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Shiga toxin-producing *Escherichia coli* plasmid diversity reveals virulence potential and control opportunities in animal hosts

Ali Nemati, Mahdi Askari Badouei✉, Gholamreza Hashemitabar & Maryam Hafiz

Shiga toxin-producing *Escherichia coli* (STEC) is a significant public health concern, with plasmids playing a key role in its pathogenicity. This study investigates the relationship between plasmid types, virulence genes, and host specificity in 96 STEC strains isolated from cattle, sheep, goats, and pigeons in Iran. We examined the distribution of plasmid-encoded virulence genes, including *astA*, *cma*, *cba*, *rhsA1*, *rhsA2*, *rhsC*, *stcE*, *katP*, *toxB*, *espP*, *subAB*, *ihaG*, *LAA iha*, *saa*, *etpD*, *cif*, and *epeA*, along with *ehxA* subtypes and plasmid replicon types using PCR and RFLP analysis. The most frequently detected virulence factors, *rhsC* and *ihaG*, were associated with *ehxA* subtype A and IncFIB/IncK/B plasmids. Pigeon-derived isolates had a unique virulence profile, including *cif* but lacking *ehxA*, suggesting distinct pathogenic mechanisms. *stcE* and *etpD* were exclusive to positive controls O157 isolates, linked to *ehxA* subtype B and IncFIB plasmids. The predominance of *ehxA* subtype A in ruminants highlights potential host-specific associations. The widespread presence of IncFIB plasmids suggests their broad role in STEC pathogenicity. These findings highlight the critical role of plasmid-encoded virulence factors in STEC pathogenesis and underscore the need for targeted surveillance and control strategies tailored to specific host populations.

Keywords Shiga toxin-producing *Escherichia coli*, STEC, Plasmid, Virulence factors, Animal

The primary microorganisms responsible for food contamination are members of the Enterobacterales, with *Escherichia coli* being a significant species[1]. This bacterium is one of the most thoroughly researched and was among the first to have its genome sequenced[2]. It includes six intestinal pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC)[1]. These pathotypes are often implicated in foodborne outbreaks, with STEC being particularly critical for public health due to its potential to cause diarrhea, bloody diarrhea (hemorrhagic colitis), hemolytic uremic syndrome (HUS), and kidney damage[3,4].

STEC have been found in several animal species, which act as natural reservoirs for these bacteria[1]. The primary reservoirs of STEC are cattle, sheep, and goats, which can harbor the bacteria in their gastrointestinal tracts without showing clinical signs[1,5]. Additionally, other animals like pigeons have also been implicated as potential carriers of STEC[6,7]. STEC's disease-causing ability is linked to the production of Shiga-like toxins (Stx), which come in two main types, Stx1 and Stx2, encoded by the *stx1* and *stx2* genes[8]. *stx1* has four variants: *stx1a*, *stx1c*, *stx1d*, and *stx1e*; *stx2* is more diverse, with 15 variants: *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, *stx2g*, *stx2h*, *stx2i*, *stx2j*, *stx2k*, *stx2l*, *stx2m*, *stx2n*, and *stx2o*[9]. Research has shown a link between the *stx* subtype and disease severity, with strains producing *stx2*, especially the *stx2a* subtype, being more associated with severe disease and HUS[10]. This knowledge has informed differential case management and public health strategies based on *stx* profile-derived STEC pathotype[8,11]. Additionally, the *eae* gene, located on the locus of enterocyte effacement (LEE), encodes intimin, an adhesin crucial for gut colonization. Furthermore, many disease-causing STEC strains, such as those belonging to O157 and serogroups O26, O103, O111, and O145, contain mobile genetic elements like plasmids, transposons, phages, and pathogenicity islands. These elements also play a significant role in the severity of the disease[12].

STEC plasmids contain various genes involved in cell adhesion, proteases, toxins, and other potential virulence factors[13–15]. These combinations of virulence factors can increase the risk of severe symptoms like HUS[16]. Large plasmids encoding enterohemolysin (*ehxA*) are found in most STEC isolates, including *E. coli*

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O157 and non-O157 strains such as O26, O103, O113, and O145, all commonly associated with diarrheal disease and HUS[17]. The *ehxA* gene, part of the hemolysin operon on these plasmids, has six subtypes (A to F) and is used as a diagnostic marker for highly pathogenic STEC like the O157 serogroup[16,18]. Plasmid replicon typing is another method for classifying the plasmids based on the variations in replication loci[14]. To date, 17 different plasmid replicon types have been identified in *E. coli*, with the IncF family being among the most frequent in STEC strains that cause severe symptoms[19].

