Cyclor (Eppendorf, India) to determine the presence of virulence marker genes of avian pathogenic *E. coli* in bacteria for *cvi* and *tsh* genes. The following primer sequences: for *tsh* gene

Forward primer– 5' ACTATTCTCTGCAGGAAGTC 3' and Reverse– 5' CTTCCGATGTTCTGAACGT 3' while for *cvi* gene Forward–5' TGGTAGAATGTGCCAGAGCAAG 3' and Reverse– 5' GAGCTGTTTGTAGCGAAGCC 3' were used for amplification (Ewers *et al.*, 2004). The PCR reaction were carried out as given protocol: Nuclease free water (9.5 µl), GoTaq® Green Master Mix 2X (12.5 µl), Forward primer 20pmol (1.0 µl), Reverse primer 20pmol (1.0 µl), Template DNA (1.0 µl) with the total of 25 µl. The cycling conditions in Master gradient cyclor for *tsh* and *cvi* genes were initial denaturation at 94°C for 3 mins, 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec (*tsh*) and 68°C 3 min (*cvi*) respectively and extension at 72°C for 10 min, then hold it at 4°C. Finally the amplified PCR product was loaded in agarose gel (1.5% agarose in 0.5X Tris borate-EDTA buffer, ethidium bromide (0.5 µg/ml) along with standard molecular size marker (100 bp DNA ladder). The gel was electrophoresed (Horizontal gel electrophoresis system, Genaxy). Amplified product were separated on agarose gel and observed by ultraviolet transilluminator and photographed in a gel documentation system (Syngene, USA).

Genotyping by ERIC-PCR: Strains were characterized by PCR using ERIC primers (Ranjba *et al.* 2017) [11]. Two primers with sequence ERIC (F) 5'ATGTAAGCTCTGGGGATTAC 3' and ERIC (R) 5'AAGTAAGTACTGGGGTGAGCG 3' were used to amplify repetitive sequence contained in the chromosomal DNA of *E. coli* isolates. ERIC PCR was carried with 25µl of the PCR mixture comprised of 100ng of *E. coli* DNA, 1µl (10pmol) of each ERIC-F, and ERIC-R', GoTaq® Green Master Mix 12.5µl and 9.5µl of Nuclease free water. The cycling conditions in Master gradient cyclor for ERIC primers were initial denaturation at 95°C for 7 min, 35 cycles of denaturation at 95°C for 1min; annealing at 56°C for 1min; extension at 72°C for 90 sec and final extension at 72°C for 7 min, then hold it at 4°C. The amplicons were electrophoresed on 1.5% agarose gel, along with 100 bp DNA ladder, and the 6X gel, loading dye containing 0.5 mg/mL of ethidium bromide was added. The image was captured by using gel documentation system (Syngene, USA).

Results

Broiler birds suspected for cellulitis showed brownish-red discoloration of skin around the inflamed area (Fig.1). On opening the carcass, petechial hemorrhages localised to tip of sternum and abdominal wall were observed along with yellowish semisolid masses on inner aspect of thigh muscle, wings, breast, keel bone area, back and flank region (Fig.2). Thickening of the diseased skins was self-evident finding. Yellowish plaques were observed in the subcutaneous tissues. In addition to cellulitis, the birds had pericarditis, airsacculitis, perihepatitis (Fig.3), polyserositis (a combination of pericarditis, perihepatitis and airsacculitis (Fig.4), usually birds with such combination appeared emaciated and with poor musculature.

Microscopic examination revealed necrosis of muscular layer attached to skin (Fig. 5), along with fibrinocaseous exudates and inflammatory cells in between muscles fibers (Fig.6). The muscle layer was submerged by fibrinous exudate mixed with inflammatory cells and bacteria (Fig.7). Subcutaneous fascia revealed congestion and infiltration of inflammatory cells heterophils and mononuclear cells in between adipocytes (Fig.8). Mild hyperkeratosis and hyperplasia of epidermal epithelial cells, vasculitis overloaded with accumulation of inflammatory cells was evident (Fig. 9).

