10.21608/AVMJ.2025.402873.1796 Assiut University web-site: www.aun.edu.eg

ANTIMICROBIAL RESISTANCE,SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF ENTEROPATHOGENIC *E. COLI* K99 ISOLATED FROM NEWBORN CALVES SCOURS

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Received: 10 August 2025; **Accepted:** 18 Sebtember 2025

ABSTRACT
This study highlights the significance of *E. coli* infection, the most prevalent bacterial disease causing diarrhoea in calves, the rise of multidrug-resistant strains, and the threat it poses to the bovine industry, particularly in developing countries. This study aimed to isolate and identify *E. coli* with their antibiogram and virulence gene detection. A total of 50 faecal samples were collected from diarrheic calves at Assiut Governorate. A total of 33 *E. coli* (66%) was isolated. Eight different antibiotic groups were tested. All the *E. coli* isolates (100%) were multidrug-resistant, such as ampicillin, amoxicillin, amoxicillin-clavulanic, cefotaxime, and rifampin. All *E. coli* isolated were 100% sensitive to norfloxacin and trimethoprim-sulfamethoxazole. Biofilm formation was confirmed in 30 isolates (90.9% of total pathogens, 60.0% of total samples) using the Congo red agar method. Serological identification of *E. coli* revealed the presence of O128:H21 (K99) (6) strains, O101 (K99) (4), O128:H2 (4), and O26:H11 (3), predominantly belonging to enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enterohemorrhagic *E. coli* (EHEC). Serologically, K99 was exhibited in 13 isolates out of 30 (43.3%). Five of the 11 isolates positive for the K99 gene were positive for *stx2*, *hlyA, blaTEM, ampC,* and *fimH*, while three isolates were positive for *stx1, eaeA,* and *blaCTX-M*. The results demonstrated that genotypic detection of the biofilm virulence gene (*fimH*) was consistent with phenotypic detection of biofilm formation in multidrug-resistant isolates. In conclusion, these findings suggest that multidrug-resistant and virulent strains of *E. Coli* K99 play a role in calf scours and continuously emerge, posing serious threats to human and animal health. Antimicrobial susceptibility testing should be done regularly on drugs that are commonly used.

Keywords: Antimicrobial Resistance, *E.coli* k99, virulence genes, calves' scours

INTRODUCTION
Diarrhoea is a major cause of morbidity and mortality, and it costs a lot of money to treat. It also makes calves more susceptible to other illnesses, slows their growth, and causes their deaths. The two primary causes of calf diarrhea are classified as infectious and non-infectious. Around the world, the leading cause of illness and death among newborn dairy calves is infectious diarrhea. Group A bovine rotavirus, coronavirus, and bovine viral diarrhea virus are the major viral infectious agents (Selles *et al.,* 2018).

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Colibacillosis is the most serious and dangerous disease that affects newborn animals, caused by several infectious agents (Lee *et al.,* 2023). *Escherichia coli* is the main culprit. It causes enteritis in calves, which is a significant health problem (Tutija *et al.,* 2022) in the livestock industry worldwide; also, it is one of the leading causes of illness and death in calves. Additionally, it reduces the development of the animal and increases the costs associated with treatment and control (Algammal *et al.,* 2020).

Bacterial pathogens are still responsible for more than half of all cases of diarrhea in newborn calves. For example, *E. coli* was found in the intestinal contents of calves during their first three weeks of life (Subhash Malik *et al.,* 2012). *E. coli* is particularly significant due to its role in causing severe watery diarrhea within the first four days of a newborn calf's life, potentially leading to death within 24 hours (Cho *et al.,* 2010). Furthermore, bacterial agents in calves less than two months old have been identified as *Salmonella* spp., *E. coli K99*, and *Clostridium* species (Acha *et al.,* 2004; Smith, 2009).

The primary cause of calf mortality in Egypt is neonatal calf diarrhea (NCD) (Younis *et al.,* 2009). The condition can result from various infectious and non-infectious factors. The calves' age has positively influenced susceptibility to infections. During the first fourteen days of age, calves are susceptible to bovine rotavirus(BRV) infections; from the 5th to the 20th days of age, to bovine coronavirus (BcoV); and during the first four days of life, to enterotoxigenic *E. coli* (ETEC) (Brunauer *et al.,* 2021). In Egypt, bovine newborn *E. coli* infection is the primary cause of calf death, which ranges between 27.4% and 55% of the mortality in newborn calves (Ahmed *et al.,* 2009). The ability of any pathogenic agent to produce gut disturbance depends on its ability to colonize the GIT and destroy the host tissue. *E.coli,* to produce diarrhea, relies on its adhesion antigens that facilitate the bacteria hanging on to enterocytes. The entero-toxigenic *E.coli* (ETEC) was suspected of being the main cause of diarrhea in calves during their first four days of life as a result of oral exposure to coliform-contaminated faeces. This causes commensal flora to colonize the GIT and continue to travel caudally with the ingesta. The ingesta with a high ETEC load with the virulence factors K99 fimbria and heat-stable toxin will produce diarrhea. The fimbrial antigen K99 enables the ETEC to attach to and colonize the intestinal wall (Foster and Smith, 2009).

The presence of fimbrial antigens like F5 and enterotoxins facilitates the colonization of ETEC in the intestinal tract, leading to watery diarrhea (Andrade *et al.,* 2012). Detecting *E. coli* resistance genes such as *blaTEM* and *aadA1* is essential for confirming virulence and assessing antibiotic susceptibility (Belete *et al.,* 2022; Pokharel *et al.,* 2023; Naderi *et al.,* 2024). According to Röhlicher *et al.* (2008) and Huehn *et al.* (2010), diarrhea in both human and animals has been associated with *E. coli* pathogenicity genes, including *iutA, iroN, iucD, papC, hlyF, ompT,* and *astA*. The most commonly observed fimbriae on ETEC from calves with diarrhea are F5, also named K99, and F41(Nagy and Fekete, 2005). The intimin (*eae*) gene, which encourages attachment to and effacement of intestinal epithelial cells, is a characteristic of enteropathogenic *E. coli* (EPEC). The presence of one or more heat-stable (*stx1* and *stx2*) and labile (*ltI* and *ltII)* toxin genes characterizes ETEC strains. STEC and EHEC are characterized by the presence of two toxin genes (*stx1* and *stx2*). The intimin gene (*eae*) is also an important virulence factor for the characterization of EHEC (Gyles and Fairbrother, 2010).