Plasmids play crucial roles in the pathogenesis of STEC; often carrying key virulence genes such as *ehxA*, *espP*, *katP*, and *etpD* that contribute to the severity of human infections[20,21]. Despite increasing knowledge of plasmid-encoded factors, significant gaps remain in our understanding of the diversity of these plasmids, their evolutionary trajectories, and their functional associations with host specificity and pathogenic potential[15,22,23]. Notably, it remains unclear whether specific *ehxA* subtypes or replicon types are consistently associated with certain plasmid-borne virulence genes, or whether particular plasmids exhibit host-specific properties linked to animal reservoirs. Previous studies have characterized plasmids in select STEC lineages, but a comprehensive understanding of plasmid diversity across different hosts is still lacking[22]. Addressing these gaps is critical, as improved knowledge of the interplay between plasmid content, host origin, and virulence could enhance surveillance, support risk assessment, and inform targeted public health interventions[24]. In this study, we conducted a comprehensive analysis of STEC strains from various animal hosts to explore these crucial connections, aiming to advance our understanding of STEC pathogenesis and ultimately reduce the burden of STEC-related diseases.

Results

STEC isolates

Among the 96 STEC isolates analyzed in this study—including 50 previously collected and 46 newly obtained strains—the sources included cattle (35 isolates), sheep (34), pigeons (18), and goats (9), reflecting a diverse range of animal hosts. Regarding the genes *stx1*, *stx2*, *eae*, and *ehxA*, we found the following: *stx1* was present in 29/96 (30.2%, $p<0.05$) isolates, *stx2* in 42/96 (43.7%, $p<0.05$), both *stx1* and *stx2* in 25/96 (26.0%), *eae* in 30/96 (31.2%), and *ehxA* in 56/96 (58.3%). *stx2* subtyping revealed that, among the 42 *stx2*-positive isolates, 18/42 (42.8%, $p<0.05$) had the *stx2f* subtype only, 13/42 (30.9%) had the *stx2a* + *stx2c* + *stx2d* profile, 5/42 (11.9%) had *stx2a* + *stx2c*, 1/42 (2.3%) had *stx2c* + *stx2d*, and 5/42 (11.9%) did not have detectable *stx2* subtypes. The strains were classified into the serogroups O5 ($n=13$), O26 ($n=6$), O80 ($n=2$), O91 ($n=3$), O103 ($n=12$), O111 ($n=3$), O113 ($n=13$), and O128 ($n=9$). For 35 isolates, the O-groups were not determined (Table S1 in supplementary materials).

Virulence genes

The analysis revealed that the most frequently identified virulence genes were *rhsC* (88.5%, 85/96, $p<0.05$), *IhaG* (67.7%, 65/96, $p<0.05$), and *subAB* (48.9%, 47/96). These were followed by *cba* (47.9%, 46/96, $p<0.05$), *LAA iha* (42.7%, 41/96), *cma* (37.5%, 36/96), *cif* (30.2%, 29/96, $p<0.05$), *astA* (29.1%, 28/96, $p<0.05$), *espP* (23.9%, 23/96), *saa* (22.9%, 22/96), *rhsA1* (12.5%, 12/96), *epeA* (11.4%, 11/96), *katP* (9.3%, 9/96), *rhsA2* (6.2%, 6/96), and *toxB* (4.1%, 4/96) (Fig. 1). The genes *stcE* and *etpD* were not detected in any of the isolates, except for the positive control (O157:H7 strain Sakai), which contained both genes.

EhxA subtypes

By *ehxA* subtyping, of 56 *ehxA*-positive isolates, we identified the subtypes A (83.9%, 47/56, $p<0.05$), C (12.5%, 7/56), and D (3.5%, 2/56) in the studied isolates (Fig. 2). *ehxA* subtype B was only detected in the positive control (*E. coli* O157:H7 295 EC-TMU). Subtypes E and F were not observed in any of the isolates. Regarding the association between *ehxA* subtypes and virulence genes, we found that subtype A was linked to three different patterns: *rhsC*, *subAB*, *IhaG*, *LAA iha*; *rhsC*, *espP*, *subAB*, *IhaG*, *saa*, *epeA*; and *cma*, *cba*, *rhsC*, *subAB*, *IhaG*, *LAA iha*. Additionally, subtype C was associated with the pattern *cba*, *rhsA1*, *rhsC*, *katP*, *toxB*, *espP*, *IhaG*, *cif* (Table 1).