Swab samples from 28 broiler carcasses were taken for isolation and identification of bacteria at the time of post mortem. Twenty six cases showed bacterial growth. While, no bacteria were isolated from cellulitis lesions of 2 birds. Metallic sheen on EMB agar plate was seen in 26 cases. The results of Biomerieux Vitek® 2 System and MALDI-TOF MS revealed that all 26 cases harboured *E. coli*, out of which in 19 cases (67.85 %) *E. coli* was the only bacterial species found. In one case (3.57%) *E. coli* and *Staphylococcus xylosus* was present. In one case (3.57%) *E. coli* and *Staphylococcus saprophyticus* was isolated. Additionally, *E. coli* along with *Enterococcus cecorum* and *Enterococcus faecium* were observed in 5 (17.85%) cases. Antibiotic

sensitivity results of isolated *E. coli* is presented in Table.1. All isolates showed 100% resistant towards Enrofloxacin, Ciprofloxacin, Furazolidone, Levafloxacin, Oxytetracycline and Bacitracin. While 60-70% resistance by Chlortetracycline, Doxycycline, Neomycin, Chloramphenicol, Cephalaxin and 15.38% least resistance was observed by Colistin sulphate.

Confirmed 26 isolates of *E. coli* (by MALDI-TOF) were future investigated for presence of virulence associated genes i.e., *cvl* and *tsh* gene, which are associated with cellulitis. It was observed that 16 iso-

lates were positive for both *cvl* and *tsh* genes, while 8 isolates were positive for only *tsh* genes, and only 2 isolates were positive for *cvl* gene alone (Fig 10).

The ERIC-PCR generated distinct amplification products that were differentiated by agarose gel electrophoresis (Fig.11). Amplicons of 26 isolates produced 1-7 bands ranged between 100 > 3,000 bp (Fig.12). Cluster analysis of *E. coli* strains showed that they were classified into 6 major groups. The generated banding profile showed highly polymorphic DNA fragments in all these isolates.



Figure 1. Thickened and brownish-red discolouration of broiler skin with cellulitis



