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Original article

PHENOTYPIC EVALUATION OF ANTIBIOTIC RESISTANCE AND PATHOTYPING OF *ESCHERICHIA COLI* ISOLATED FROM HEALTHY CAMELS IN MASHHAD

M. AMIRI[1], M. JAMSHIDIAN-MOJAVER[2], H. FARZIN[2] & M. ALIMOLAEI[3]

[1]Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran; [2]Mashhad Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran; [3]Research and Development Department, Research and Production Department of Biological Products, Kerman branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran

Summary

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This study aimed to investigate the antibiotic resistance and pathotyping of *Escherichia coli* isolated from healthy camels in farms around Mashhad, Iran. A total of 80 faecal swab samples were collected over a three-month period. Samples were cultured on MacConkey agar, and isolates were confirmed through biochemical tests. Pathotyping was conducted using virulence genes stx_1, stx_2, *eaeA*, *STI*, *LTI*, and *ipaH*. Results showed the following distribution among the 80 isolates: 14 isolates (17.5%) positive for the *stx1* gene, 9 isolates (11.25%) positive for the *stx2* gene, 15 isolates (18.75%) positive for the *eae* gene, 2 isolates (2.5%) positive for the *LT* gene, 6 isolates (7.5%) positive for the *ST* gene. None of the isolates (0%) tested positive for the *ipaH* gene. Antibiotic susceptibility testing revealed neomycin as the most effective antibiotic with 100% sensitivity. In contrast, complete resistance (100%) was observed to tetracycline and florfenicol. Streptomycin and chloramphenicol were highly effective, while gentamicin and erythromycin showed low sensitivity. Ceftazidime and ampicillin displayed moderate efficacy. The findings highlighted the pathogenic diversity of *E. coli* strains in camels and the alarming levels of antimicrobial resistance. The presence of multiple virulence genes suggests the involvement of a mix of pathotypes with potential implications for both animal and human health. The results also emphasise the need for targeted antimicrobial strategies based on specific resistance profiles.

Key words: antibiotic resistance, camel, disk diffusion method, *Escherichia coli*, Mashhad

INTRODUCTION

The presence of antibiotic residues occurs when, after the use of antibiotics, traces of these substances remain in animal products, which can then be consumed by

humans. These residues may enter the human body and, over time, lead to bacterial resistance and other health issues. Excessive or inappropriate use of antibiotics can significantly increase the prevalence of antibiotic residues that may be found in various pathogenic agents affecting livestock, parasites, and other harmful organisms (Amiri, 2019).

Consumption of diverse animal products derived from both large and small ruminants has led to the development of various animal production industries. This, in turn, has drawn attention to the impact of such practices on both public health and the economy. From a public health perspective, ruminants can serve as reservoirs that facilitate the transfer of antibiotic residues through animal-derived food products, thus playing a role in the spread of resistance. From an economic standpoint, this problem can cause serious harm to the livestock and food industries, resulting in substantial financial losses (Amiri, 2017).

Escherichia coli (*E. coli*) plays a crucial role in the gut microbiome of humans and other warm- blooded animals. Most *E. coli* strains are non-pathogenic, while the pathogenic strains are classified based on the presence or absence of virulence factors (Msolo *et al*., 2020). Among these, the enteric pathogenic strains include six pathotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) which are responsible for different gastrointestinal infections and diseases (Pokharel *et al*., 2023). Enterotoxigenic *Escherichia coli* (ETEC) are Gram-negative bacteria, particularly recognised as a major cause of diarrhoea in humans, infants, and animals. ETEC is prevalent in developing countries and is a significant factor in causing traveler's diarrhoea as well as an estimated 380,000 annual deaths in these regions (Pokharel *et al.*, 2023).

ETEC causes illness by producing two types of enterotoxins. The heat-labile enterotoxin (*LT*) is similar to the cholera toxin and has a molecular weight of approximately 80,000 Da. The genes responsible for producing this toxin are located on a plasmid and are encoded by the *eltAB* gene. The toxin consists of two subunits, A and B. Subunit A activates the enzyme adenylate cyclase in intestinal cells, leading to increased cyclic AMP (cAMP) production (Youmans *et al.*, 2014). This, in turn, causes excessive secretion of water and electrolytes into the intestines, resulting in diarrhoea. Subunit B binds the toxin to the ganglioside GM1 receptor located on the surface of epithelial cells in the small intestine. The heat-stable enterotoxin (*ST*) has a molecular weight between 1,500 and 4,000 Da and unlike LT, is resistant to high temperatures (Habets *et al.*, 2020). It is also genetically regulated and induces changes in the function of intestinal epithelial cells, promoting fluid secretion. These toxins directly cause an increase in water and electrolyte secretion in the intestines, leading to severe diarrhoea (Badouei *et al.*, 2024). One of the groups of *Escherichia coli* is Shiga toxin-producing *Escherichia coli* (STEC), which includes the subgroup enterohemorrhagic *Escherichia coli* (EHEC). Shiga toxins (*Stx1* and *Stx2*) produced by these bacteria cause serious diseases in humans by inhibiting protein synthesis in the host cells, leading to cellular damage.