Replicon types

Plasmid Inc typing revealed that, among the 76 STEC isolates harboring plasmids, the most common replicon type was FIB (82.8%, 63/76, $p<0.05$), followed by IncK/B (27.6%, 21/76, $p<0.05$), IncB/O (14.4%, 11/76), IncP (10.5%, 8/76), IncY (7.8%, 6/76), IncFIA (3.9%, 3/76), IncI1 (3.9%, 3/76), and IncL/M (1.3%, 1/76) (Fig. 2). Notably, some plasmids contained more than one replicon type. The virulence gene patterns associated with these replicon types were as follows: *astA*, *cma*, *cba*, *rhsC*, *cif* were most commonly detected in IncFIB plasmids; *rhsC*, *subAB*, *IhaG*, *LAA iha* were associated with IncK/B and IncFIB; *rhsC*, *espP*, *subAB*, *IhaG*, *saa*, *epeA* were linked to IncK/B; and *cba*, *rhsA1*, *rhsC*, *katP*, *toxB*, *espP*, *IhaG*, *cif* were found in IncFIB and IncI1 plasmids (Table 1; Fig. 3).

Discussion

The highly variable nature of STEC plasmids prompted an examination of the correlations between their *ehxA* subtypes and replicon types with the virulence genes they carry. These plasmids harbor genes for adhesion, toxins, and other virulence factors, significantly influence the severity of illnesses such as HUS[25]. This investigation aims to elucidate the intricate relationship between specific plasmid types and virulence factors in STEC strains, with a particular focus on whether certain *ehxA* subtypes or plasmid replicon types are associated with the plasmid-encoded virulence genes, as revealed through a comprehensive analysis of STEC strains from various animal hosts.

