International Journal of Bio-resource and Stress Management https://ojs.pphouse.org/index.php/IJBSM

Crossref

IJBSM October 2025, 16(10): 01-10 **Article** AR6368

Research Article *Natural Resource Management*

DOI: HTTPS://DOI.ORG/10.23910/1.2025.6368

Haemolysis, Biofilm and Virulence Determinants of *Escherichia coli* Isolated from Bovine Calf Diarrhea

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ABSTRACT

The research was conducted during July, 2021 to April, 2022 in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Mhow (M.P.), India, with the objective of isolating and characterizing *Escherichia coli* strains from diarrheic calves using both phenotypic and molecular techniques. A total of 250 diarrheic fecal samples were obtained from calves exhibiting clinical symptoms such as dehydration, watery feces, and poor appetite. These samples were then immediately transported to the laboratory under aseptic and cold conditions for the isolation and further identification of *E. coli*. Nutrient agar, EMB agar and Blood agar were used for the isolation and characterization. From the processed samples, 124 *E. coli* isolates were obtained, indicating an overall incidence rate of 49.6%. Out of the 124 isolates, 15 (12.09%) showed haemolysis on blood agar, whereas 109 (87.9%) were non-hemolytic. Biofilm formation was demonstrated in 60 isolates (48.38%) by the tube method and in 63 isolates (50.80%) using the Modified Congo Red Agar method. Among the 124 isolates, 90 (72.58%) tested positive for invasiveness through Congo red dye binding assay (CR+). In serotyping, multiple O-types including O126, O17, O2, O7, O111, O101, O57, O135, O120, O63, O88, O121, O26, O11, O157, and O18 were identified, while six isolates were untypable for the "O" antigen. Singleplex PCR detected the *fimH* gene in 70% and the *ompT* gene in 100% of the isolates.

KEYWORDS: Calve diarrhea, *E. coli*, haemolysis, biofilm, virulence

***Citation* (VANCOUVER):** Sikrodia et al., Haemolysis, Biofilm and Virulence Determinants of *Escherichia coli* Isolated from Bovine Calf Diarrhea. *International Journal of Bio-resource and Stress Management,* 2025; 16(10), 01-10. HTTPS://DOI.ORG/10.23910/1.2025.6368.

Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

Conflict of interests: The authors have declared that no conflict of interest exists.

RECEIVED on 16th May 2025 RECEIVED in revised form on 19th August 2025 ACCEPTED in final form on 04th September 2025 PUBLISHED on 10th September 2025

1. INTRODUCTION

Millions of rural households' socioeconomic development is greatly aided by livestock, which is a crucial component of every nation's agricultural production system (Radostits et al., 2000). In many developing countries, especially India, livestock farming is not just a traditional occupation but also a critical livelihood strategy that supports income generation, food security, and nutritional needs. According to estimates, nearly one billion poor people worldwide depend on livestock for their livelihoods (Salmon et al., 2020). However, low productivity and poor animal health limit the advantages of owning livestock, such as income and nutrition for the household. On dairy farms, the management of calving, colostrum, feeding, housing, and timely treatment are all considered to be extremely important for the calf (Mormede et al., 1982). According to Blood and Radostits (1989), it is estimated that a calf mortality rate of 12% can bring the negative impact on net profit down to 38%.

Among the various health issues affecting calves, neonatal calf diarrhea (NCD) remains a predominant and alarming concern in both commercial and traditional dairy setups. The prevalence of diarrheal diseases in calves results in considerable morbidity and mortality, particularly in developing regions such as India, where access to timely and effective veterinary care may be limited (Malik et al., 2012). One of the most frequently reported illnesses in calves under three months old is calf diarrhea, as stated by Svensson et al., 2003. Calves are most susceptible to developing diarrhea during their first month of life, and the occurrence of diarrhea decreases as they get older, as highlighted by Meganck et al., 2014. Diarrhea in calves may result from various bacterial infections, with *Escherichia coli* (*E. coli*) being the most prevalent among them. *E. coli* is often utilized for the surveillance of AMR in cattle and animal-based food products due to its widespread presence and preference for the digestive systems of warm-blooded animals (Faridah et al., 2020, Ramos et al., 2020). While some strains of *E. coli* are harmless and symbiotic, others possess a range of virulence factors that enable them to invade host cells, evade immune responses, and establish infections (Ramos et al., 2020; Brunauer et al., 2021). Clinically, *E. coli*-induced diarrhea is characterized by light brown, foul-smelling feces, which may progress to watery, grey, sometimes bloody stools, accompanied by abdominal discomfort, dehydration, and, in severe cases, acute toxemia that can lead to death or long-term growth retardation in surviving calves (Foster and Smith, 2009).

