**Intimin (eae) and virulence membrane protein pagC genes are associated with biofilm formation and multidrug resistance in *Escherichia coli* and *Salmonella enterica* isolates from calves with diarrhea**

Shaimaa O. Hasson1*, Hawraa K. Judi2, Hawazen H. Salih3, Ameer Al-Khaykan4, Sousan Akrami5, Sahar Sabahi6, Morteza Saki7,8* and Zahraa A. Al-Rubaie9

**Abstract**

**Objectives** This study aimed to evaluate the association of the intimin (eae) and pagC genes with biofilm formation and multidrug resistance (MDR) phenotype in *Escherichia coli* and *Salmonella enterica* collected from calves with diarrhea.

**Results** Fecal samples (n: 150) were collected from calves with diarrhea. Of 150 fecal samples, 122 (81.3%) were culture positive and 115/122 (94.2%) were Gram-negative bacteria. Among them, *E. coli* (n = 64/115, 55.6%) was the most common isolate followed by *S. enterica* (n = 41/115, 35.6%). Also, 10 (8.6%) isolates were other *Enterobacteriaceae* bacteria including *Klebsiella* and *Proteus* species. Eighty-nine isolates (77.4%) from calf diarrhea, including 52 (81.3%) *E. coli* and 37 (68.3%) *S. enterica* isolates, were MDR. The eae and pagC genes were detected in 33 (51.5%) *E. coli* and 28 (68.3%) *S. enterica* isolates, respectively. There was a strong association between these genes and biofilm formation and MDR phenotype (P-value = 0.000). All *E. coli* isolates carrying the eae gene were biofilm producers and MDR. Also, all pagC-positive *S. enterica* isolates were MDR and 25 (89.3%) isolates of them produced biofilm.

**Keywords** Biofilm formation, Calves with diarrhea, *Escherichia coli*, Multidrug resistance, *Salmonella enterica*
Introduction

Diarrheal infections in calves are among the most common diseases in small livestock and cause significant economic productivity losses for livestock producers [1]. *Salmonella* species, *Escherichia coli*, and *Clostridium perfringens* are among the infectious bacteria associated with calf diarrhea [2, 3].

When *E. coli* and *S. enterica* invade the host, they use different virulence factors to cause infection. *E. coli* is responsible for a variety of diseases in humans and animals, including intestinal and extraintestinal infections. The various virulence factors of *E. coli* include hemolysins, colicins, toxins, proteases, adhesion like fimbriae, and cell surface hydrophobicity [4]. *Salmonella* also has several factors responsible for its pathogenicity, including the invasion gene (*inva*), the fimbriae (*fimA*) gene, and the *spvABCD* system [5]. *Salmonella* is now the most common cause of bacterial gastroenteritis. Mild diarrhea is a major symptom of salmonellosis caused by *Salmonella* [5].

Farm animals such as cattle are known reservoirs for multidrug resistance (MDR) bacteria such as *E. coli* [6]. The MDR phenomenon has increased worldwide and is considered a public health threat. Several recent studies have reported the emergence of multidrug-resistant bacterial pathogens of various origins, necessitating the proper use of antibiotics, routine antimicrobial susceptibility testing to determine the antibiotic of choice, and screening of emerging MDR strains [7, 8].

The phenomenon of antibiotic resistance is often associated with the development of biofilms in bacterial pathogens such as *E. coli* and *S. enterica* [9]. Biofilms are microbial colonies that adhere to biotic or abiotic surfaces and serve as an important site for horizontal gene transfer [9, 10].

Various genes are involved in the process of biofilm production in *E. coli* and *S. enterica* bacteria [11, 12]. However, not all of them have been studied. For example, there is little information on the function of the *pagC* gene, which is widely distributed in *Salmonella* species [13]. Also, previous studies reported an inverse association between the presence of the *intimin* (*eae*) gene and biofilm formation in *E. coli* [14, 15]. Moreover, to the best of our knowledge, there are no studies that have investigated the association between these genes and the MDR phenotype in bacteria.