Bacterial cell wall antigenic determinants, such as somatic (O antigens), flagellar (H antigens), or capsular (K antigens), are used to classify *Escherichia coli* isolates into

serotypes and serogroups. The serotypes of *E. coli* found in faecal samples from sick calves were therefore O119, O111, O157:H7, O111, O126, O26, and O78 (Tamaki *et al.,* 2005, and Badouei *et al.,* 2010).

Antimicrobial resistance (AMR), which eventually turns into multidrug resistance (MDR), is increased when humans and animals take antibiotics unreasonably (Khairullah *et al.,* 2020; Sweileh, 2021). The potential for transmission of antibiotic resistance between humans, animals, and wildlife is a global problem that was studied by multidisciplinary sciences in collaboration through a "One Health" approach (Velazquez-Meza *et al.,* 2022).

Antimicrobial resistance is a major global health problem, particularly in developing countries where infectious diseases, a lack of wholesome food, and poverty are pervasive (Moges *et al.,* 2014). The main causes of antibiotic resistance include modifications, or transformations, in the microorganisms' DNA as well as the acquisition of antibiotic-blocking genes from other bacterial species via plasmid transfer. According to Moradigaravand *et al.* (2018), *E. coli* in particular could exhibit a great ability to acquire antibiotic resistance determinants, which results in a limited number of effective treatment options against its pathogenic varieties.

The capability of *E. coli* to form biofilms is another element that contributes to the development of MDR in this organism. The bacteria use the biofilm as a defense mechanism to communicate with one another and tolerate argumentative environments, developing a collective resistance to antibiotics (Saha *et al.,* 2021).

Bacteria adhere to surfaces and secrete extracellular polymeric substances (EPS) to form a protective shell. This biofilm structure makes it difficult for antibiotics to penetrate and effectively kill the bacteria (Ahmed *et al.,* 2019). Antimicrobial resistance management is becoming more difficult as a result, especially in hospital settings where the number of multidrug-resistant (MDR) pathogens is increasing (Ammenti *et al.,* 2020). *E. coli* can attain resistance genes by horizontal gene transfer (HGT) through interspecies and intraspecies routes (Poirel *et al.,* 2018). The HGT mechanism varies, including plasmid, transposon, and pathogenicity island (PAI) insertion into the recipient *E. coli* genome (Javadi *et al.,* 2017).

Extended Spectrum β-Lactamase (ESBL) is a serious problem faced by global health. The extensive usage of β-Lactam antibiotics such as third-generation cephalosporins has resulted in the ineffectiveness of these drugs, which can result in complications in the treatment of infections (Farizqi *et al.,* 2023). The ESBL enzyme can hydrolyze higher β-lactam antibiotics such as monobactam and cephalosporin but can be inhibited by β-lactam inhibitors, one of which is clavulanate (Prayudi *et al.,* 2023).

The emergence of antimicrobial resistance among pathogens is an increasing concern in veterinary medicine, posing a threat not only to animals but potentially to humans as well (Pomba *et al*., 2017). Consequently, the goal of the current study was to clarify the new characteristics of the most prevalent pathotype, *E. coli K99,* isolated from diarrheic neonatal calves regarding the presence of virulence and antibiotic-resistant genes, serotypes, and trends in antimicrobial resistance, and to update our current knowledge of antimicrobial sensitivity and the newly developed drug-resistance genes in *E. coli* isolates from diarrheic calves' faecal samples to provide veterinarians with the ability to make an efficient plan for precise controlling, managing, and treatment of neonatal calves' diarrhea in the Assiut Governorate, Egypt.

MATERIALS AND METHODS

Ethical approval :
The experiments were performed on naturally collected faecal samples; no ethical question was raised by this study. The samples were taken under the supervision of a veterinarian.

Sample collection:
Sterile 50 rectal swabs were used to collect faecal specimens aseptically between December 2024 and March 2025, collected from diarrheal calves aged one day to three weeks old from private farms in the Assiut governorate. With the minimum of delay, all the collected specimens were transferred to the bacteriological lab in an icebox for bacteriological analysis.

Isolation and Identification of *E. coli*:
All collected samples were enriched in tryptose soya broth (TSB) (HI Media, Mumbai, India) at 37°C for 18–24 h. Subsequently, a loopful of this bacterial culture was streaked onto McConkey agar (HI Media, Mumbai, India) and was grown at 37°C for 18–24 h. The pink-colored colonies (lactose fermenters) from the McConkey agar culture were picked up and transferred onto an Eosin Methylene Blue (EMB) (HI Media, Mumbai, India) agar medium and incubated under the same conditions.
The *E. coli* colonies were presumptively identified based on their appearance of a greenish metallic sheen on Eosin Methylene Blue (EMB) (HI Media, Mumbai, India) medium. Three well-isolated presumptive *E. coli* colonies were randomly selected from each EMB plate. The *E. coli* isolates were identified biochemically using the Indole, Methyl Red, Vogues–Proskauer test, and Citrate utilization (IMViC) tests, as described by Quinn *et al.* (2011).

Antimicrobial Susceptibility Testing:
All isolates of *E. coli* (33) were tested for antibiotic drug susceptibility using the conventional Kirby-Bauer disc diffusion technique (Bauer *et al.,* 1966). Twelve different antibiotic disks (Oxoid Ltd., Basingstoke, UK) representing eight distinct categories of antibiotics were used. The included antibiotics were ampicillin (AM 10), amoxicillin (AX25), amoxicillin-clavulanic (AMC30), gentamicin (CN10), neomycin (30), vancomycin (VA30), trimethoprim-sulfamethoxazole (SXT25), clindamycin (DA 2), norfloxacin (NOR 10), ceftiofur (EFT30), cefotaxime (CTX30), and rifampin (RA5). The results were interpreted in accordance with the Clinical and Laboratory Standards Institute's (CLSI,2024) guidelines. The "multidrug-resistant strains" (MDR) refer to strains that exhibit resistance to three or more distinct antibiotic classes (Magiorakos *et al.,* 2012). In this investigation, isolates exhibiting moderate susceptibility were categorized as resistant.