Some *E. coli* pathotype strains, such as Enterotoxigenic *E. coli* (ETEC), Shiga Toxigenic *E. coli* (STEC), Enteropatho genic *E. coli* (EPEC), and Enterohemorrhagic *E. coli* (EHEC) cause NCD (Dubreuil et al., 2016, Pervez et al., 2018). All bacteria possess certain virulence factors to develop a disease. Virulence factors are the molecules that help the bacteria colonize the host at the cellular level. Virulence factors can be surface-bound, secreted, or intracellular, and play a crucial role in the pathogenesis of disease (Sharma and Dashmana, 2017). Understanding the presence and expression of these virulence traits is essential for designing effective preventive strategies, such as vaccines, and for predicting disease severity (Lee, 2023; Ayenew, 2021, Ariyanti et al., 2022). Since vaccines for all *E. coli* pathotypes are not yet available, the detection of virulence factors becomes even more critical. Hence, the present study was aimed at the isolation and characterization of *E. coli* strains from diarrheic calves, with a focus on identifying their virulence determinants using both phenotypic and molecular techniques.

2. MATERIALS AND METHODS

The research was conducted during July, 2021 to April, 2022 Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Mhow, Madhya Pradesh, India. Neonatal bovine calves of different ages (1–120 days) examined clinically for diarrhea on dairy farms in and around Mhow. This region was home to a large number of organized and unorganized dairy farms that provided the town and its neighbours' residents with milk and milk-related products.

2.1. Study population

Animals included in this investigation were bovine (Cattle and buffalo) calves under 4 months of age with clinical diarrhoea, showing symptoms like poor appetite, fever, dehydration and pasty-watery feces. A total of 250 diarrhea samples were obtained from calves exhibiting clinical symptoms of diarrhea. Swabs of the rectum were taken directly from diarrhoeic calves and stored in sterile test tubes and then immediately transported to the laboratory for processing.

2.2. *Isolation and identification of E. coli*

The sample was inoculated in BHI broth and incubated aerobically at 37°C for 24 hours, followed by streaking on Nutrient agar, MacConkey agar plate and EMB agar and incubated aerobically at 37°C for 24 hrs to obtain isolated single bacterial colonies. The gross morphological characteristics of the colonies were observed. The *E. coli* was confirmed on the basis of cultural and biochemical characters (Barrow and Feltham, 1993). The bacterial colony produced characteristic purple-black colonies with dark centre with metallic sheen on EMB agar. Single colony was then picked up and inoculated on Nutrient agar slant for the further use.

2.3. DNA extraction

The isolates were inoculated in Brain heart infusion broth (Hi Media, Mumbai) and incubating at 37°C for 12–18 hrs. Extraction of DNA was done using readymade kit (GenElute™ Bacterial Genomic DNA Kit, Sigma) as per the manufacturer's instructions. The obtained DNA was maintained at -20°C for future utilization.

2.4. Molecular identification of E. coli isolates using Polymerase chain reaction (PCR)

E. coli isolates identified phenotypically as above were confirmed genotypically by PCR using primers of *16 S rRNA* gene (Table 1). The process of DNA amplification was carried out in a total reaction volume of 25 µl, which comprised 2 µL of DNA template, 12.5 µl of Master mix (SIGMA Life Science), 1 µl each of forward and reverse primers for 16S rRNA (20 pmol μl^{-1}), and 8.5 µl of PCR-grade nuclease-free water (SIGMA Life Science). The temperature and time are indicated in the table 2.

2.5. Serotyping

All the *E. coli* isolates were sent to Central Research Institute, Kasauli (H.P.) for serotyping of somatic (O) antigen.