Hence, the current study aimed to evaluate the association of the *intimin* (*eae*) and *pagC* genes with biofilm formation and MDR phenotype in *E. coli* and *S. enterica* collected from calves with diarrhea.

Main text

Materials and methods

Bacterial isolation and identification

From October 2020 to January 2021, fecal samples (*n*: 150) were aseptically collected from calves with diarrhea at Veterinary Teaching Hospital and private clinics in Babylon province in a sterile cup tube and immediately transported to the microbiology laboratory at Al-Qasim Green University. After overnight enrichment in nutrient broth (Merck, Darmstadt, Germany), samples were plated on MacConkey agar (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h. To obtain a pure culture, colonies appearing after 24 h were re-streaked and then preliminarily identified with microscopy and biochemical tests including urea, triple sugar iron (TSI), indole, motility test, lysine iron agar (LIA), methyl red/Voges-Proskauer (MR/VP), and simmons citrate. Biochemical tests were performed using the VITEK® 2 system (bioMérieux, Inc., Durham, NC, USA) according to the manufacturer’s instructions [16].

Antimicrobial susceptibility testing using VITEK® 2 system

Antibiotic susceptibility testing (AST) was performed using the VITEK® 2 system (bioMérieux, Inc., Durham, NC, USA) according to the manufacturer’s instructions with AST cards for Enterobacteriaceae (ASTN280 cards) [17]. This system performed AST based on the broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) interpretation criteria [18]. These antibiotics include ampicillin, cefazolin, ceftazidime, ceftriaxone, cepfemip, imipenem, tobramycin, ciprofloxacin, and gentamycin. Isolates that were resistant to three or more antimicrobial classes were categorized as MDR [19].

Biofilm formation detection

Biofilm production was assessed using Congo red agar (CRA) method as described previously [20]. Congo red stain (0.8 g/L) (Sigma-Aldrich, St. Louis, MO, USA), 5% sucrose (50 g/L) (Sigma-Aldrich, St. Louis, MO, USA), and agar (10 g/L) (Sigma-Aldrich, St. Louis, MO, USA) were added to brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) to prepare CRA medium [20]. Bacterial isolates were inoculated onto CRA medium and incubated at 37 °C for 24 h. When black colonies with a dry crystalline appearance were present, the isolates were classified as having a strong biofilm formation. A moderately positive biofilm producer was indicated by dark coloration of colonies in the absence of dry crystalline colony morphology. Non-biofilm producers were defined as colonies that remained pink [20].
laboratory-confirmed biofilm producer strain was used as a positive control.

Polymerase chain reaction (PCR)

Colony PCR method was used to detect the *eae* and *pagC* genes in *Enterobacteriaceae* bacteria isolated from feces of calves with diarrheal disease. Genomic DNA was extracted from the bacterial isolates using the Genomic DNA Mini Bacteria Kit (Bio Basic, Markham, Canada) according to the manufacturer’s instructions. The purity of the extracted DNA was determined using a Nanodrop instrument (Thermo Fisher Scientific, Waltham, USA) at 260/280 nm [21]. PCR was performed to amplify the *eae* and *pagC* genes (Table 1) using the specific primers (Bioneer, Daejeon, Korea) [22, 23]. PCR master mix for each gene was prepared using the Maxime PCR PreMix kit (iNtRON Bio, South Korea) according to the company’s instructions as follows: DNA template 5–50 ng in 5 µL, forward primer (10 pmol) 1µL, reverse primer (10 pmol) 1µL and PCR water 13 µL, the total volume 20 µL. PCR conditions for each gene were as follows: initial denaturation at 95 °C for 5 min for one replicate, followed by 30 cycles at 95 °C for 30 s (denaturation), annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min for one repeat. PCR products were separated by 1% agarose (Sigma-Aldrich, St. Louis, MO, USA) gel electrophoresis.