Detection of biofilm formation:
For all *E. coli* isolates (33), biofilm formation was detected by the Congo red agar method (CRA) as described by Freeman *et al.* (1989). The preparation of the CRA medium involved combining 37 g/L of brain heart infusion broth (Oxoid, UK), 50 g/L of sucrose, 10 g/L of agar No. 1 (Oxoid, UK), and 0.8 g/L of Congo red indicator (Oxoid, UK). Separately from the other medium ingredients, the Congo red stain was made as a concentrated aqueous solution and autoclaved for 15 minutes at 121°C. After that, the autoclaved brain heart infusion agar was mixed with sucrose at 55°C and stained with Congo red. Following test organism inoculation, CRA plates were aerobically incubated for 24 hours at 37°C. Biofilm formation was detected by black colonies with a dry crystalline consistency, while red or pink crystalline colonies indicated no biofilm formation.

Serological Identification of Isolated *E. coli* :
The obtained *E. coli* isolates were serologically identified in accordance with Kok *et al.* (1996), using quick diagnostic *E. coli* antisera sets (Denka Seiken Co., Ltd.,

Tokyo, Japan) for enteropathogenic type determination.

Technique:

- Two separate drops of saline were put on a glass slide, and a portion of the colony from the suspected culture was emulsified with the saline solution to give a smooth, fairly dense suspension.
- To one suspension, control, one loopful of saline was added and mixed. To the other suspension, one loopful of undiluted antiserum was added and tilted back and forward for one minute.
- Agglutination was detected under indirect illumination against a dark backdrop. When a colony showed highly positive agglutination with one of the polyvalent serum pools, a part of it was injected onto a nutrient agar slant and incubated at 37°C for 24 hours to develop as a culture for testing with monovalent sera.
- To detect the O-antigen, a heavy suspension of bacteria from each slope culture was made in saline, and slide agglutination tests were done using diagnostic sera.

Testing of isolated *E. coli* for K99 antigen (Acha *et al.*, 2004):

E. coli isolates were streaked on low-glucose agar to determine the expression of the K99 antigen. The inoculation plates were incubated at 37°C for 24 hours. As a result, one suspicious colony was utilized for slide agglutination with K99 antiserum, and the agglutination was examined using a light microscope.

Molecular characterization of *E. coli*:

Twenty multidrug-resistant *E. coli* isolates were chosen at random from 30 biofilm-producing isolates and checked for the K99 gene using polymerase chain reaction (PCR). Five positive K99 isolates were tested for virulence and β-lactam antibiotic resistance genes (*STX1&2, eaeA, hlyA, fimH, blaTEM, blaCTXM,* and *ampC*) using Metabion primers (Table A).

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

PCR amplification: Primers were utilized in a 25-µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler.

Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. For gel analysis, 20 µl of the products was loaded in each gel slot. A general 100 bp ladder (Fermentas, Thermo) was used to determine the fragment size. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed through computer software. Application of the PCR assay was performed in the Reference Lab for veterinary quality control on poultry production at the Animal Health Research Institute, Doki, Giza, Egypt.

Statistical Analysis: statistical analysis of data was performed with the Chi-square test to analyze the data and whether there was a significant difference between antibiotics, and a "P" value of less than 0.05 was deemed statistically significant ($p < 0.05$). Data were analyzed using GraphPad Prism 9.5.1 software **(GraphPad Software Inc., San Diego, CA, USA).**

Table A: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondar denaturation	Annealing	Extension		
K99	TATTATCTTAGGTGG TATGG GGTATCCTTTAGCA GCAGTATTTC	314	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Franck *et al.*, 1998
blaTEM	ATCAGCAATAAACC AGC CCCCGAAGAACGTT TTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Colom *et al*., 2003
BlaCTX-M	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Archambault *et al*., 2006
ampC	TTCTATCAAMACTG GCARCC CCYTTTTATGTACCC AYGA	550	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Srinivasan *et al.* 2005
eaeA	ATGCTTAGTGCTGG TTTAGG GCCTTCATCATTTCG CTTTC	248	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	Bisi-Johnson *et al*., 2011
fimH	TGCAGAACGGATAA GCCGTGG GCAGTCACCTGCCC TCCGGTA	508	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Ghanbarpour and Salehi, 2010
hlyA	GCATCATCAAGCGT ACGTTCC AATGAGCCAAGCTG GTTAAGCT	534	94˚C 5 min.	94˚C 30 sec.	65°C for the first 10 cycles, decrementing to 60°C by cycle 15 40 sec.	72˚C 45 sec.	72˚C 10 min.	Paton and Paton, 1998
Stx1	ACACTGGATGATCT CAGTGG CTGAATCCCCCTCC ATTATG	614	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Dipineto *et al.*, 2006
Stx2	CCATGACAACGGAC AGCAGTT CCTGTCAACTGAGC AGCACTTTG	779	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	