Figure 2. Petechial hemorrhages localised to tip of sternum and abdominal wall



Figure 3. Perihepatitis



Figure 4. Combination of pericarditis, perihepatitis and airsacculitis

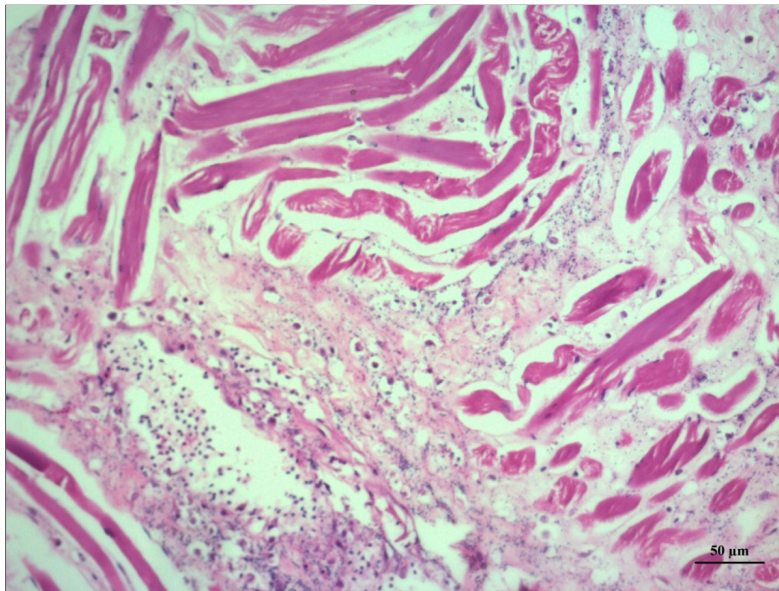


Figure 5. Necrosis of the muscular layer of skin. H&E

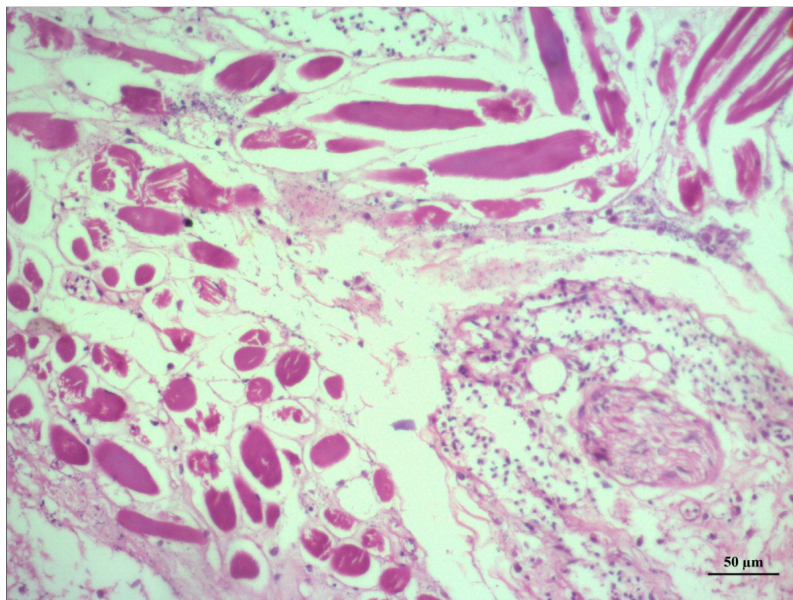


Figure 6. Necrotic cellular debris, fibrinocaseous exudates and inflammatory cells in between muscles fibers. H&E

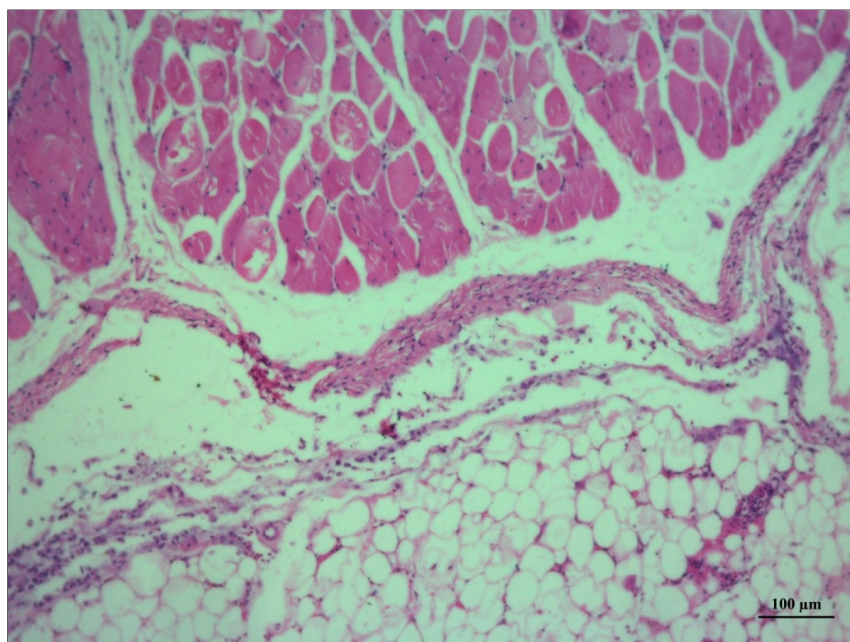


Figure 7. Muscle covered with fibrinous exudate mixed with inflammatory cells and bacteria, Subcutaneous fascia revealed congestion and infiltration of inflammatory cells in between adipocytes. H&E