Table 1 Details of the oligonucleotides used for polymerase chain reaction in this study

| PCR Primer | Sequence | Product Size (bp) | Reference |
|------------|----------|------------------|-----------|
| *eae* gene for *Escherichia coli* | F: TCAATGCAAGTCCGGTTATCAGTT  R: GTAAGTCGATCACCACCTG | 482 | 22 |
| *pagC* gene for *Salmonella enterica* | F: CGCCTTTTCCGGGAGTATGC  R: GAAGCGGTTATTATTGTAGGAGGATGGT | 454 | 23 |

### Results

**Phenotypic characteristics of the recovered isolates using VITEK® 2 system**

Of the 150 fecal samples collected from calves with diarrhea, 122 (81.3%) were culture positive, while 28 (18.6%) samples were negative. On MacConkey agar, 115/122 (94.2%) were Gram-negative bacteria. Using the VITEK® 2 system, *E. coli* (n=64/115, 55.6%) was the most common isolate with the following characteristics: motility (+), MR (+), VP (-), indole (+), citrate and urea (-), TSI (acid/acid without H2S), and LIA (+). *S. enterica* (n=41/115, 35.6%) with the following characteristics: motility (+), MR (+), VP (-), indole (-), citrate (+), urea (-), TSI (alkaline/acid with H2S), and LIA (+) was the second most abundant isolate. The remaining 10 (8.6%) isolates included *Klebsiella* (5, 4.3%) and *Proteus* (5, 4.3%) species.

**Antibiotic resistance rates and MDR phenotype**

Imipenem and ciprofloxacin were the most effective antibiotics with a sensitivity rate of 100.0%. Ampicillin and cefazolin with resistance rates of 100.0% were the less effective antibiotics (Table 2). Eighty-nine isolates (77.4%) from calf diarrhea, including 52 (81.3%) *E. coli* and 37 (90.2%) *S. enterica*, were found to be MDR. Of these, more than 80.0% of the isolates were simultaneously resistant to ampicillin, ceftriaxone, ceftazidime, cefepime, and cefazolin.

### Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software version 24.0 software (IBM Corporation, Armonk, NY, USA) [24]. All kinds of data can be processed and analyzed with SPSS software. Significant associations were considered as *P*-value ≤0.05 using Chi-square test [25].

**Table 2** Antibiotic resistance rates and multidrug resistance profiles in *Escherichia coli* and *Salmonella enterica* isolates

| Antibiotics | *Escherichia coli* n: 64 | *Salmonella enterica* n: 41 |
|-------------|--------------------------|---------------------------|
|             | Resistant (n, %) | Intermediate (n, %) | Susceptible (n, %) | Resistant (n, %) | Intermediate (n, %) | Susceptible (n, %) |
| Gentamicin  | 7 (10.9) | 0 (0.0) | 57 (89.1) | 2 (4.9) | 0 (0.0) | 39 (95.1) |
| Tobramycin  | 3 (4.7) | 0 (0.0) | 61 (95.3) | 0 (0.0) | 0 (0.0) | 41 (100.0) |
| Imipenem    | 0 (0.0) | 0 (0.0) | 64 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) |
| Cefazolin   | 64 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 0 (0.0) | 0 (0.0) |
| Ceftazidime | 61 (95.3) | 0 (0.0) | 3 (4.7) | 41 (100.0) | 0 (0.0) | 0 (0.0) |
| Ceftriaxone | 61 (95.3) | 0 (0.0) | 3 (4.7) | 40 (97.6) | 0 (0.0) | 1 (2.4) |
| Cefepime    | 59 (92.2) | 0 (0.0) | 5 (7.8) | 40 (97.6) | 0 (0.0) | 1 (2.4) |
| Ciprofloxacin | 0 (0.0) | 0 (0.0) | 64 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) |
| Ampicillin  | 64 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 0 (0.0) | 0 (0.0) |
| Multidrug resistance | 52 (81.3%) | | | 37 (90.2%) | | |
Biofilm formation

In the current study, all bacterial isolates were evaluated for biofilm formation using the CRA method. The results showed that 40 (62.5%) E. coli isolates and 32 (87%) S. enterica isolates had the ability to form biofilms (Fig. 1). The black and rough colony on CRA showed the strong biofilm formation activity. All the biofilm producers were MDR isolates. There was a significant association between biofilm formation and MDR phenotype of the isolates (P-value=0.000).