RESULTS

Out of 50 faecal swabs, 33 *E. coli* isolates were detected and identified using morphological characters (Gram-negative non-sporulated motile bacilli), cultural characters (lactose fermenter pink colonies on MacConkey's agar and green metallic sheen colonies on EMB agar, and biochemical identification (positive results with Indole and Methyl Red tests but negative results with Voges-Proskauer, Citrate, Urease, and H2S tests). All *33 E. coli* isolates were multidrug resistant, with 100% resistance to ampicillin (AM), amoxicillin (AX), and amoxicillin-clavulanic acid (AMC). In contrast, the sensitivity to trimethoprim-sulfamethoxazole (SXT-25) and norfloxacin (NOR) was 100% (Table 1). Out of 33 isolates, 30 were strongly positive for biofilm formation (Fig. 1). Serological identification of *E. coli* revealed the presence of O128:H21(K99) (6) strains, O101 (K99) (4), O128:H2 (4), and O26:H11, (3).O20:H7(K99) (2), O119:H6 (2), O91:H21 (2), O125:H21 (2), and O14(K99) (1) were the most predominantly belonging to ETEC, EPEC, and EHEC

pathotypes. Serologically, K99 was exhibited in 13 isolates out of 30 (43.3%) (Table 2). Out of 30 biofilm formation-identified *E. coli,* twenty isolates were randomly selected and subjected to gene-specific polymerase chain reaction (PCR) assays to identify the characteristic K99 gene for *E. coli;* 11 isolates were positive for the K99 gene. Five of the 11 positive K99 gene isolates were examined for stx1, *stx2, eaeA, hlyA, fimH, blaTEM, blaCTX-M,* and *ampC (Table 2).* All five isolates were positive for *stx2, hlyA, blaTEM, ampC,* and *fimH, while* three isolates were positive for *stx1, eaeA,* and *blaCTX-M* (Table 3). The findings revealed that phenotypic identification of biofilm formation and multidrug-resistant isolates was consistent with genotypic molecular detection of biofilm virulence *fimH* and antibiotic resistance genes.

Table 1: Antibiotic Susceptibility of *E. coli* isolates (n = 33).

Antibiotics	Antimicrobial Classes	Resistant **		Sensitive**	
		No	%	No	%
Ampicillin (AM) 10µg (µg)	β-lactamase	33	100	0	0
Amoxicillin (AX) 25 µg	β-lactamase	33	100	0	0
amoxicillin-clavulanic acid (AMC) 30 µg	β-lactamase	33	100	0	0
Gentamicin (CN)10 µg	Aminoglycosides	5	15.2	28	84.8
Neomycin (N)30 µg	Aminoglycosides	26	78.8	7	21.2
Vancomycin (VA) 30 µg	Glycopeptides	24	72.7	9	27.3
Rifampin (RA) 5 µg	Rifamycin	33	100	0	0
Trimethoprim-Sulfamethoxazole (SXT-25) 1.25/23.75 µg	Sulfonamides	0	0	33	100
Clindamycin (DA) 2 µg	Lincosamides	30	90.9	3	9.1
Norfloxacin (NOR) 10 µg	Fluoroquinolones	0	0	33	100
Ceftiofur (EFT) 30 µg	Cephalosporin	25	75.8	8	24.2
Cefotaxime (CTX) 30 µg	Cephalosporin	33	100	0	0

** High significant statistical difference between different types of antibiotics ($p< 0.0001$, $chi^2 = 277.8$).
% was calculated based on the total number of isolates of *E. coli* (n = 33).

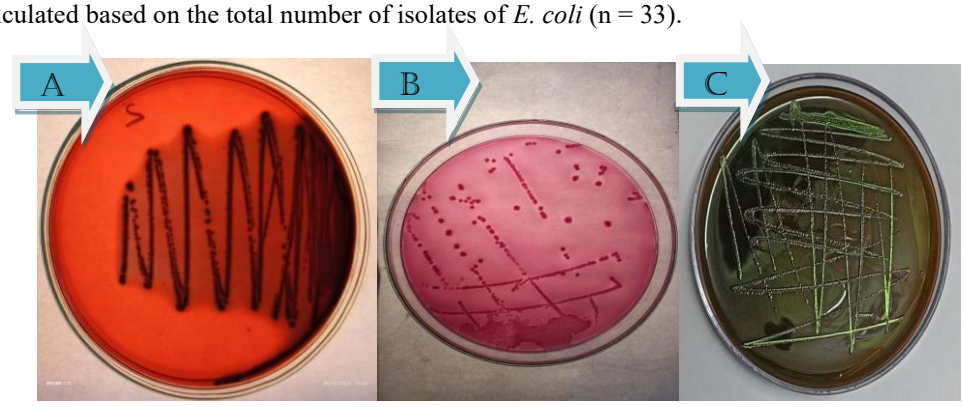

Fig. (1): A) biofilm formation on the Congo red agar. The isolates appeared as dry or shiny black as a result of biofilm formation. B) the pink color colonies (lactose fermenter) on the McConkey agar. C) *E.coli* K99 cultured on EMB agar shows characteristic green metallic sheen colonies

Table 2: Frequency distribution of *E. coli* serotypes (n = 30).

E. coli serotypes	No.	%	Strain characterization
O8 : H21 (K99)	6	20	ETEC
O128 : H2	4	13.3	ETEC
O20 : H7 (K99)	2	6.7	ETEC
O14 (K99)	1	3.3	ETEC
O101 (K99)	4	13.3	EPEC
O125 : H21	2	6.7	EPEC
O15 : H2	2	6.7	EPEC
O44 : H18	1	3.3	EPEC
O153 : H2	1	3.3	EPEC
O26 : H11	3	10	EHEC
O119 : H6	2	6.7	EHEC
O91 : H21	2	6.7	EHEC
Total	30	100	

Table 3: PCR results for detection of *K99* genes in twenty isolates of *E. coli* and *stx1, stx2, eaeA, hlyA, blaTEM, blaCTXM, ampC* and *fimH*, from five isolates of *E. coli* k99 positive gene.