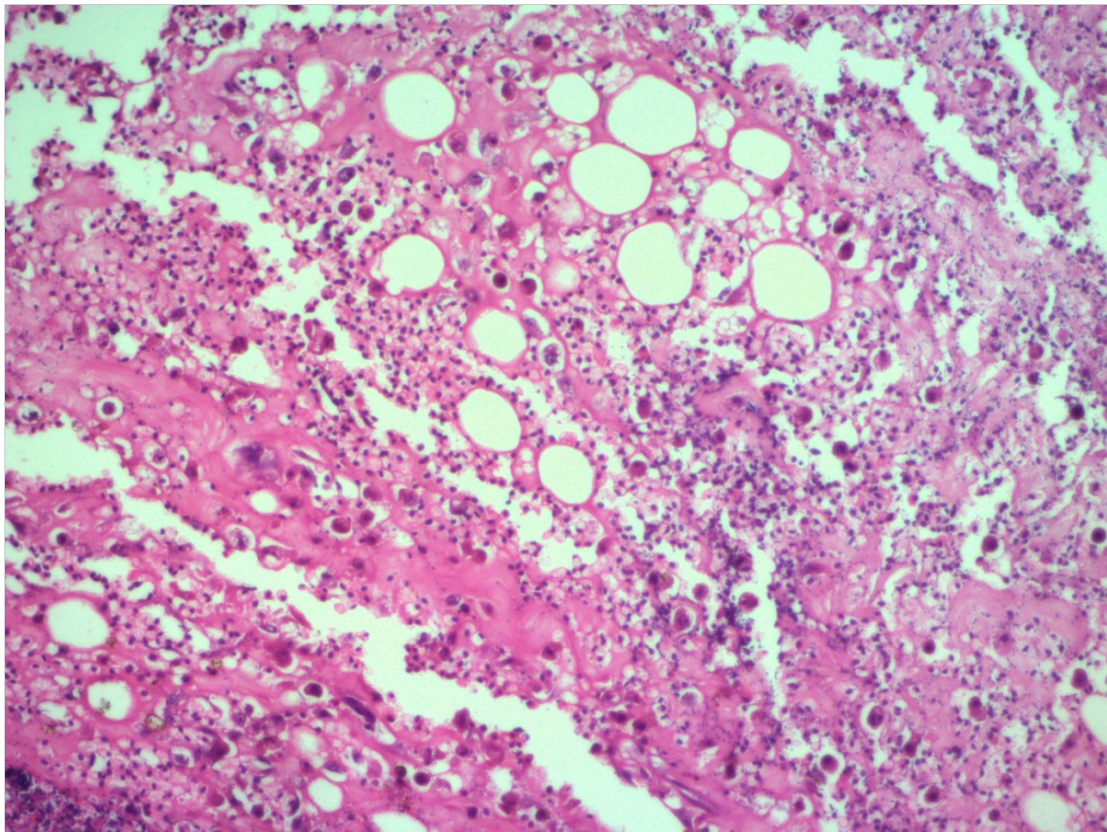


Figure 8. Marked accumulation of heterophils and mononuclear cells in the subcutaneous fatty tissue. H&E