Table 1: Details of primers of *E. coli* used for PCR reaction

Target gene	Primer sequence	Product size (bp)	Reference
16S rRNA	F: GACCTCGGTTTAGTTCACAGA R: CACACGCTGACGCTGACCA	585	Sobur et al., 2019
fimH	Forward 5'- TGCAGAACGGATAAGCCGTGG Reverse 5'- GCAGTCACCTGCCCTCCGGTA	508	Fernandes et al., 2011
ompT	F:TCATCCCGGAAGCCTCCCTCACTACTAT R:TAGCGTTTGCTGCACTGGCTTCTGATAC	496	Subedi et al., 2018

Table 2: Thermocyclic conditions for *16sRNA*, *fimH* and *ompT* gene of *E. coli*

Sl. No.	Steps		*16sRNA*	*fimH*	*ompT*	No. of cycles
1.	Initial denaturation	Temp.	95°C	94°C	94°C	1
		Time	5 min	5 min	7 min	
2.	Denaturation	Temp.	94°C	94°C	94°C	35
		Time	30 sec	45 sec	30 sec	
3.	Annealing	Temp.	58°C	60°C	51°C	
		Time	30 sec	45 sec.	30 sec	
4.	Extension	Temp.	72°C	72°C	72°C	
		Time	2 min	1 min	30 sec	
5	Final extension	Temp.	72°C	72°C	72°C	1
		Time	7 min	7 min	7 min	

2.6. Haemolysis

Haemolytic activity was demonstrated on blood agar plate (Ready prepare, Hi-media) as per the method of Shiva Shankar et al. (2010). The plates were then examined for "greening" or clearing of the agar around areas of bacterial growth as an indication of alpha or beta hemolytic activity (Rodriguez-Siek et al., 2005).

2.7. Biofilm formation

A qualitative assessment of biofilm formation by tube and modified congo red agar methods was determined as per method described by Dadawala et al. (2010) and Nachammai et al. (2016).

2.8. Tube method

The culture was inoculated into 5 ml of Tryptone soya broth (TSB) and incubated for a duration of 12 hours at a temperature of 37°C. Following this incubation period, 50% of the spent media from each tube was carefully aspirated and substituted with an equal volume of fresh TSB that contained 0.25% sucrose. The tubes were re-incubated at 37°C for 12 hrs. Subsequently, broth was decanted and tubes were gently washed twice with PBS, kept inverted for drying, and stained with 0.1% safranin solution. Excess stain was removed and tube was gently washed with de-ionized water. The tubes were subsequently dried in an inverted

orientation and monitored for the presence of biofilm. Biofilm formation was deemed positive when a discernible film coated the inner wall and base of the tube. The presence of a ring at the liquid interface was not regarded as a sign of biofilm formation. The level of biofilm formation was assessed as-

a. 0- Non weak
b. ++ moderate
c. +++ Strong

2.9. Modified congo red agar method

A concentrated aqueous solution of Congo red stain was prepared and subsequently autoclaved at 121˚C for a duration of 15 min. Glucose underwent sterilization through filtration. Both components were then incorporated into the autoclaved blood agar base at a temperature of 55˚C. The plates were inoculated with the test organism and incubated aerobically at 37˚C for a period of 24 to 48 hrs. Black colonies with a dry crystalline consistency indicated biofilm production, non-biofilm producers usually remained pink (Nachammai et al., 2016).

2.10. Congo red dye binding assay

The test was performed to determine the congo red binding ability of the *E. coli* isolates as an indicative of invasiveness Yadav et al. (2014). The isolates were streaked on tryptone soya agar media containing 0.03% Congo red dye, and incubated for 24–72 hrs at both 37˚C and 25˚C. The presence of bright orange or red colonies was documented as a positive response. Conversely, negative colonies failed to take up the dye, resulting in a white or grey appearance.

2.11. Molecular detection of virulence genes for E. coli

The PCR was standardized for the detection of virulence genes viz. *fimH* and *ompT* using published primers (Table 1). It was carried out in final reaction volume of 25 µl using 0.2 ml thin wall PCR tube. Amplification was performed in a total reaction volume of 25 µl, which comprised 2 µl of DNA template, 12.5 µl of Master mix (SIGMA Life Science), and 1 µl each of forward and reverse primers for 16S rRNA, at a concentration of 20 pmol μl^{-1}, and 8.5 µl PCR-grade nuclease free water (SIGMA life science). The DNA amplification reaction was performed in the Thermocycler (Applied Biosystem) with a pre-heated lid. The cycling conditions for PCR included the steps shown in Table 2. The amplified products were separated by electrophoresis through 1.5% agarose (wt vol^{-1}), stained with 0.5 µg ml^{-1} ethidium bromide, visualized under UV illumination, imaged with a GelDoc 1000 fluorescent imaging system (UviTech).