The STEC strains are classified into five seropathotypes, from A to E (Reshadi *et al.*, 2021). Bacterial resistance to anti-

biotics typically occurs through mechanisms such as drug-degrading enzymes, changes in bacterial permeability to the drug, alterations in drug receptors on the bacterial surface, modifications in the bacterial cell wall structure, and the acquisition of alternative metabolic pathways that compensate for the drug-inhibited reaction (Croxen *et al.,* 2010). This resistance can arise either through spontaneous mutations in the bacterial genetic material or through the transfer of plasmids from one bacterium to another (Lyimo *et al.,* 2016). Due to various factors such as the widespread and excessive use of antibiotics by humans, the use of antibiotics to treat viral infections, failure to complete antibiotic treatment courses, the preference for monotherapy over combination therapy, the lack of rapid diagnostic tests to identify infectious agents, and the misuse of antibiotics as feed additives or prophylactics in the livestock industry (including aquaculture, animal husbandry, and agriculture), antibiotic resistance has rapidly increased (Lyimo *et al.*, 2016). If antibiotic resistance is not controlled and continues to rise, we will face an increase in antibiotic-resistant bacterial species and higher treatment failure rates in managing infections caused by resistant microbes (Naderi *et al*., 2024).

MATERIALS AND METHODS

A total of 80 faecal swab samples were collected over a period of three months from healthy camels on various farms in Mashhad, Iran. Initially, the samples were streaked on MacConkey agar (Merck, Germany) and incubated at 37 °C. After 24 hours, in the presence of lactose-positive, smooth, round, and convex colonies of *Escherichia coli*, confirmation was performed through biochemical tests, including urease, Simmons citrate, TSI, and SIM (Merck, Germany) tests.

Antimicrobial resistance phenotyping

Phenotypic antibiotic resistance was determined for all strains using the Kirby-Bauer disk diffusion method, following the guidelines of CLSI (2025). To determine the sensitivity and resistance of *Escherichia coli* strains isolated from 80 camels against the antibiotics ceftazidime-clavulanic acid (CZA; 30/10 µg), ampicillin (AMP; 30 µg), gentamicin (GM; 10 µg), streptomycin (S; 10 µg), kanamycin (K; 30 µg), enrofloxacin (ENR; 5 µg), tetracycline (TE; 30 µg), oxytetracycline (OTC; 30 µg), florfenicol (FF; 30 µg), chloramphenicol (C; 30 µg), and cefotaxime (CTX; 30 µg) (Padtan Teb, Iran), antibiogrammes were performed using the agar disk diffusion method. The target bacterium was cultured on a specific medium, and after incubation, a colony from each isolate is selected and transferred to Mueller-Hinton broth (Merck, Germany). Once the turbidity reached the 0.5 McFarland standard, a sterile swab was used to transfer the bacterial suspension onto Mueller-Hinton agar plates (Merck, Germany) by lawn streaking. The antibiotic disks were then placed at appropriate distances on the agar surface using sterile forceps. After 24 h of incubation at 37 °C, resistance or sensitivity was determined by measuring the inhibition zone, and the results were interpreted according to CLSI (2025) guidelines. The quality control of antimicrobial susceptibility testing was performed using the *E. coli* ATCC 25922 standard strain.

DNA extraction

In this study, the boiling method was used for DNA extraction. The bacteria were cultured on Luria- Bertani agar (Merck,

Table 1. Specific primers for detection of pathotypes genes in *E. coli*

Gene	Sequence (5′–3′)	PCR condition	Product size (bp)	Reference
stx_1	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	35 cycles, starting with 95°C for 60 s, followed by 65 °C for 120 s in the first 10 cycles, gradually reducing to 60 °C by cycle 15. The extension at 72 °C lasts 90 s for cycles 1–25, and 150 s for cycles 25–35	180	Vidal *et al.* (2005)
stx_2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	Same as above	255	Vidal *et al*, (2005)
eae	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	Same as above	384	Askari Badouei *et al.* (2015)
st	ATTTTTGTGCGCAACATGCTT CACCCGGTACAAGCAGGATT	30 cycles: 95°C, 30 s; 60°C, 30 s; 72°C, 30 s	190	(Alizade *et al*., 2017)
lt	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTTT	30 cycles: 95°C, 30 s; 60°C, 30 s; 72°C, 30 s	450	Alizade *et al*. (2017)
ipaH	CTCGGCACGTTTTAATAGTCTGG GTGGAGAGCTGAAGTTTCTCTGC	35 cycles: 94°C (90 s), 60°C (90 s), 72°C (90 s)	600	Naderi *et al.* (2024)