Sample No.	K99	Stx1	Stx2	eaeA	hlyA	blaTEM	BlaCTX-M	ampC	fimH
1	+	-	+	-	+	+	+	+	+
2	-	ND	ND	ND	ND	ND	ND	ND	ND
3	+	+	+	-	+	+	+	+	+
4	-	ND	ND	ND	ND	ND	ND	ND	ND
5	-	ND	ND	ND	ND	ND	ND	ND	ND
6	-	ND	ND	ND	ND	ND	ND	ND	ND
7	+	-	+	+	+	+	-	+	+
8	+	+	+	+	+	+	-	+	+
9	-	ND	ND	ND	ND	ND	ND	ND	ND
10	-	ND	ND	ND	ND	ND	ND	ND	ND
11	+	+	+	+	+	+	+	+	+
12	-	ND	ND	ND	ND	ND	ND	ND	ND
13	-	ND	ND	ND	ND	ND	ND	ND	ND
14	+	ND	ND	ND	ND	ND	ND	ND	ND
15	-	ND	ND	ND	ND	ND	ND	ND	ND
16	+	ND	ND	ND	ND	ND	ND	ND	ND
17	+	ND	ND	ND	ND	ND	ND	ND	ND
18	+	ND	ND	ND	ND	ND	ND	ND	ND
19	+	ND	ND	ND	ND	ND	ND	ND	ND
20	+	ND	ND	ND	ND	ND	ND	ND	ND

ND: not detected

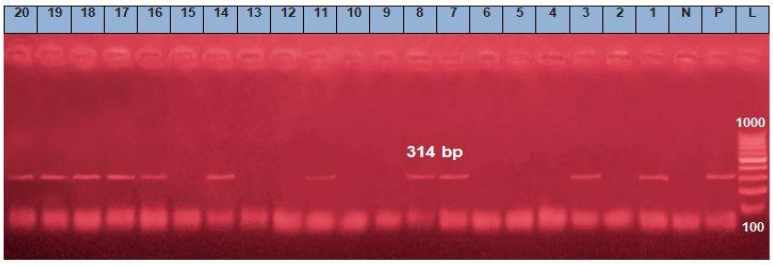

Fig. (2): Amplification profiles for *E. coli* K99 gene. Lane L, DNA ladder marker (100 bp). Lane P, control positive *E. coli* K99 gene. (314 bp). Lane N, negative control. Lanes 1,3,7,8,11,14,16,17,18,19 and 20 positive *E. coli* isolates for K99 gene.

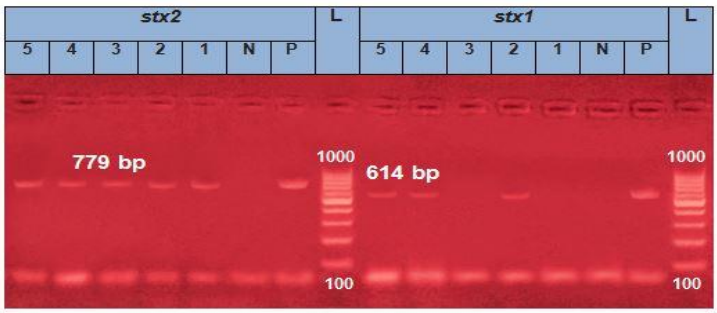

Fig. (3): Amplification profiles for the *E. coli* K99 stx1 & stx2genes. Lane L, DNA ladder marker (100 bp). Lane P is the control positive *E. coli* K99 stx1 & stx2genes (614 & 779 bp). Lane N, control negative. Lanes 2, 4and 5 positive isolates for the *stx1*gene, Lanes 1, 2,3,4 and 5 positive isolates for the *stx2*gene.

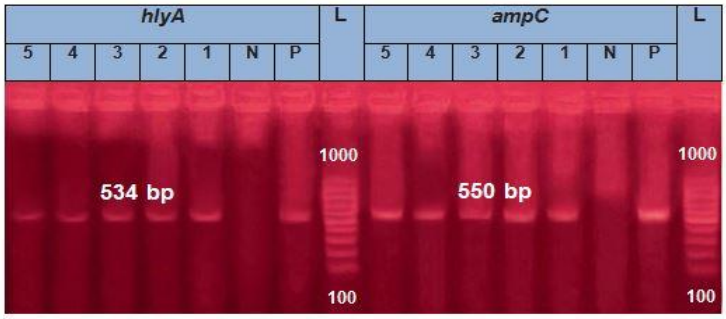

Fig. (4): Amplification profiles for the E. coli K99 hylA & ampC genes. Lane L, DNA ladder marker (100 bp). Lane P is the control positive E. coli K99 hylA & ampC genes (534 & 550 bp). Lane N, control negative. Lanes 1, 2,3,4 and 5 positive isolates for both the hylA & ampC genes.

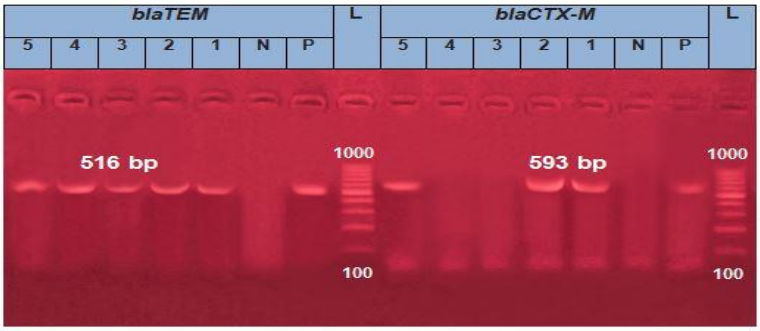

Fig. (5): Amplification profiles for the E. coli K99 blaTEM & blaCTX-M genes. Lane L, DNA ladder marker (100 bp). Lane P is the control positive E. coli K99 blaTEM & blaCTX-M genes (516 & 593 bp). Lane N, control negative. Lanes 1, 2,3,4 and 5 positive isolates for the blaTEM gene and Lanes 1, 3 and 5 positive isolates for the blaCTX-M gene.

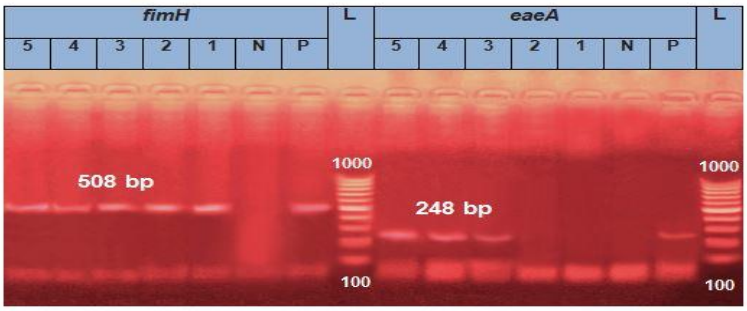

Fig. (6): Amplification profiles for the E. coli K99 fimH & eaeA genes. Lane L, DNA ladder marker (100 bp). Lane P is the control positive E. coli K99 fimH & eaeA genes (508 & 248 bp). Lane N, control negative. Lanes 1, 2,3,4 and 5 positive isolates for the fimH gene and Lanes 3, 4 and 5 positive isolates for the eaeA gene.