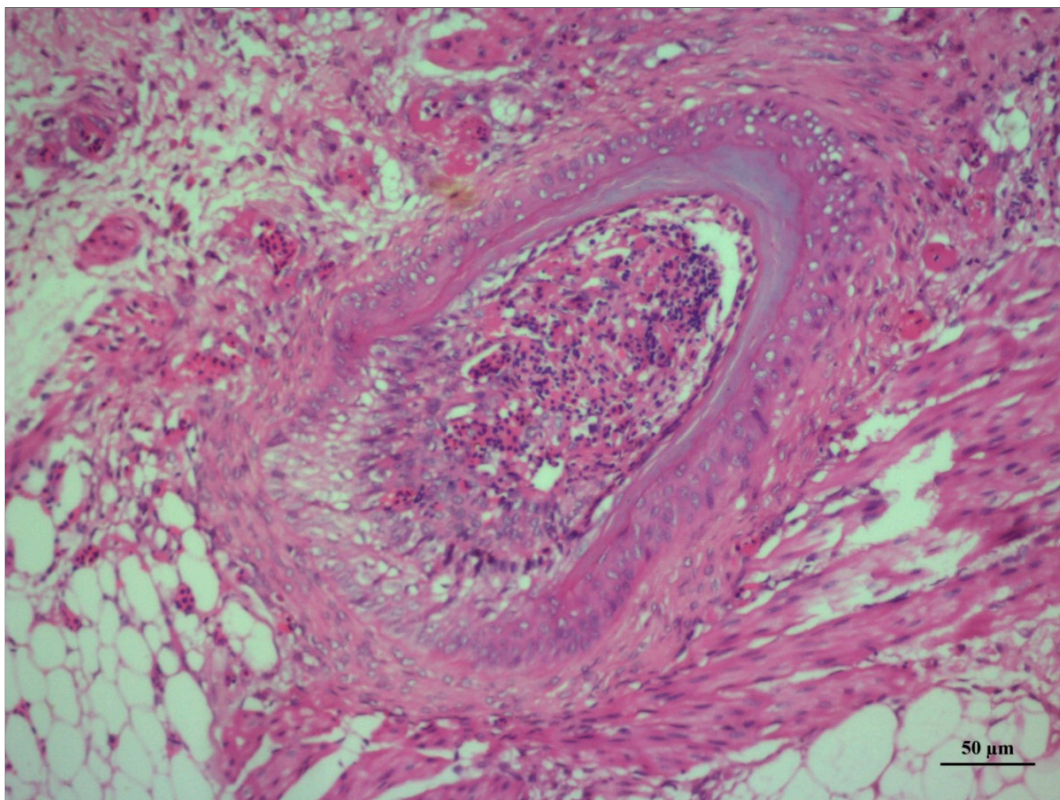


Figure 9. Vasculitis overcommed with inflammatory cells and also damaged peripheral architecture of muscle. H&E

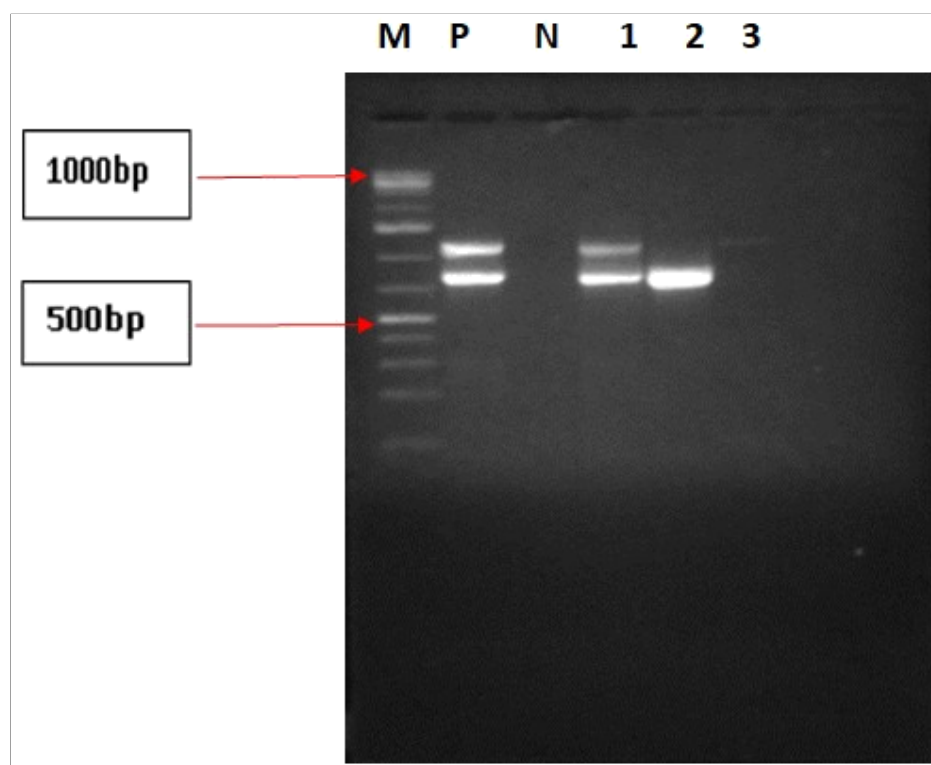


Figure 10. PCR amplification of *cvi* and *tsh* genes of avian pathogen *E. coli*. M) 100bp DNA Ladder marker (Invitrogen®); B) Positive control of *cvi* (1181 bp) and *tsh* (824 bp) gene; N) Negative control; 1) Field sample positive for both *cvi* and *tsh*; 2) Field sample for *tsh* gene; 3) Field sample for *cvi* gene.