Germany) and incubated at 37 °C for 24 hours. After the incubation period, the obtained single colonies were used for DNA extraction. First, 400 μL of sterile distilled water was transferred into sterile microtubes. Then, a single colony was dissolved in the water. The microtubes were placed in a heating block at 98 °C for 10 min and were then cooled in a freezer at −20 °C for 5 min. Finally, the microtubes were centrifuged at 13,000 rpm for 2 min (Amiri *et al*, 2019).

PCR for detection of enteropathogenic E. coli

For performing PCR, total volume of 20 μL, containing 10 μl of master mix (Denmark-Ampliqon), 0.1 μl of each primer (Sinaclon-Iran), 3 μl of DNA from the desired samples, and deionised water were used to make a reaction volume of 20 μl. The specific primers and optimal thermocycler conditions for performing PCR to identify the genes responsible for the ETEC, EIEC, EHEC, STEC, and EPEC pathotypes are provided in Table 1.

The *stx1, stx2, eae, LT-I, ST-I,* and *ipaH* genes were analysed. The *E. coli* strains Sakaï (*eae*$^+$, *stx1*$^+$, *stx2*$^+$), *E. coli* 85b (*ipaH*$^+$), *E. coli* H10407 (*LT-I*$^+$, *ST-I*$^+$), were used as positive controls, while the *E. coli* strain MG1655 was applied as a negative control in virulence profiling.

RESULTS

Based on the results obtained in this study, Table 2 illustrates the effectiveness of various antibiotics against specific bacterial strains, as determined by the percentage of sensitive, intermediate, and resistant responses. Neomycin showed 100% sensitivity with no intermediate or resistant strains, making it the most effective antibiotic in this study. Streptomycin and chloramphenicol also demonstrated strong effectiveness, with 80% sensitivity and relatively low resistance rates. Tetracycline and florfenicol displayed 100% resistance, indicating that they are completely ineffective against the tested bacteria. Gentamicin and erythromycin also showed very low sensitivity (0% and 7%, respectively), suggesting limited clinical value in this context. Ceftazidime and ampicillin exhibited moderate levels of

Table 2. The rate of antibiotic resistance in *E. coli* isolates from healthy camels (n=80)

Chemotherapeutics	S (%)	Confidential limits (CL)	R+I (%)	Confidential limits (CL)
Cefotaxime-clavulanic acid	52.5	41.6%–63.4%	17.5	10.7%–27.7%
Ceftazidime	40	29.3%–50.7%	60	49.6%–70.4%
Ampicillin	50	39.0%–61.0%	50	39.0%–61.0%
Gentamicin	0	0	20	12.7%–29.6%
Streptomycin	80	70.6%–89.4%	20	12.7%–29.6%
Clindamycin	60	49.6%–70.4%	40	29.3%–50.7%
Neomycin	100	95.5%–100%	0	0
Erythromycin	7	2.9%–14.0%	43	32.9%–53.8%
Tetracyclin	0	0	100	95.5%–100%
Florfenicol	0	0	100	95.5%–100%
Chloramphenicol	80	70.6%–89.4%	20	12.7%–29.6%

S: sensitive; R: resistant; I: intermediate.

sensitivity and resistance, suggesting they may still be viable options depending on the specific infection and patient response.

A total of 80 *E. coli* isolates were collected from 80 rectal swab samples in this study. The virulence gene-positive isolates were categorised into different pathotypes based on their virulence gene patterns: STEC (stx_1^+ and/or stx_2^+), EHEC (eae^+ and stx_1^+ and/or stx_2^+), EIEC ($ipaH^+$), and ETEC ($LT\text{-}I^+$ and/or $ST\text{-}I^+$). The distribution of various genes among the 80 *E. coli* isolates was as followed: 14 isolates (17.5%) were positive for the stx_1 gene, 9 isolates (11.25%) were positive for the stx_2 gene, 15 isolates (18.75%) for the *eae* gene, 2 isolates (2.5%) for the *LT* gene, 6 isolates (7.5%) for the *ST* gene while none tested positive for the *ipaH* gene (Table 3; Fig. 1 and 2).