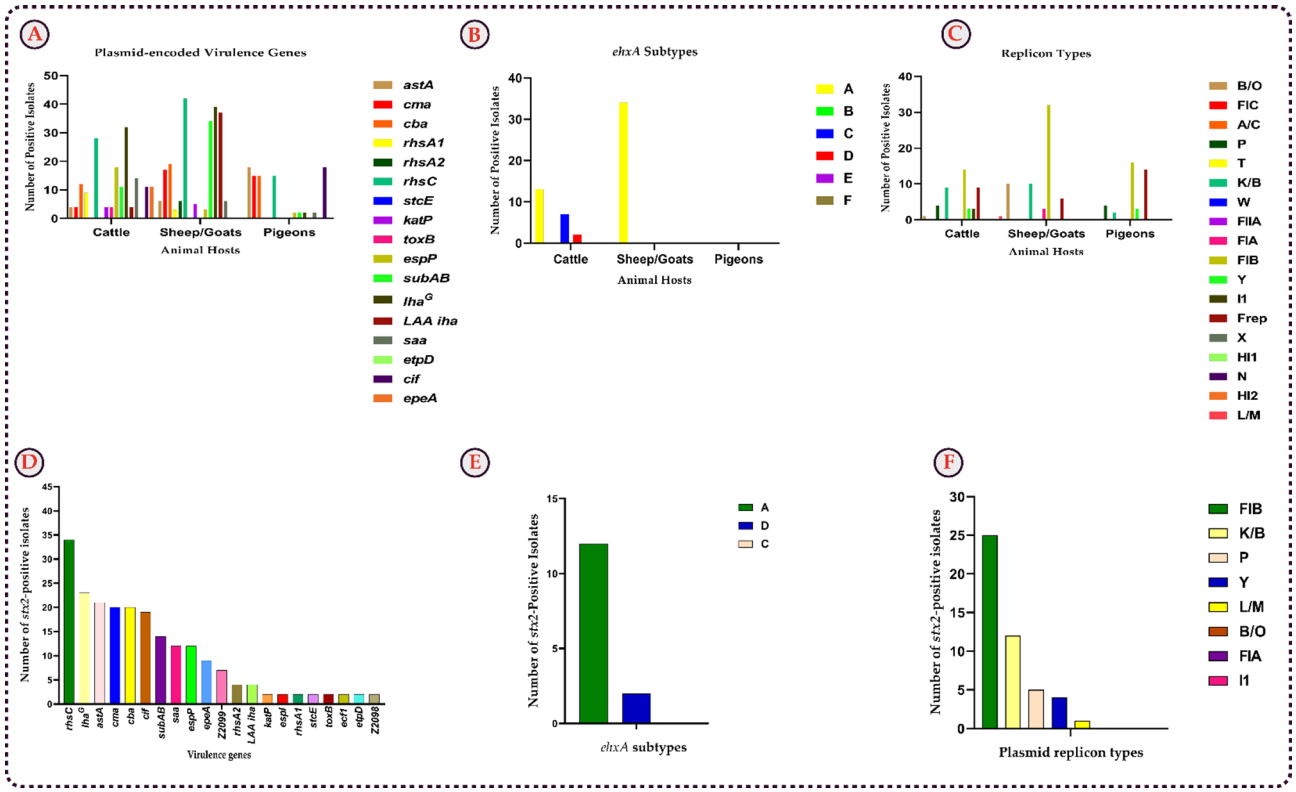

Fig. 1. Distributions of the plasmid-encoded virulence genes, *ehxA* subtypes, and plasmid replicon types in different animal hosts (**A**, **B**, **C**) and *stx2*-positive isolates (**D**, **E**, **F**), respectively. The most prevalent virulence genes detected were *IhaG* in cattle (91.4%, 32/35), *rhsC* in sheep/goats (97.6%, 42/43), and *cif* in pigeons (100%, 18/18) isolates (**A**). Subtype A of the *ehxA* gene was the most common *ehxA* subtype in cattle (37.1%, 13/35) and sheep/goats (79%, 34/43), while pigeons tested negative for the *ehxA* gene (**B**). The highest rates of replicon types were found to be IncFIB in cattle (40%, 14/35), sheep/goats (74.4%, 32/43), and pigeons (88.8%, 16/18) isolates (**C**). Among the 42 *stx2*-positive isolates, *rhsC* (80.9%, 34/42) virulence gene (**D**), subtype A (28.5%, 12/42) of *ehxA* gene (**E**), and FIB (59.5%, 25/42) replicon type (**F**), were detected as the highest rate.

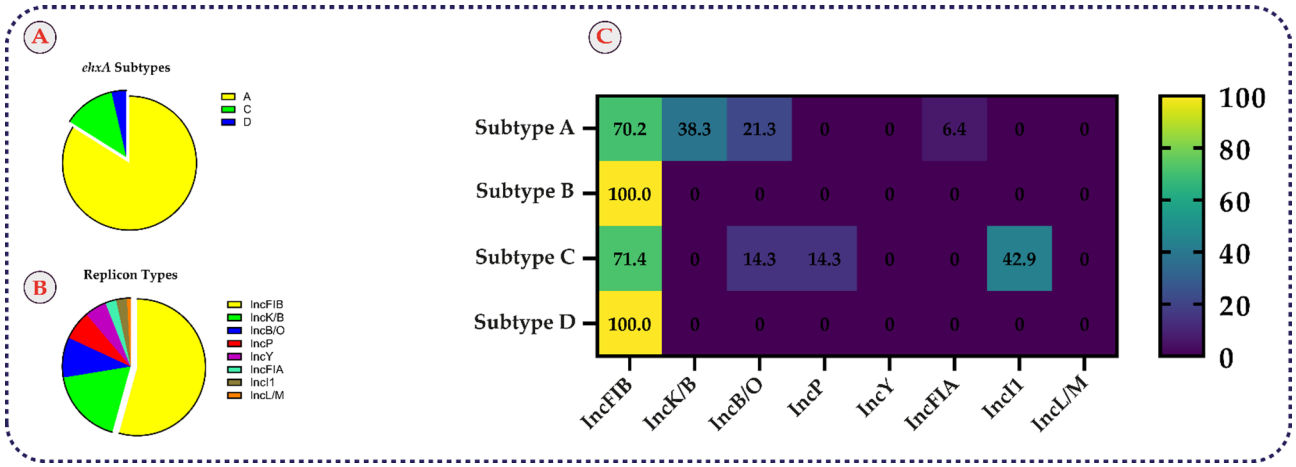

Fig. 2. Dissemination of *ehxA* subtypes, replicon types, and their associations in the 96 STEC isolates in Iran. A total of 56 *ehxA*-positive isolates were identified, with the majority (83.9%) classified as subtype A, followed by subtypes C (12.5%), and D (3.5%) (**A**). Among the 76 STEC isolates containing plasmids, the most common replicon type was FIB (82.8%), with additional types including IncK/B (27.6%), IncB/O (14.4%), IncP (10.5%), and others (**B**). The heat map illustrates the co-occurrence of *ehxA* subtypes and replicon types in these isolates (**C**).