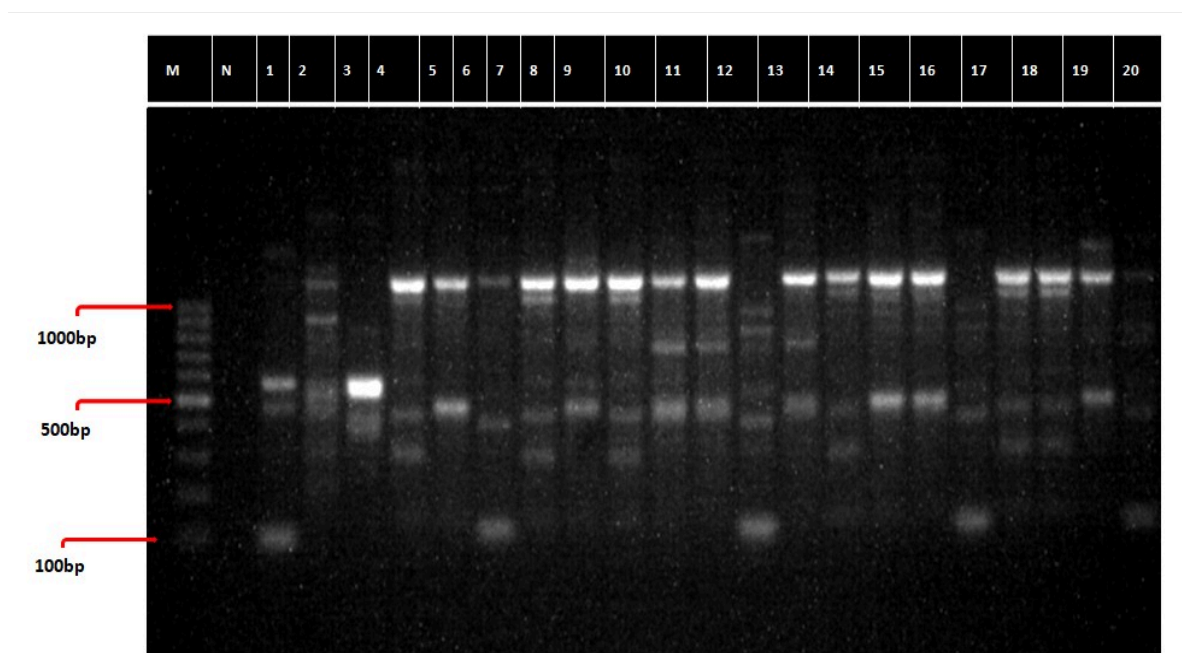


Figure 11. The ERIC-PCR generates distinct amplification products that can be differentiated by agarose gel electrophoresis. 28 isolates Amplicons ranged between 100 > 3,000 bp, with 1-7 bands produced, M, 100 bp DNA ladder marker; 1-20, ERIC-PCR amplification results; N, negative controls.

Discussion

Owing to the importance of cellulitis in broiler industry various attempts have been made worldwide to know the prevalence of cellulitis in broiler chickens (Gomis *et al.*, 2000; Xavier *et al.*, 2010; Bianco *et al.*, 2016) [3, 12, 2]. The reports of avian cellulitis from Indian sub-continent is missing so the study was undertaken to know causes of cellulitis in broiler chickens, for the first time across Nagpur region in Central, India. As reported worldwide, we also report the cases of cellulitis in broilers from central India. The cellulitis associated lesions were present on inner aspect of thigh muscle, wings, breast, keel bone area, back and flank region. The development of this infection is asso-

ciated with skin surface injuries, often caused by close contact among birds and litter quality (Xavier *et al.*, 2010, Mellata 2013) [12]. We report that cellulitis is often accompanied by septicaemia-toxaemia i.e., pericarditis, airsacculitis, perihepatitis, polyserositis. These lesions leads to the slowed processing lines, carcass down grading and total financial losses due to condemnation of carcasses (Norton *et al.*, 2000) [4]. Microscopically severe hyperkeratosis and thickening of the dermis along with evidence of infiltration of mononuclear cells and heterophils as observed occur due to weak innate immune response of the broilers (Olkowski *et al.*, 2005, Mellata 2013) [13]. The lesions further worsen to necrotic cellular debris and fibrinocaseous exudates due to growth of bacteria (Bianco *et al.*, 2016) [2].