DISCUSSION

Antimicrobial agents are essential drugs for human and animal health and welfare. The World Organization for Animal Health (OIE) recognizes the need for access to antimicrobial agents in veterinary medicine, where antimicrobial resistance is a global public and animal health concern that is influenced by the usage of antimicrobial agents in humans, animals, and elsewhere. Antimicrobial agents are essential for treating and controlling infectious diseases in animals (Ferreira *et al.,* 2022). Antimicrobial agents are considered popular to fight diarrhea in calves. Nevertheless, their wide spectrum of activity and the emergence of microbial tolerance of different antimicrobial agents have become well-known phenomena, which represent a major concern (Hajipour *et al.,* 2012).

Diarrhea is one of the most prominent causes of morbidity and mortality, resulting in economic losses owing to the expense of treatment, prevention, increased susceptibility to other illnesses, lower growth rates, and death of calves globally (Selles *et al.,* 2018). In the present investigation, the most prevalent bacterial cause of newborn calf scours was *E. coli,* which was isolated at a rate of 66%. Nearly identical findings with high percentage isolation rates of *E. coli* were reported before at 80% (El-Seedy *et al.,* 2016), 70.7% (Aref *et al.,* 2018), 75.6% (Awad *et al.,* 2020), 62% (Shehta *et al.,* 2022), and 62.5% (Asmaa and Adel, 2024), respectively. In Egypt, Algammal *et al.* (2020) reported a 28.8% isolation rate for *E. coli*, whereas Esmail *et al.* (2023) and Coşkun and Şahin (2023) reported 34% and 43.5% isolation rates, respectively. While Abeer *et al.* (2022) reported that *E. coli* is the most common isolate from newborn diarrheic buffalo calves, with a prevalence rate of 48%. This present study's findings are also consistent with prior reports that *E. coli* is the most common bacterial infection causing diarrhea in newborn calves (Cho and Yoon 2014). This discrepancy between the rates of *E. coli* isolation from different localities and countries may be attributed to the differences in management and control programs and environmental conditions. It also indicates the uncontrolled and hazardous use of antibiotics in food animal management and prophylaxis programs in Egypt. It stresses the bacteria, pushing it to develop drug resistance to overcome routine management and control measures and guarantee their presence and multiplication. It also should represent an alarming problem that necessitates nationwide, strictly implemented rules to reduce antibiotics' uncontrolled and hazardous use (Esmail *et al.*, 2023).

The most common pathogen associated with diarrhea in calves is E. coli K99 (Prescott et al., 2022). In the current investigation, 55% of the diarrheic calf samples were positive for *E. coli* K99 (Table 2 & Fig. 2). This was virtually identical to those reported by Acha *et al*. (2004) in Mozambique (40%) and Langoni *et al*. (2004) in Brazil (35%). Previous studies have reported lower prevalences of *E. coli* K99, including 12.3%, 20.74%, 23.53%, 22.6%, 18.17%, 28.4%, 16%, 5.3%, 11.3%, 11.42%, and 12.9%, which were reported by Afify *et al*. (2024), Yosef *et al*. (2024), Esmail *et al.* (2023), Coşkun and Şahin (2023), Sharieff (2015), Shahrani *et al.* (2014), and Selles *et al*. (2018). Kumar *et al.* (2022), Pourtaghi & Sodagari (2016), Lacroute (2016), and Picco *et al*. (2015), respectively.

Conversely, Keykhaei *et al.* (2021) didn't record K99 in the *E. coli* isolates from diarrheal calves. The differences in prevalence and frequency rates among the studies may be attributed to regional variations, management and hygienic conditions where *E. coli* K99 infection occurs mainly through ingestion of contaminated food and water, the age of the animals, vaccination of the dam, colostrum intake, or different diagnostic methods (Picco *et al*., 2015*).*

The misuse of antibacterial drugs for prophylaxis and infection treatment in food animals and poultry has led to the emergence of a global high threat of the spread of antimicrobial-resistant bacterial strains (Abavisani *et al.,* 2023). *E. coli* can work not only as a recipient of horizontal gene transfer from other bacteria but also as a donor of resistance genes to other bacteria (Poirel *et al.,* 2018). As a result, it plays a significant part in the spread of drug-resistant genes among pathogenic bacteria, compared to recent research conducted in Egypt on *E. coli* diarrhea (Algammal *et al.,* 2020).

Owing to the antibiotic susceptibility test findings, all *E. coli* isolates from diarrheal calves (100%) exhibited the MDR pattern (Table 1). One hundred percentage of *E. coli* isolates were shown to be resistant to amoxicillin (AX), ampicillin (AM), and amoxicillin-clavulanic acid (AMC). The results obtained closely match those of Haque *et al.* (2022) and Esmail *et al.* (2023); they discovered that 100% of the *E. coli* isolates from diarrheal calves had the MDR pattern and were resistant to both amoxicillin (AX) and amoxicillin-clavulanic acid (AMC). Similar results were also reported by Ansari *et al.* (2014), who found that 100% resistance to amoxicillin (AX) was present. While all of the *E. coli* isolates in this investigation were MDR, 83.33% of the isolates in Gupta *et al.'s* (2019) study were MDR. Constable (2004) only recommended amoxicillin (AX) to treat calf diarrhea based on previously published evidence supporting oral administration of these antimicrobial medicines.