Number	Plasmid-encoded Virulence Genes Pattern	ehxA Subtype	Replicon Type	Host
14	*astA*, *cma*, *cba*, *rhsC*, *cif*	-	FIB*, Frep*	Pigeon*/Cattle*
8	*rhsC*, *subAB*, *IhaG*, *LAA iha*	A	K/B, FIB	Sheep/Goat
8	*rhsC*, *espP*, *subAB*, *IhaG*, *saa*, *epeA*	A*	K/B*	Cattle*
3	*cma*, *cba*, *rhsC*, *subAB*, *IhaG*, *LAA iha*	A	FIB	Sheep
3	*cba*, *rhsA1*, *rhsC*, *katP*, *toxB*, *espP*, *IhaG*, *cif*	C*	FIB, I1*	Cattle

Table 1. Association between plasmid-encoded virulence genes with *EhxA* subtypes and their replicon types in respective plasmids. * Statistically significant (p-value < 0.05).

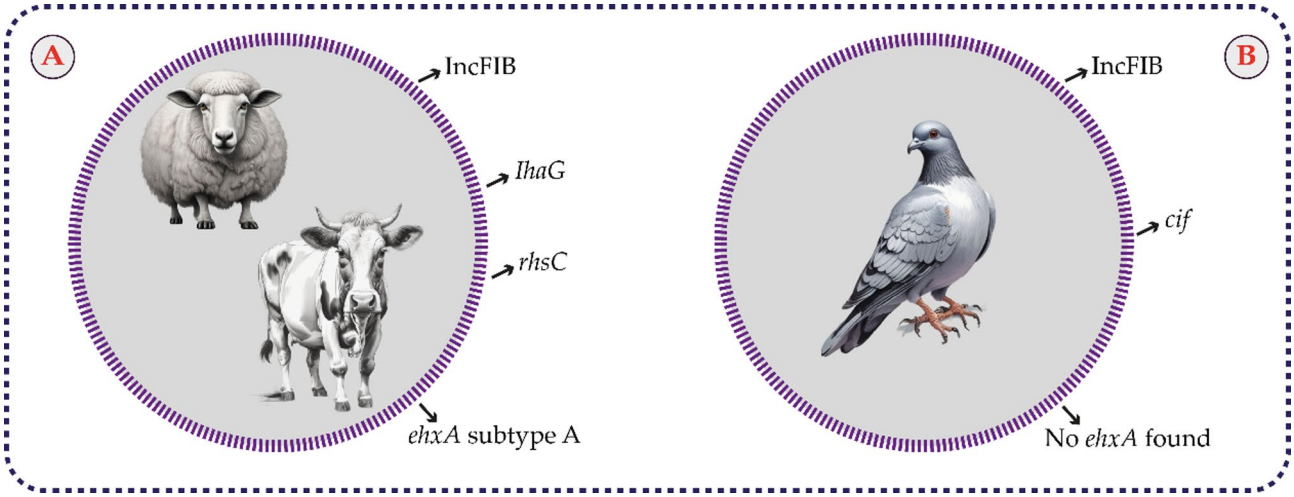

Fig. 3. Features of the plasmids based on the most detected profiles in STEC isolates from cattle, sheep/goats (**A**), and pigeons (**B**).

The analysis revealed that the *rhsC* (88.5%, $p < 0.05$) and *IhaG* (67.7%, $p < 0.05$) genes were the most frequently identified virulence genes among the isolates. The *rhsC* gene is associated with the type VI secretion system (T6SS) in pathogenic *E. coli*, including STEC, which mediates bacterial competition and enhances pathogenicity by aiding intracellular survival against human macrophages[26–28]. Meanwhile, the *IhaG* gene encodes an adhesin crucial for STEC adherence to epithelial cells, facilitating toxin delivery, evasion of peristaltic clearance, and access to host nutrients[29–31]. These two genes were prevalent among the highly pathogenic *stx2*-positive isolates ($p < 0.05$), predominantly linked to plasmids containing *ehxA* subtype A ($p < 0.05$) and IncFIB/IncK/B replicons ($p < 0.05$). Notably, some alleles of the *ihaG* gene may also be encoded on the chromosome, indicating that its presence is not exclusively plasmid-mediated[16]. This finding underscores the significant role of plasmids harboring the *rhsC* gene and, in particular, those encoding the *IhaG* gene in the pathogenesis of STEC strains and emphasizes the necessity for specific approaches to reduce the dangers posed by these highly pathogenic types.