The main cause for broiler chicken cellulitis in Nagpur region seems to be *E. coli* which was confirmed by observing metallic sheen on EMB agar and MALDI-TOF result. In 26.92 percent cases it was also complicated with other bacterial pathogens. Earlier Norton *et al.*, (2000) [4], Fallavena *et al.*, (2000) [14], Johnson *et al.*, (2001) [15] also claimed that *E. coli* is the major pathogen which causes avian cellulitis. The prevalence of cellulitis with other systemic manifestations of

E. coli in broilers has been reported earlier by Gomis *et al.*, (2000) [3]. All the *E. coli* isolates harbors both or either of pathogenic *cvi* and *tsh* genes. The role of *cvi* and *tsh* genes in the virulence profiling of Avian Pathogenic *E. coli* has been initiated by Ewers *et al.*, (2004) later on confirmed by Kemmett *et al.*, (2014) [16]. These genes are usually absent in the non chicken source of *E. coli*.

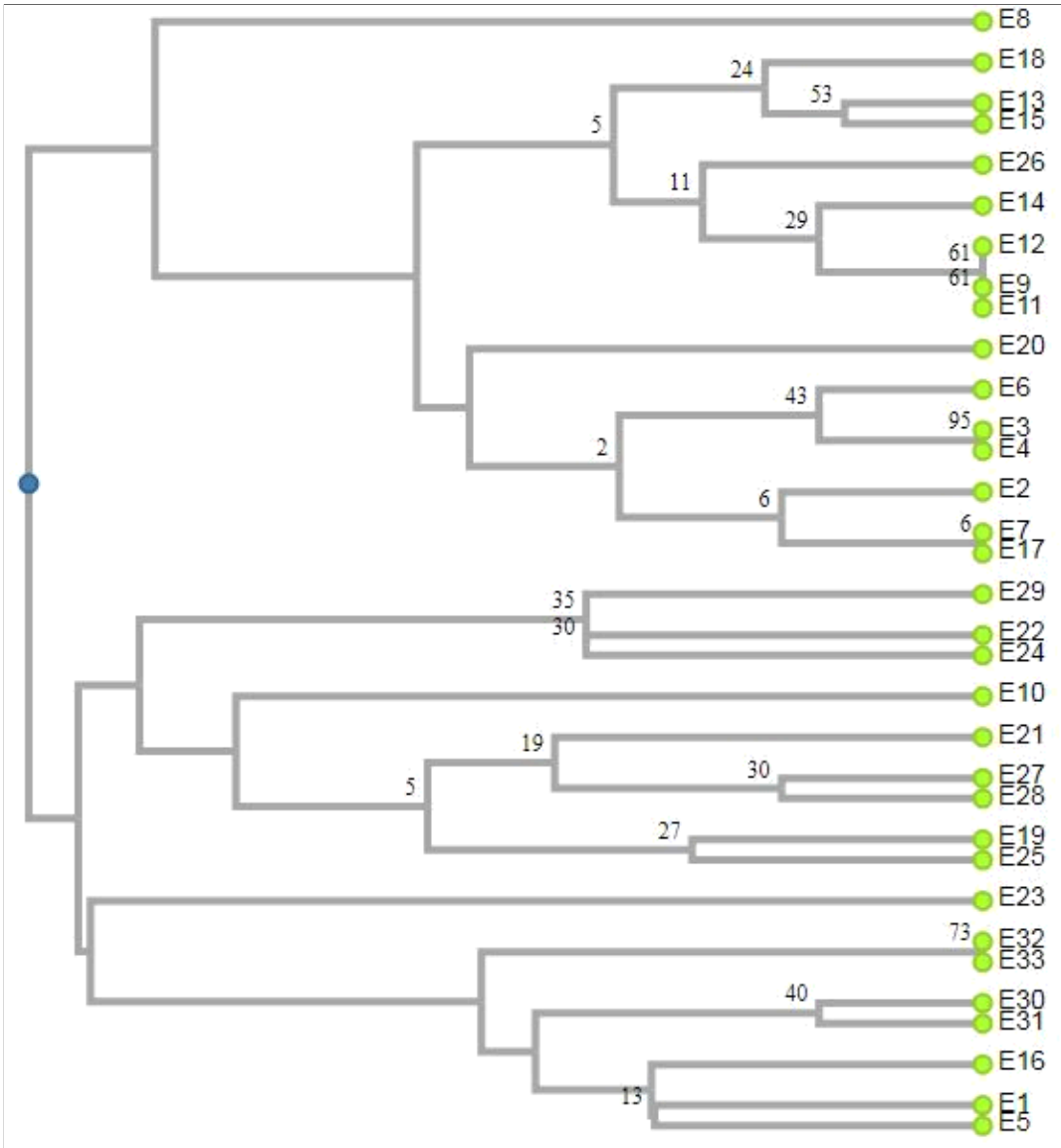


Figure 12. Dendrogram of *E. coli* fingerprint by cluster analysis. Identification Profiles, Bands and Clusters in Association with *Escherichia coli* Strains

The ERIC-PCR method is recently being used as molecular epidemiological tool and analyses different bacteria (Ahmed *et al.*, 2012) [17]. This technique is used mostly to know the source of the isolate and to know the genetic homology of the isolates. We used the ERIC-PCR typing technique to analyze the source of pathogenic APEC in all isolates along with *E. coli* isolated from dogs, cow, and buffalo. The pat-

tern of bands observed by ERIC PCR was different in chicken isolates as compared to *E. coli* from other animal species. The present results indicates that APEC present in the chicken environment or one infected chicken is source of infection to the broilers and exhibit cellulitis and responsible for economic losses.