All our isolates in this study were found to be resistant to AMX, indicating that it was ineffective for those suffering calves. This could be because of the development of microbial drug resistance or because *E. coli* is under different antibiotic stress due to variations in management and preventive control measures in other studied localities. *E. coli* bacteria exhibiting advanced resistance to the majority of antibiotic classes have been identified by Xiao *et al.* (2019) and Zhang *et al.* (2021). The multidrug resistance of the isolated bacteria in the current study is an example of this situation, since all antibacterial medications were either completely or partially ineffective when trimethoprim-sulfamethoxazole (SXT-25) and norfloxacin (NOR) were the only medications that could effectively inhibit the growth and multiplication of *E. coli*. Furthermore, compared to the recently reported 21.5% resistant *E. coli,* the isolates under study have a 100% resistance to amoxicillin/ clavulanic acid (Algammal *et al.,* 2020). Globally, MDR *E. coli* is known to pose a serious threat to public health. As a result, treating both humans and animals in hospitals becomes costly and would undoubtedly lengthen the course of therapy. For this reason, choosing an appropriate antibiotic to treat calf scours requires examining the antibiotic sensitivity pattern.

Table (1) showed that there were highly significant differences in the antimicrobial sensitivity of various antibiotics ($p < 0.0001$, $chi^2 = 277.8$) and antimicrobial resistance of various antibiotics ($p < 0.0001$, $chi^2 = 277.8$). The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognized by the World Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, an increasingly troublesome situation because of the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and even commensal organisms (Ferreira *et al.,* 2022).

Regarding the Congo red agar method (CRA) for detecting the production of biofilms, 30 of the 33 *E. coli* isolates showed a high prevalence rate of 90.9% for biofilm

formation (Fig. 1), which is consistent with the findings of Hasnawi and Al-Hilu (2025), Molina *et al.* (2025), and Algammal *et al.* (2020), who reported the higher prevalence rates of 100%, 99%, and 89.8%, respectively. Conversely, Azarakhsh and Aminnezhad (2025) observed a lower incidence (26.7%) of *E. coli* isolates that tested positive for biofilm development.

One of the pathogenicity characteristics that bacteria utilize to counteract the effectiveness of antibacterial medicines is biofilm (Mlugu *et al*., 2023). Treatment expenses are rising as a result of the increase in germ resistance to antibiotics. Their capacity to produce biofilms, one of their virulent traits, was connected to their multidrug resistance. This allowed them to disrupt the antibiotic's target site, change the molecules present, and eliminate the antibiotics that were already attached. According to Seifi *et al.* (2016), one of the virulence features that makes infections resistant to therapy is their capacity to build biofilm. The WHO has identified antimicrobial resistance as the greatest danger to global health, and experts have referred to it as a "shadow pandemic" (Ranjbar and Alam, 2023).

Biofilms significantly enhance the virulence of *E. coli* by providing a protective environment that promotes its survival and persistence under adverse conditions. The extracellular polymeric substance (EPS) matrix of biofilms acts as a barrier against environmental stressors, including anti-biotics and host immune responses, thereby increasing resistance and promoting chronic infections. There was a significant correlation between biofilm production and multiple resistance to antibiotics. The global burden of antimicrobial resistance is a major public health emergency.

The presented data in Table (2) showed the serological identification of the obtained isolates, and it revealed the detection of 12 serotypes: O128:H21(K99) (6 strains), O101 (K99) (4), O128:H2 (4), O20:H7(K99) (2), O26:H11 (3), O119:H6 (2), O91:H21 (2), O125:H21 (2), and O14(K99) (1). They predominantly belonged to ETEC, EPEC, and EHEC pathotypes. The identified serotypes in this study are not the same as those previously reported (Esmail *et al.,* 2023; Algammal *et al.,* 2020, Enany *et al.,* 2019). However, this result was in line with those of Wani *et al*. (2013) and Haggag *et al.* (2016), who discovered that ETEC was the most common isolate from the diarrheal calves. The prevalence of *E. coli* pathotypes varied by season (El-Azzouny *et al.,* 2020). To discover zoonotic and human-harmful serotypes, screening of *E. coli* isolated from ill animals should always be of interest, preferably in line with the One Health strategy (Croxen *et al.,* 2013).

The molecular screening *of E.coli* K99's virulence and antibiotic resistance genes revealed that all five isolates under investigation expressed *stx2, hlyA, blaTEM, ampC*, and *fimH*, while three isolates tested positive for *stx1, eaeA*, and *blaCTX-M* (Table 3 and Fig. 3, 4, 5, and 6). These results are almost identical to those reported by El-Azzouny *et al.* (2020), who concluded that out of ten *E. coli* isolates examined by PCR, there were *stx1* (80%) and *stx2* (90%), and *eaeA* was found in all isolates (100%). Conversely, the detection rate of *stx1* was very low, and *stx2,* as well as intimin, were not detected at all among the diarrheic calves' isolates (Feuerstein *et al.,* 2021). However, the most common Shiga-toxin gene, according to Algammal *et al.* (2020), is the stx1 gene, which may be found either by itself, in conjunction with the stx2 gene, or in combination with the *eaeA* and *stx2* genes. But, according to Asmaa and Adel (2024), the *eaeA* gene (88%), *Stx1* (84%), and *Stx2* (64%) were identified using molecular identification of *E. coli* strains.

Nevertheless, Salama *et al.* (2023) demonstrated the presence of *eaeA* in all isolates (100%), *stx1* (16.7%), and *stx2* (11%) in isolates of *E. coli.*

Shiga toxin-producing E. coli (STEC) and enteropathogenic E. coli (EPEC) are linked to several virulence factors, such as Shiga toxins 1 and 2, intimin (eaeA), and enterohaemolysin (hlyA). The intimin gene *(eae)* is also a crucial virulence factor for the identification of enterohemorrhagic *E. coli* (EHEC) (Abu-Ali *et al*., 2010; Gyles and Fairbrother, 2010). According to Kang *et al.* (2004), the genes *stx*(1) and *stx*(2) seem to be important in the pathophysiology of hemolytic-uremic syndrome and hemorrhagic colitis, and STEC strains primarily carry either *stx1* or *stx2* or both. In the current investigation, the *stx2* gene (100%) is detected more frequently than the *stx1* gene (60%) (Table 2 and Fig. 3). This is in the same line as Kuyucuoğlu *et al.* (2011). The size of the sample, the way the samples were handled, the samples' place of origin, the number of strains analyzed, and the type of virulence genes found by PCR could be some variation factors as a consequence in the presence of virulence, enterotoxin, and Shiga toxin genes between the other studies and our findings (Franz *et al.,* 2007).