3. RESULTS AND DISCUSSION

Calf diarrhea represented a significant health challenge within dairy farming, resulting in elevated rates of morbidity and mortality, which obstructed the sustainable advancement of the dairy industry. This condition incured considerable economic losses related to labor and capital, alongside calf mortality and veterinary expenses (Pereira et al., 2011; de Verdier et al., 2012).

The bacterial analysis resulted in the identification of 124 *E. coli* isolates from a total of 250 diarrheic samples, with the incidence of 49.6% (Figure 1 and 2). This identification process utilized biochemical and molecular assays, with PCR specifically targeting the 16S rRNA gene (Plate 1). The use

Figure 1: White colonies of *E. coli* on nutrient agar

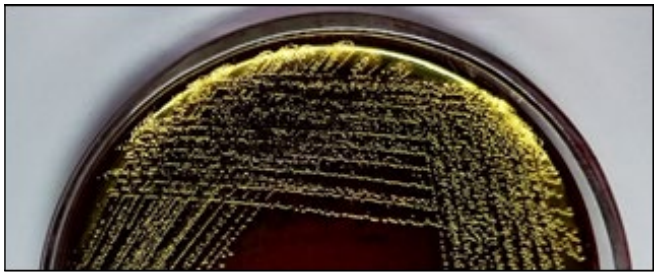

Figure 2: Colonies of *E. coli* on EMB agar

Plate 1: Results of PCR showing 16 S rRNA gene of *E. coli* Lane 1: 100 bp ladder; Lane 2: Positive control; Lane 3-5: Positive samples (Size 585 bp)

of 16S rRNA for the genotypic confirmation of *E. coli* had also been documented in studies by Gamal et al., 2019; and by Mousa and Abo Shama, 2021.

Overall, *E. coli* was found to be a significant contributor to calf diarrhea, acting either independently or alongside other bacterial agents. Our investigation showed that *E. coli* was isolated most frequently from calves suffering from diarrhea. This was in agreement with the observations of several researchers who have noted the extensive distribution of *E. coli* strains in cases of infectious calf diarrhea (Arya et al., 2008; Shahrani et al., 2014). The elevated occurrence of calf diarrhea attributed to *E. coli* in this research might be a result of substandard management conditions on the farms. Key issues included inadequate or poor-quality colostrum consumption by the calves, limited awareness among farmers, and predisposing factors such as overcrowding and malnutrition, which were believed to significantly contribute to immunosuppression. Contrast to the present finding (49.6%), lower percent of the *E. coli* was recorded viz. 21.33%, and 28.8% by Abdeen et al. (2019) and Algammal et al. (2020), which was against our findings.

Bacterial infection capability was augmented by a wide range of virulence factors that were encoded by virulence-associated genes (VAG). These factors, including adhesins, invasins, toxins, haemolysins, iron acquisition systems (siderophores), and protectins, were integral to the processes of colonization, adhesion, invasion, and resistance to host immune responses (Janben et al., 2001; Jeong et al., 2012). As a result, they may contribute to the distinct pathogenic profiles exhibited by various bacterial types.

Haemolytic activity had the potential to act as a phenotypic marker for assessing the virulence factors of *E. coli.* All the 124 isolates of *E. coli* were tested for qualitative haemolysis. Out of the 124 isolates, only 15 (12.09%) isolates were showing haemolysis, while 109 (87.9%) were nonhemolytic (Figure 3 and 4). These results were contradictory to findings of Fakruddin et al. (2013), Mailk et al., 2013 and AL-Saiedi and Al-Mayah (2014), who reported high percentage of *E. coli* to produce haemolysin in 37.03, 100, 44.6 and 53.3% isolates, respectively. The production of different types of haemolysins has been frequently contributed to *E. coli* from intestinal and extraintestinal diseases. It triggered the release of Ferro from cells, supplying the necessary iron for bacterial growth (Salehi et al., 2007).