Table 3. The rate of virulence genes in *E. coli* isolates from healthy camels

Genes	Number (%) of *E. coli* isolates
stx_1	14 (17.5)
stx_2	9 (11.25)
eae	15 (18.75)
LT	2 (2.5)
ST	6 (7.5)
ipaH	0

Table 4. Virulence gene profiles (pathotypes) in *E. coli* isolates from healthy camels

Pathotypes	Number (%)
eae (EPEC)	15 (18.75)
stx_1 (STEC)	14 (17.5)
stx_2 (STEC)	9 (11.25)
stx_1/stx_2 (STEC)	2 (2.5)
stx_1/*eae* (EHEC)	2 (2.5)
stx_2/*eae* (EHEC)	5 (6.25)
stx_1/stx_2/*eae* (EHEC)	1 (1.25)
lt/*st* (ETEC)	6 (7.5)
eae/*st* (EPEC/ETEC)	3 (3.75)

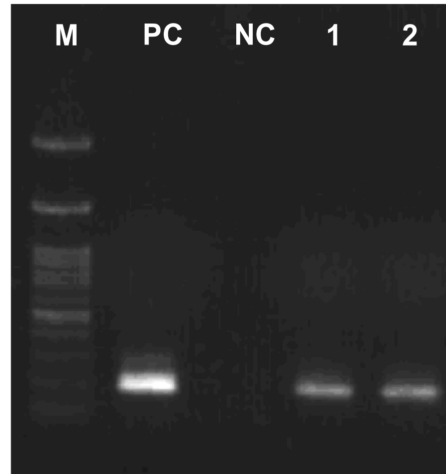

Fig. 1. Electrophoresis of PCR products associated to *stx1* genes (180 bp). M: DNA ladder (100 bp), PC: positive control (*E. coli* strain Sakaï); NC: negative control for *stx1* gene; lanes 1–2: positive samples for the *stx1* gene.

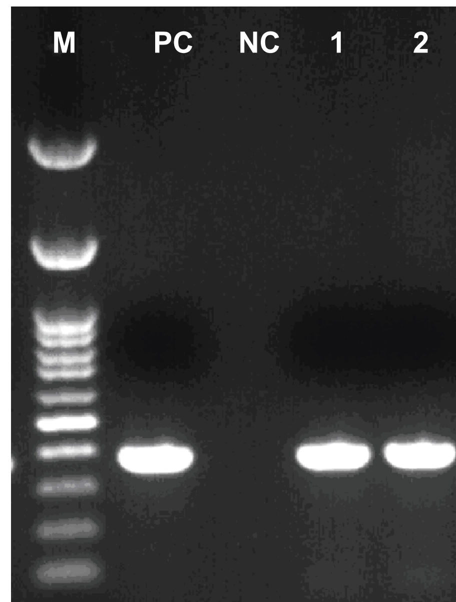

Fig. 2 Electrophoresis of PCR products associated to *eae* genes (384 bp). M: DNA ladder (100 bp), PC: positive control (*E. coli* strain Sakaï); NC: negative control for *eae* gene; lanes 1–2: *eae* gene positive samples.

The examination of different virulence gene profiles of *E. coli* isolates showed that the most prevalent virulence gene profile was *eae* (EPEC) with 15 isolates (18.75%), followed by stx_1 (STEC) with 14 isolates (17.5%) and stx_2 (STEC) with 9 isolates (11.25%). Combination profiles were also common. For example, 2 isolates (2.5%) were stx_1/stx_2 (STEC). Each of combinations stx_1/eae (EHEC) and *stx* /*eae* (EHEC) were encountered also in 2 isolates (2.5%). The combination of all three genes, $stx_1/stx_2/eae$ (EHEC) was seen in 1 isolate (1.25%) only (Table 4). Other profiles included *lt*/*st* (ETEC) with 6 isolates (7.5%) and *eae*/*st* (EPEC/ ETEC) with 3 isolates (3.75%). These results indicate a high diversity in the virulence gene profiles among these isolates.

DISCUSSION

Escherichia coli is a symbiotic bacterium and a part of the normal microflora of the gastrointestinal tract in humans and warm-blooded animals. In healthy humans, it colonises the intestine within a few hours after birth. *E. coli* has various pathotypes (Braz *et al*., 2020); some of them can cause severe diseases and even death in individuals. Pathogenic strains may act as intestinal pathogens, damaging in the intestinal area and causing diarrhoea, or as extra-intestinal pathogens, leading to various infections such as urinary tract infections, neonatal meningitis, and septicaemia in humans and young individuals. These strains are transmitted through contaminated water, food, and contact with infected individuals or animals (Kaper *et al*., 2004).