Methodology	Unrepeated profiles	Repeated profiles	Bands (Minimum)	Bands (Maximum)	Diversity of bands	No. of cluster
ERIC -PCR	22	4	3	7	24	6

* DendroUPGMA software was used for generating dendrogram by using ERIC-PCR data.

(continued on next page)

Table 1. Antibiotic sensitivity results

Antibiotics	Resistance (%)	Intermediate (%)	Sensitive (%)
Neomycin (N) 30 mcg	61.53%	23.07%	15.38%
Ciprofloxacin (CIP) 5 mcg	100%	0	0
Doxycycline (DO) 30 mcg	73.07%	15.38%	11.53%
Chlortetracycline (CT) 30mcg	69.23%	23.07%	7.69%
Furazolidone (FR) 100mcg	100%	0	0
Levofloxacin (LE) 5mcg	100%	0	0
Cephalexin (CN) 30mcg	65.38%	15.38%	19.23%
Oxytetracycline (O)30mcg	100%	0	0
Basitracin (B) 10units	100%	0	0
Enrofloxacin (EX) 10mcg	100%	0	0
Colistin sulphate (CL) 10mcg	15.38%	7.69%	76.92%
Chloramphenicol ©30mcg	69.23%	15.38%	15.38%

In this present study we report the cases of avian cellulitis from central India, majorly caused by *E. coli*. These isolates were sensitive towards Colistin sulphate and resistant towards Enrofloxacin, Ciprofloxacin, Furazolidone, Levofloxacin, Oxytetracycline and Bacitracin. These *E. coli* harbors pathogenic genes *cvl* and *tsh*. These isolates has distinct different pattern of ERIC PCR and can be well differentiated from *E. coli* of other species.

Acknowledgements

Authors are thankful to Associate Dean, Nagpur Veterinary College, MAFSU, Nagpur, Maharashtra for providing necessary help and facilities for the successful completion of this work.

Statement of animal rights

The manuscript does not contain clinical studies or patient data. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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How to cite this article: Thorat S, Sonkusale P, Kurkure N. Etiopathology of cellulitis in broilers from Nagpur region of central India. *International Journal of Advanced Biochemistry Research*. 2026;10(9):590-599. doi: <https://www.doi.org/10.33545/26174693.2026.v10.i9h.10129>