The current investigation found that the presence of the virulence genes *blaTEM, blaCTX-M*, and *ampC* in *E. coli* was linked to phenotypic resistance to one or more antimicrobials and the capacity to withstand several drugs (Table 1, Fig 4 and 5). This outcome was consistent with Esmail *et al.* (2023). The expression of the multi-drug-resistance genes *ampC, blaTEM,* and *blaCTX-M* was examined in all isolates. All of the *E. coli* isolates in this investigation showed a high prevalence rate of biofilm formation (Fig.1) that was accompanied by the presence of the virulence gene for biofilm formation, *fimH* (Table 2 & Fig. 6). The higher the stress the *E. coli* are facing by improperly using antibacterial drugs, the more advanced and new mechanisms the bacteria develop to overcome this challenge.

CONCLUSION

The obtained results indicated that *E. coli* infection remains the major bacterial disease causing diarrhea in calves, with the emergence of multi-drug-resistant strains making treatment increasingly challenging with high risk to human health, as they can transmit antibiotic resistance genes through the food chain. This study confirms the presence of virulent and multidrug-resistant *E. coli* serotype E. *coli K99* in diarrheic calves. Antimicrobial susceptibility is important to formulate a suitable treatment for *E. coli*. Therefore, it is important to check the antibiotic sensitivity pattern regularly on the commonly used drugs to select a suitable antibiotic for the treatment of calf diarrhea.

CONFLICT OF INTEREST:

There is no conflict of interest declared by the authors.

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المقاومة للمضادات الحيوية والتوصيف الجزئيى والسيرولوجى لميكروب اى كولاى المعوى الممرض (ك 99) المعزول من اسهالات العجول حديثة الولادة

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الملخص العربى

يعتبر ميكروب إى كولاى واحد من اهم الميكروبات المسببة للاسهالات فى العجول حديثة الولادة بالاضافة الى مقاومته للعديد من المضادات الحيوية. فكان من الضرورى اجراء هذه الدراسة لعزل الميكروب من العجول المصابة واختبار مقاومة هذا الميكروب للمضادات الحيوية والكشف عن بعض الجينات المسئولة عن ضراوة و مقاومة الميكروب للمضادات الحيوية. حيث ان مشكلة الاسهالات و عدم الاستجابة للمضادات الحيوية و نفوق العجول اهم المشاكل التى تواجه المربى و مزارع الانتاج الحيوانى . تم جمع عدد خمسين عينة اسهال من عجول حديثة الولادة فى عمر يوم حتى ثلاثة اسابيع. اظهرت نتائج الفحص البكتريولوجى ايجابية عدد 33 عينة لميكروب إى كولاى بنسبة عزل 66٪. جميع عزلات اى كولاى كانت عديدة المقاومة للمضادات الحيوية حيث اظهرت مقاومة بنسبة 100٪ لكل من الامبسيلين والاموكسيسيلين والاموكسيسيلين كلافيولانيك والريفاميسين بينما اظهرت حساسية عالية بنسبة 100٪ لكل من الترايميسوبريم سلفا والنوروفلوكساسين . كذلك اظهرت النتائج ان عدد 30 عزلة من 33 إى كولاى بنسبة 90.9 ٪ كانت ايجابية لتكوين الغشاء الحيوى (البيوفيلم) . بينما اظهرت نتائج الفحص السيرولوجى تنوع عالى من الانماط الممرضة المسببة للاسهال التى تنتمى فى الغالب الى ETEC , EPEC, EHEC وحيث كانت المجموعات المصلية الاكثر تكرارا فى سلالات إى كولاى هى $O_8:H_{21}(K_{99})$: عدد 6 عزلات وO_{101} (K_{99}) عدد 4 عزلات و $O_{128}:H_2$ عدد 4 عزلات و $O_{20}:H_7(K_{99})$ عدد2 و$O_{26}:H_{11}$ عدد 3 و $O_{119}:H_6$ عدد 2 و$O_{91}:H_{21}$ عدد 2 و $O_{125}:H_{21}$ عدد2 وO_{14} (K_{99}) عزلة واحدة حيث وجد عدد 13 عزلة حاملة لجين K_{99} من اجمالى العدد 30 بنسبة 43.3 ٪ . هذا و قد اوضح الفحص الجزئيى لعدد 20 عزلة تم اختيارها بطريقة عشوائية من عدد 30 عزلة التى اظهرت ايجابية لتكوين الاغشية الحيوية ايجابية 11 عزلة لجين (**ك 99)** بنسبة 55 ٪ من اجمالى عدد العزلات التى تم فحصها . كذلك اظهر الفحص الجزئيى لعدد خمسة من إى **كولاى (ك 99)** ايجابية كل العزلات لجينات **stx2, hlyA , blaTEM, ampC** and **fimH,** بنسبة 100 ٪ بينما كانت ثلاث عزلات حاملة لجينات **stx1, eaeA** and **blaCTX-M** بنسبة 60 ٪ لكل من جينات الضراوة و المقاومة للمضادت الحيوية.

حيث أظهرت النتائج التي تم الحصول عليها أن الكشف الظاهري للعزلات متعددة المقاومة للمضادات البكتيرية و تكوين الاغشية الحيوية يتفق مع الكشف الجزيئي الجيني لجينات مقاومة المضادات الحيوية بيتا لاكتاماز والجين المسئول عن تكوين الاغشية المخاطية **fimH**. تؤكد هذ ه النتائج على الحاجة إلى تحسين تدابير التحكم لمكافحة مقاومة المضادات الحيوية وتسلط الضوء على أهمية الاستراتيجيات العلاجية البديلة وكذلك ينبغى رفع مستوى الوعى حول الاجراءت الصحية الصارمة فى تربية العجول حديثى الولادة .