Association of eae and pagC genes with biofilm formation and MDR.

The eae and pagC genes were detected in 33 (51.5%) E. coli and 28 (68.3%) S. enterica isolates, respectively (Fig. S1 and S2). There was a strong association between these genes and biofilm formation and MDR phenotype (P-value=0.000). All E. coli isolates carrying the eae gene were biofilm producers and MDR. Also, all pagC-positive S. enterica isolates were MDR and 25 (89.3%) isolates of them produced biofilm.

Discussion

In this study, E. coli (64/150, 42.6%) and S. enterica (41/150, 27.3%) were the most prevalent bacteria. In a previous study from Egypt, El-Seedy et al. [26] reported a frequency rate of 18.1% and 75.6% for Salmonella serovars and E. coli in diarrheic calves, respectively. Also, in line with the current findings, Mousa et al. [27] and Shekhar et al. [28] reported a frequency rate of 40.0% and 41.6% for E. coli isolates in samples from calves with diarrheal disease, respectively. On contrary, the current result was lower than previous studies by Tadesse et al. [29] and Mohammed et al. [30], who reported prevalence rates of 49%, 85%, and 46.4% for E. coli, respectively. This difference in E. coli incidence could be due to differences in calf age, study location, sample size, farm size, and hygiene parameters [30].

According to the antimicrobial susceptibility test in this study, the tested strains showed sensitivity to ciprofloxacine, imipenem, tobramycin, and gentamicin with a percentage close to 100%. Meanwhile, resistance to cefazolin, ampicillin, ceftazidime, ceftriaxone, and ceftepime exceeded 80%. Similar findings were reported by Mohammed et al. [30] and Manjushree et al. [31]. Eighty-nine isolates from calf diarrhea, including 52 (81.3%) E. coli and 37 (90.2%) S. enterica, showed the MDR phenomenon. However, Bandyopadhyay et al. [6] reported a lower MDR rate (12.3%) of E. coli strains isolated from calves with diarrhea than in the current study. Moreover, in line with the current study, Gebeyehu et al. [32] from Ethiopia reported a 100.0% resistance rate to ampicillin and a 100.0% susceptibility rate to ciprofloxacine among Salmonella strains isolated from raw cow milk samples. They also noted a high MDR rate (100.0%) among the isolates [32].

This high prevalence of resistant or multidrug-resistant isolates in the current study may be attributed to the widespread and indiscriminate use of antimicrobials in animals for the treatment, prevention, and control of infectious diseases, and as growth promoters for potential livestock production [32, 33]. Indiscriminate use of these conventional antibiotics without veterinarian prescription and misuse or abuse of veterinary antimicrobials by dairy farmers resulted in the emergence of resistance genes of public concern because they could be transmitted to humans [34]. In recent years, numerous MDR isolates of E. coli and S. enterica have been reported from several countries [31–33]. Both pathogens utilize multiple antibiotic resistance mechanisms including mobile genetic elements, integrons, plasmids, and efflux pumps [35, 36].

Another mechanism of antibiotic resistance used by some bacteria is biofilm production mediated by several genes [9–12]. In this study, we investigated the association of the eae gene of E. coli and the pagC gene of S. enterica with biofilm formation and MDR phenotype to elucidate probable link. To the best of our knowledge, this issue has not been investigated in previous studies from Iraq.