A notable presence of the *cif* virulence gene was found in pigeon-derived STEC isolates, with all such isolates testing positive for *cif* ($p < 0.05$). *cif*, a member of the cysteine protease superfamily, is known to inhibit cell-cycle progression in eukaryotic hosts, suggesting it provides specific advantages in the STEC pathogenic process[32–35]. This observation is consistent with findings by van Hoek et al., who also reported *cif* in all pigeon-derived STEC isolates, indicating a strong correlation between *cif* and the pigeon host[36]. Additionally, plasmids in these pigeon-STEC isolates were of the IncF family, specifically the IncFIB subtype, and notably, these plasmids lacked the *ehxA* gene, making *ehxA* subtyping unfeasible. This points to a distinctive plasmid profile in pigeon-derived STEC, emphasizing unique pathogenic mechanisms that may be involved in these isolates.

The identification of the virulence genes *stcE* and *etpD* was exclusive to positive controls O157 isolates. These genes, encoded on the plasmid O157 (pO157), play a crucial role in STEC pathogenesis. Specifically, O157 utilizes the etp type II secretion system to secrete the metalloprotease *stcE*, which cleaves the serpin C1 esterase inhibitor during human infection[13,37]. Consistent with previous studies, *stcE* and *etpD* were found only in O157 STEC isolates, highlighting their specific association with this serogroup[38,39]. Additionally, these genes were carried on STEC plasmids with *ehxA* subtype B and IncFIB replicon type, emphasizing the distinct plasmid profile of O157 strains and aligning with these reports by Lorenz et al., which identified *ehxA* subtype B specifically in O157 STEC strains[18,40]reinforcing the unique genetic and pathogenic characteristics of these isolates.

The prevalence of *ehxA* subtype A was significantly higher in STEC isolates from sheep ($p < 0.05$) and goats ($p < 0.05$) compared to other animal hosts. Due to limited data on this specific association, further research is strongly recommended to better understand the relationship between *ehxA* subtypes and different animal hosts.

In terms of plasmid characteristics, the IncFIB replicon type was the most frequently detected across all studied animal hosts ($p < 0.05$), including cattle, sheep, goats, and pigeons. This high distribution of IncFIB plasmids in STEC strains aligns with previous studies highlighting their prevalence not only in STEC but also in other *E. coli* pathotypes, such as avian pathogenic *E. coli* (APEC), fecal *E. coli* (AFEC), and uropathogenic *E. coli* (UPEC)[19,41,42]. These findings underscore the widespread occurrence of IncFIB plasmids in *E. coli* strains and their potential role in the pathogenicity of various *E. coli* pathotypes, including STEC.

The most common plasmid-encoded virulence gene profile in pigeon isolates included the IncFIB ($p < 0.05$) and Frep ($p < 0.05$) replicons, along with the *astA*, *cma*, *cba*, *rhsC*, and *cif* ($p < 0.05$) virulence genes, but lacked the *ehxA* hemolysin gene. Conversely, the IncFIB/IncK/B replicons were predominantly found in sheep ($p < 0.05$), goat ($p < 0.05$), and cattle ($p < 0.05$) hosts, along with *ehxA* subtype A ($p < 0.05$) and various other virulence gene profiles. Interestingly, despite differing ecological niches, the plasmid-encoded virulence gene profiles in sheep, goat, and cattle hosts were more similar to each other than to those found in pigeons. Although more research is needed to confirm this, it points to a complex and surprising plasmid landscape. This highlights the importance of further studies to better understand plasmid diversity and its role in STEC pathogenicity across various animals. The similarity in plasmid profiles among sheep, goats, and cattle, compared to pigeons, suggests that these animals can be grouped by their plasmid-related traits, even though the plasmids themselves vary.