Escherichia coli was identified as one of the most prevalent bacteria capable of forming biofilms across diverse environments. The capacity to form biofilms might enhance the persistence and survival of *E. coli* in both environmental contexts and within host organisms. Additionally, they provided protection against the host's immune responses. A total of 124 *E. coli* isolates were evaluated for their biofilm-

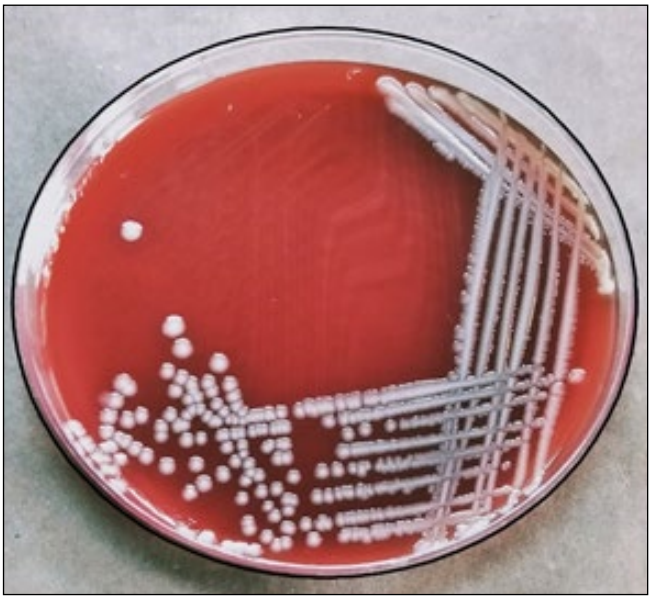

Figure 3: Non-haemolytic colonies of *E. coli* on blood agar

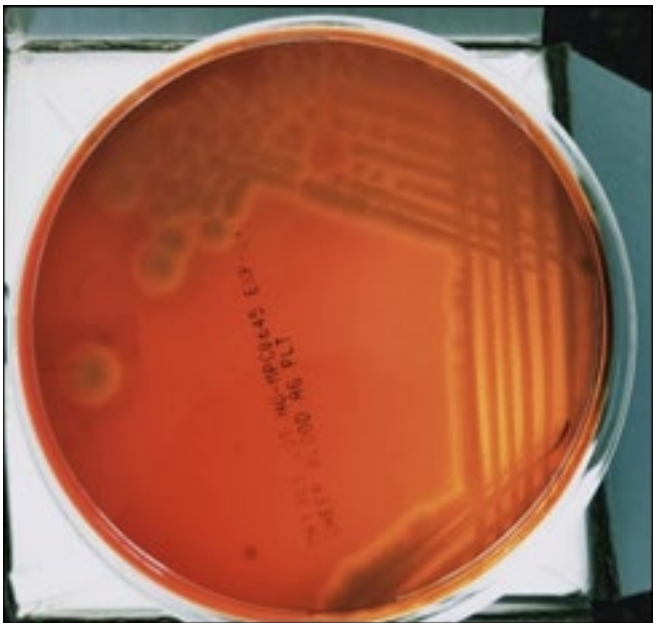

Figure 4: Haemolytic colonies of *E. coli* on Blood agar

forming capabilities using the tube method and modified congo red agar media. Out of these, 60 isolates, representing 48.38%, demonstrated positive results for biofilm formation via the tube method (Figure 5). Additionally, 63 (50.80%) isolates of the total, were found to be positive for biofilm formation when assessed using the modified congo red agar method (Figure 6). In our investigation, the percentage of biofilm formation in *E. coli*, assessed through two different methodologies, yielded results that were remarkably similar. Our findings align closely with those of Skyberg et al. (2007) and Rodrigues et al. (2019), who documented biofilm formation rates of 55.8% using the Congo red method in *E. coli* isolates. Conversely, Singh (2018) reported a