In our study, a strong association was found between the presence of the eae and pagC genes and biofilm formation and MDR in E. coli and S. enterica isolates. The pagC gene helps Salmonella survive macrophage phagosomes [13]. However, in a previous study by Lu et al. [13] from China, deletion of the pagC gene was found to promote biofilm formation in S. enterica subspecies enterica.
serovar Pullorum. According to their study, deletion of the \( \text{pagC} \) gene reduces the production of outer-membrane vesicles (OMVs) in \( \text{Salmonella} \) isolates, which in turn promotes biofilm stability and bacterial colonization [13]. Moreover, in contrast to this study, Nesse et al. [14] and Stanford et al. [15], claimed an inverse association between the presence of the \( \text{intimin} \) (\( \text{eae} \)) gene and biofilm formation in \( \text{E. coli} \) isolates. Until now, it was not known whether intimin plays a role in biofilm formation in \( \text{E. coli} \) [14]. It seems that more in depth studies are needed to clarify the exact role of the \( \text{pagC} \) and \( \text{eae} \) genes in biofilm formation in \( \text{Salmonella} \) and \( \text{Escherichia} \) species.

In conclusion, this study revealed the association of \( \text{eae} \) and \( \text{pagC} \) genes with biofilm formation and MDR phenomenon in \( \text{E. coli} \) and \( \text{S. enterica} \) species isolated from calves with diarrhea. More in depth molecular based studies are recommended to reveal the detailed mechanisms behind this observations.

**Limitations**

No investigation of other related virulence genes and small sample size were the major limitations of this study. Also, the detection of virulence and antimicrobial resistance genes and the correlation between phenotypic and genotypic MDR in the recovered isolates were not be performed.

**Abbreviations**

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| CRA          | Congo red agar                                   |
| MDR          | Multidrug resistance                             |
| PCR          | Polymerase chain reaction                        |
| SPSS         | Statistical package for the social sciences      |

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13104-022-06218-6.

**Acknowledgements**

None.

**Authors’ contributions**

SOH, HKJ, and HHS: conceptualisation, data curation, formal analysis, investigation, methodology, project administration, writing—original draft preparation, writing—review and editing. AAK and SA: data curation, formal analysis, writing—original draft preparation, writing—review and editing. All authors read and approved the final manuscript.

**Funding**

None.