In this study, *Escherichia coli* isolates obtained from healthy samples were evaluated to assess their antibiotic resistance patterns, presence of virulence genes, and distribution of different pathotypes. The results indicated a significant presence of potentially pathogenic strains even in apparently healthy camels, highlighting the importance of ongoing surveillance and monitoring of these bacteria. Regarding antibiotic resistance, the highest sensitivity was observed against neomycin (100%), while complete (100%) resistance was noted to tetracycline and florfenicol. The high levels of resistance to commonly used antibiotics raise serious concerns about the effectiveness of conventional treatments and the spread of resistant strains. The notable rates of intermediate or complete resistance to certain antibiotics, such as gentamicin, further emphasise the need for optimisid antibiotic stewardship programmes. The analysis of virulence genes revealed important findings. The *eae* gene, associated with intestinal epithelial adherence, was the most frequently detected virulence factor (18.75%). The stx_1 and stx_2 genes, responsible the production of Shiga toxins, were found in 17.5% and 11.25% of isolates, respectively. These findings suggest that even asymptomatic carriers could harbour strains capable of causing severe illnesses. In terms of pathotype distribution, EPEC (enteropathogenic *E. coli*, characterized by *eae* presence) was the most common. STEC (Shiga toxin-producing *E. coli*), with either stx_1, *stx2*, or both. The prevalence of EHEC (enterohemorrhagic *E. coli*, carrying both *stx* and *eae*) strains was also significant. ETEC (enterotoxigenic *E. coli*), characterised by the presence of *lt* and *st* genes, was identified in 7.5% of isolates. The detection of mixed pathotypes (such as *eae*/*st*) highlights the genetic diversity and pathogenic potential among the isolates. Overall, the findings demonstrate that healthy animals can serve as reservoirs for antibiotic-resistant

and potentially pathogenic *E. coli* strains. This underscores the necessity for rigorous hygiene control measures, rational antibiotic use, and continuous screening to minimise the risk of disease outbreaks. Further studies employing advanced molecular techniques and more detailed phenotypic and genotypic characterisation would provide deeper insights into the epidemiology and risk management of *E. coli* infections.

The findings from the present study is in line with the report of Naderi *et al.* (2024) in diarrhoeic and healthy calves in Iran, where the prevalence of virulence genes was higher in healthy calves (21.17%) compared to diarrhoeic ones (8.82%). In both cases, the presence of potentially pathogenic pathotypes in asymptomatic hosts underscores the importance of continuous surveillance.

In terms of antibiotic resistance, the observed 100% susceptibility to neomycin along with complete resistance to tetracycline and florfenicol (100%) differ from the findings of Naderi *et al.* (2024) affirming highest resistance to trimethoprim-sulfamethoxazole (49.01%) and lack of resistance to florfenicol (0%). Such discrepancies may be attributed to differences in host species, geographic region, and selective pressure from local antibiotic usage. The comparison with camel isolates from the study of Diab *et al.* (2021) showed that the highest resistance was to oxytetracycline (53.8%) and the lowest to amoxicillin and clindamycin (0%). The full resistance to tetracycline observed in our study parallels these findings and suggests that frequent veterinary use of this drug may drive resistance in *E. coli* from animal hosts.

Regarding virulence genes, the *eae* gene was the most frequent (18.75%) in the present study, consistent with fingings of Asadi *et al.* (2024) in human patients, who identified EPEC as one of the dominant pathotypes. The presence of stx_1 (17.5%) and stx_2 (11.25%) in our isolates is comparable to the findings of Diab *et al.* (2021) in camels, although their focus was mainly on antibiotic resistance rather than virulence profiles.

Overall, evidence from different hosts and geographic settings consistently indicates that pathogenic and antibiotic-resistant *E. coli* can occur in healthy carriers, acting as reservoirs for disease. This is particularly important in camels, which have close contact with humans and other livestock, facilitating cross-species transmission. Rigorous hygiene management, prudent antibiotic use, and continuous monitoring are therefore essential. Future research should apply advanced molecular techniques and broader genotypic characterisation to identify transmission pathways and factors influencing resistance development.

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Correspondence:

Mohadese Amiri
Razi Vaccine and Serum Research Institute,
Agricultural Research, Education and
Extension Organization (AREEO),
Mashhad, Iran
e-mail: mohadeseamiri1990@gmail.com