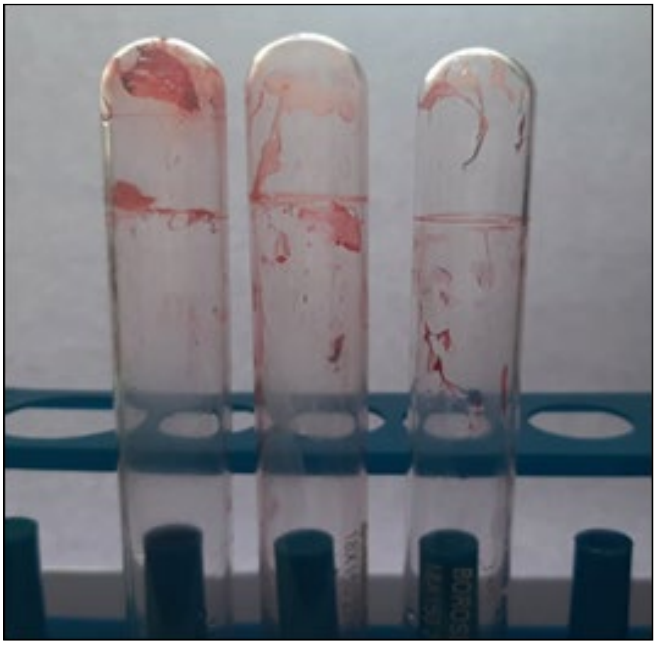

Figure 5: Tubes showing result of biofilm formation

Figure 6: Modified CRA plate showing black colonies

significantly higher percentage of isolates exhibiting biofilm production via the Congo red agar (CRA) method (70.37%) compared to the tube method (33.33%). In contrast to our results, lower percentages of isolates positive for biofilm production using both CRA and tube methods were noted by Dhanawade et al. (2010), and Vijay et al. (2015). The discrepancies in the reported percentages might arise from various factors associated with the establishment of a model biofilm system. These factors included the composition of the media, temperature, the presence of antimicrobial agents, the specific organism involved, the amount of inoculum, hydrodynamic forces, and the properties of the substratum (Donlan and Costerton, 2002).

Congo red dye binding assay (CRBA) was employed to differentiate between invasive and non-invasive *E. coli* isolates. Among the 124 *E. coli* isolates examined, 90 (72.58%) tested positive for the Congo red binding dye (CR+) and exhibited brick red colonies (Figure 7). Previous studies by Yadav et al. (2014), and Eid et al. (2016) reported higher percentages of Congo red dye binding positive *E. coli*, with findings of 92.86%, and 91.7%, respectively. In contrast, lower percentages were noted in another research, such as 60% by AL-Saiedi and Al-Mayah (2014), and 60.4% by Zahid et al. (2016). Moawad et al, 2008 also used the Congo red dye binding assay for the study of *E. coli.*

Figure 7: Congo red agar showing brick red colonies

In serotyping a total of fourteen isolates of *E. coli* were identified as O126, representing the highest numbers among the isolates. In contrast, there was one isolate each for O17, O2, O7 and O111, indicating a lower prevalence. Additionally, the serotypes O101, O57, O135, O7, O120, O63, O88, O121, O26, O11 and O18 were also detected in the diarrhea cases. Furthermore, two isolates were classified as O157. A total of six isolates were not able to be identified with respect to the "O" antigen. Mona et al., 2020 reported O111 and O26 as most prevalent serotype in their finding which is contrast to our finding. Nguyen et al., 2011 reported O15, O20, O103 and O157 serotypes in the sample from the calve diarrhea. Coskun and Sahin, 2023 reported most common serotype O101 and O9 in their finding and *27 E. coli* strain were not typed. Moawad et al., 2008 reported O111, O115, O165, O25 and O1 as most prevalent serotype

Single plex PCR was employed to amplify the virulence genes *fimH and ompT.* The amplification resulted in the detection of a 508 bp amplicon for the *fimH* gene in 70% of the *E. coli* isolates, and a 496 bp amplicon for the *ompT* gene was observed in all (100%) isolates (Plate 2 and 3). Similarly, El-Seedy et al. (2016) identified the *fimH* gene in over 90% of isolates, while El Ashmawy et al. (2016) and Mousa et al. (2021) reported that all isolates tested were positive for the *fimH* gene, indicating a higher prevalence than observed in the current study.