**References**

1. Tamrat H, Mekonnen N, Ferede Y, Cassini R, Belayneh N. Epidemiological study on calf diarrhea and coccioides in dairy farms in Bahir Dar, North West Ethiopia. Ir Vet J. 2020;73(1):1–8.
2. Whon TW, Kim HS, Shin NR, Sung H, Kim MS, Kim JY, et al. Calf diarrhea caused by prolonged expansion of autochthonous gut Enterobacteriaceae and their lytic bacteriophages. Mysterms. 2021;6(2):e00816-20.
3. Ngileka M, Godson D, Vanier G, Desmarais G, Wojnarowicz C, Sayi S, et al. Frequency of \( \text{Escherichia coli} \) virotypes in calf diarrhea and intestinal morphologic changes associated with these virotypes or other diarrheagenic pathogens. J Vet Diagn Invest. 2019;31(4):611–5.
4. Abd El-Baky RM, Ibrahim RA, Mohamed DS, Ahmed EF, Hashem ZS. Prevalence of virulence genes and their association with antimicrobial resistance among pathogenic \( \text{E. coli} \) isolated from Egyptian patients with different clinical infections. Infect Drug Resist. 2020;13:1221–36.
5. Nikkama ME, Kakou-Ngazoa S, Sylla A, Bako E, Addablah AL, Ouoba JR, et al. Characterization of virulence factors of \( \text{Salmonella} \) isolated from human stools and street food in urban areas of Burkina Faso. BMC Microbiol. 2021;21:338.
6. Bandypadhyay S, Bhattacharyya D, Samantara I, Banerjee J, Habib M, Dutta TK, et al. Characterization of multidrug-resistant biofilm-producing \( \text{Escherichia coli} \) and \( \text{Klebsiella pneumoniae} \) in healthy cattle and cattle with diarrhea. Microb Drug Resist. 2021;27(11):1457–69.
7. Kareem SM, Al-Kadmy IM, Kazal SS, Ali AN, Aziz SN, Mahkanta RR, et al. Detection of \( \text{gyrA} \) and \( \text{parC} \) mutations and prevalence of plasmid-mediated quinolone resistance genes in \( \text{Klebsiella pneumoniae} \). Infect Drug Resist. 2021;14:555–63.
8. Hetta HF, Al-Kadmy I, Khazaal SS, Abbas S, Shuaib A, El-Mokhtar MA, et al. Anti-biofilm and antivirulence potential of silver nanoparticles against multidrug-resistant \( \text{Acinetobacter baumannii} \). Sci Rep. 2021;11(1):10751.
9. Uruen C, Choop-Escuin G, Tommassen J, Mainair-Jaime RC, Arenas J. Biofilms as promoters of bacterial antibiotic resistance and tolerance. Antibiot (Basel). 2020;10(1):3.
10. Bai X, Nakatsu CH, Bhunia AK. Bacterial biofilms and their implications in pathogenesis and food Safety. Foods. 2021;10(9):2117.
11. Holden ER, Yasir M, Turner AK, Wain J, Charles KG, Webber MA. Massively parallel transposon mutagenesis identifies temporally essential genes for biofilm formation in \( \text{Escherichia coli} \). Microb Genom. 2021;7(11):000673.
12. Dantas ST, Rossi BF, Bonsaglia EC, Castilho IG, Hernandez RT, Fernandes A, et al. Cross-contamination and biofilm formation by \( \text{Salmonella enterica} \) serovar Enteritis on various cutting boards. Foodborne Pathog Dis. 2018;15(2):81–5.
13. Lu J, Li L, Pan F, Zuo G, Yu D, Liu R, et al. \( \text{pagC} \) is involved in \( \text{Salmonella pullorum} \) OMVs production and affects biofilm production. Vet Microbiol. 2020;247:108778.
14. Nesse LL, Sekse C, Berg K, Johannessen KC, Solheim H, Vestby UK, et al. Potentially pathogenic \( \text{Escherichia coli} \) can form a biofilm under conditions relevant to the food production chain. Appl Environ Microbiol. 2014;80(7):2042–9.

**Data Availability**

The data of the current study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

This study were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. All experimental protocols of this study were approved by the Al-Qasim Green University, Babylon, Iraq, and all methods were carried out in compliance with the ARRIVE guidelines.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Received:** 22 June 2022 / **Accepted:** 20 September 2022

**Published online:** 11 October 2022
15. Stanford K, Tran F, Zhang P, Yang X. Biofilm-forming capacity of *Escherichia coli* isolated from cattle and beef packing plants: Relation to virulence attributes, stage of processing, antimicrobial interventions, and heat tolerance. Appl Environ Microbiol. 2021;87(23):e0112621.

16. Desouky EM, Deif N, Eljakee HJK. Isolation and identification of the most common bacteria isolated from intestine of broiler chickens in Egypt. J Appl Vet Sci. 2021;6(4):23–7.

17. Angaali N, Vemu L, Padmarsi C, Mamidi N, Teja VDO. Direct identification and susceptibility testing of Gram-negative bacteria from turbid urine samples using VITEK2. J Lab Physicians. 2018;10(03):299–303.

18. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 31st ed. CLSI supplement M100-S31. Wayne: Clinical and Laboratory Standards Institute; 2021.

19. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268–81.

20. Rakhi L, Ganagshettappan N, Shantala GB, Nandan BR, Sinha D. Study of biofilm formation in bacterial isolates from contact lens wearers. Indian J Ophthalmol. 2020;68(1):23.