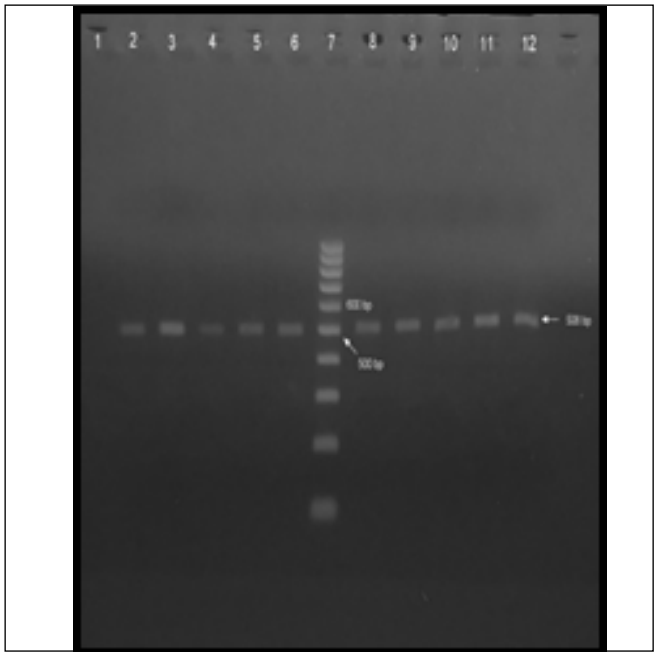

Plate 2: Results of PCR showing fimH gene of *E. coli*; Lane 7: 100 bp ladder; Lane 2: Positive control; Lane 1: Negative control; Lane 3-6 and 8-12: Positive samples (Size 508 bp)

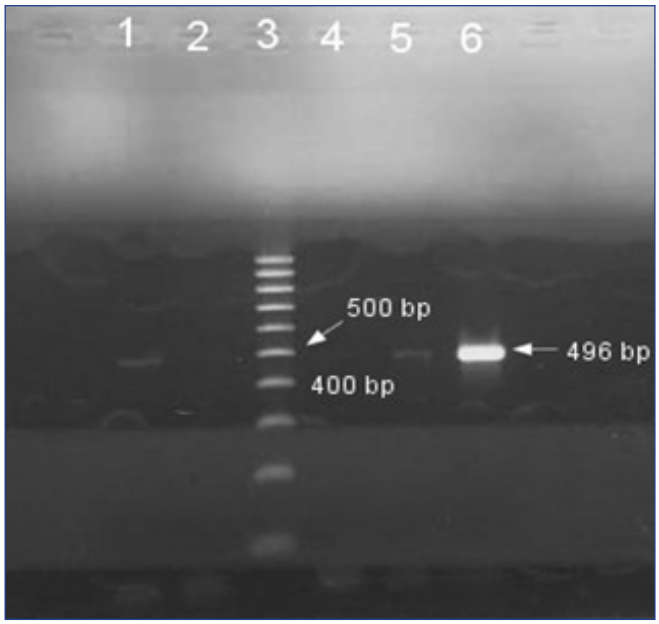

Plate 3: Results of PCR showing ompT gene of *E. coli*; Lane 3: 100 bp ladder;Lane 6: Positive control; Lane 1 and 5: Positive samples (Size 496 bp); Lane 2: Negative control; Lane 4: Negative sample

The initiation of host tissue colonization was believed to be facilitated by fimbrial adhesins. Type 1 fimbriae were encoded by a fim cluster gene that comprises nine related genes (A, B, C, D, E, F, G, H and I), which are essential for their biosynthesis (Bryan et al., 2006; Pusz et al., 2014). The *fimH* gene was believed to contribute to the defense of *E. coli* against host heterophils, as indicated by Mellata et al. (2003). Furthermore, the *fimH* gene was a significant virulence factor linked to the expression of curli fimbriae and cellulose, influencing biofilm formation (Dubravka et al., 2015).

4. CONCLUSION

Escherichia coli as a major pathogen associated with neonatal calf diarrhea. Phenotypic assays revealed significant haemolytic activity, biofilm formation, and invasiveness among the isolates. Molecular analysis showed a high prevalence of key virulence genes, including *fimH* and *ompT*. Serotyping highlighted considerable antigenic diversity, with O126 being the most common. The combined use of phenotypic and genotypic methods strengthened the characterization of virulent strains. The pathogenic potential of *E. coli* was contingent upon the synergistic action of various virulence factors. Therefore, enhancing management practices, ensuring timely colostrum administration, and maintaining proper hygiene could increase the likelihood of calf survival.

5. ACKNOWLEDGEMENT

The authors are thankful to College of Veterinary Science and A.H., Mhow and NDVSU University officials for providing necessary facilities for research.

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