21. Al-Ouqaili MTS, Majeed YH, Al-Ani SK. SEN virus genotype H distribution in β-thalassemic patients and in healthy donors in Iraq: Molecular and physiological study. PLoS Negl Trop Dis. 2020;14(6):e0007880.

22. Yang X, Sun H, Fan R, Fu S, Zhang J, Matussek A, et al. Genetic diversity of the intimin gene (*eae*) in non-O157 *Shiga toxin-producing Escherichia coli* strains in China. Sci Rep. 2020;10(1):3275.

23. Brahmanianfard H, Derakhshandeh A, Nazir Z, Khatibadi Farahani R. Prevalence, virulence factor and antimicrobial resistance analysis of *Salmonella enteritidis* from poultry and egg samples in Iran. BMC Vet Res. 2021;17(1):196.

24. Hussen RA, Al-Ouqaili MT, Majeed YH. Detection of Helicobacter *Pylori* infection by invasive and non-invasive techniques in patients with gastrointestinal diseases from Iraq: A validation study. PLoS ONE. 2021;16(8):e0256593.

25. Al-Ouqaili MT, Musleh MH, Al-Kubaisi SMA. Depending on HPLC and PCR, detection of aflatoxin B1 extracted from *Aspergillus flavus* strains and its cytotoxic effect on AFB treated-hematopoietic stem cells obtained from human umbilical cord. Asian J Pharm. 2018 (Suppl);12(3):S1048.

26. El-Seedy FR, Abed AH, Yanni HA, Abd El-Rahman SA. Prevalence of *Salmonella* and *E. coli* in neonatal diarrheic calves. Beni Suef Univ J Basic Appl Sci. 2016;5(1):45–51.

27. Mousa WS, Shams UH. Prevalence, antimicrobial resistance and substantial virulence-associated genes of *Escherichia coli* isolated from colibacillosis in neonatal calves in Egypt. J Microbiol Biotechnol Food Sci. 2020;9(6):1145–50.

28. Sekhar S, Ranjan R, Singh CV, Kumar P. Prevalence. Clinicohaemato-Biochemical alterations in colibacillosis in neonatal calves. Int J Curr Microbiol App Sci. 2017;6:3192–8.

29. Tadesse N. Prevalence and multidrug resistance profiles of *Escherichia coli* in Dairy Farms. Int J Vet Sci Res. 2020;6(2):142–7.

30. Mohammed SA, Marouf SA, Erfana AM, El JK, Hessain AM, Dawoud TM, et al. Risk factors associated with *E. coli* causing neonatal calf diarrhea. Saudi J Biol Sci. 2019;26(5):1084–8.

31. Manjushree TR, Sethulekshmi C, Latha C, Mathew B, Gleeja VL. Characterization and antibiogram profile of Enterocaggregative *Escherichia coli* isolated from calves with diarrhoea and environmental sources in Emakulam, Kerala. J Pharm Innov. 2019;8(9):34–8.

32. Gebekeyhu A, Taye M, Abebe R. Isolation, molecular detection and antimicrobial susceptibility profile of *Salmonella* from raw cow milk collected from dairy farms and households in southern Ethiopia. BMC Microbiol. 2022;22:284.

33. Aworh MK, Kwaga JK, Hendriksen RS, Okolocha EC, Thakur S. Genetic relatedness of multidrug resistant *Escherichia coli* isolated from humans, chickens and poultry environments. Antimicrob Resist Infect Control. 2021;10:58.

34. Ma E, Xu S, Tang Z, Li Z, Zhang L. Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. Biosaf Health. 2021;3(01):32–8.

35. Rozwadowski M, Gawel D. Molecular factors and mechanisms driving multidrug resistance in uropathogenic *Escherichia coli*—An update. Genes. 2022;13(8):1397.

36. Algarni S, Ricke SC, Foley SL, Han J. The dynamics of the antimicrobial resistance mobilome of *Salmonella enterica* and related enteric bacteria. Front Microbiol. 2022;13:859854.