STEC carry large plasmids of various sizes, often 75–100 kb. These plasmids are highly heterogeneous, harboring different collections of genes[43]. Our study underscores the critical role of plasmid-encoded virulence factors in the pathogenicity of STEC strains across various animal hosts. We identified a significant prevalence of the *rhsC* and *IhaG* genes in highly pathogenic *stx2* positive isolates, predominantly associated with plasmids containing the *ehxA* subtype A and IncFIB/IncK/B replicons. The exclusive detection of the *cif* gene in pigeon-derived isolates, with a unique plasmid profile lacking the *ehxA* gene, suggests distinctive pathogenic mechanisms in these strains. Similarly, the *stcE* and *etpD* genes were found solely in O157 isolates, linked to plasmids with the *ehxA* subtype B and IncFIB replicon type, reinforcing their specific association with this serogroup. Our findings also reveal that the *ehxA* subtype A is more prevalent in STEC isolates from sheep and goats, highlighting the need for further investigation into the relationship between *ehxA* subtypes and different animal hosts. The widespread occurrence of IncFIB plasmids across all studied hosts aligns with their known prevalence in other *E. coli* pathotypes, suggesting a broad role in *E. coli* pathogenicity. The observed similarities in plasmid-encoded virulence gene profiles among sheep, goat, and cattle hosts, despite their differing ecological niches, indicate a complex and heterogeneous plasmid landscape. These insights necessitate further research to fully elucidate the diversity of plasmid-encoded virulence factors and their implications for STEC pathogenicity, essential for developing targeted strategies to mitigate the risks posed by highly pathogenic STEC variants.

Methods

STEC isolates investigated

A total of 96 STEC strains were investigated in this study. Of these, 50 collection strains were obtained from various provinces in Iran, including Tehran, Razavi Khorasan, Semnan, Mazandaran, and Khuzestan. These isolates were collected from different animal hosts (cattle, sheep, goats, and pigeons) between 2015 and 2018, as documented in our previous studies[6,44]. In addition, 46 new STEC strains were included, which were isolated from 75 fecal samples from cattle, 70 from sheep, and 15 from goats in Razavi Khorasan province between April and June 2022 (strain characteristics are provided in Table S1).

DNA extraction

All *E. coli* isolates were confirmed by culturing on MacConkey agar and Eosin-Methylene Blue (EMB) agar, followed by biochemical tests. Each isolate was then cultured on Luria-Bertani (LB) agar and incubated for 24 h at 37 °C. After overnight incubation, total genomic DNA was extracted using a boiling method. Briefly, a loopful of confluent growth from the LB agar culture was suspended in 350 µL of molecular-grade water in sterile microtubes and boiled for 10 min. The samples were then centrifuged at 15,000×g for 15 min, and the supernatants were used as templates for end-point PCR assays. DNA samples were stored at − 20 °C until further analysis.

Strains' characterisations

To confirm the STEC genotype for both collection and new strains, we utilized a multiplex PCR targeting *stx1*, *stx2*, *eae*, and *ehxA*, as previously described[45]. For detecting *stx* in pigeon isolates, a single primer pair was used to amplify *stx2f*[7]. Additionally, we performed three separate PCR assays to identify the key *stx2* subtypes associated with human disease: *stx2a*, *stx2c*, and *stx2d*[46]. *E. coli* O157 strain (ATCC 35218) and *E. coli* O157 strain Sakai (ATCC BAA-460) served as positive controls, while the strain T5B-Ir (KJ397538) from the Ferdowsi University of Mashhad (FUM) collection was used as a control for *stx2f*.

Our study focused on 14 important serogroups associated with human diseases: O26, O45, O55, O80, O91, O103, O104, O111, O113, O121, O128, O145, O146, and O157. We used PCR to identify these serogroups specifically in new STEC isolates using the various panels described earlier[47,48]. Additionally, we double-checked the collection strains for the same serogroups. We also tested all isolates for the O80 and O146 serogroups using methods outlined in previous studies[49,50]. *E. coli* O157 (295 EC-TMU) and FUM collection strains were used as positive controls.

PCR for plasmid-encoded virulence genes

We conducted various PCR assays to detect the key plasmid-associated virulence genes in STEC strains, as outlined in previous studies[26,29,38,51–59]. The genes examined included: *astA*, Colicin M (*cma*), Colicin B (*cba*),

T6SS (*rhsA1*, *rhsA2*, *rhsC*), *stcE*, *katP*, *toxB*, *espP*, *subAB*, *IhaG*, *LAA iha*, *saa*, T2SS (*etpD*), *cif*, and *epeA* (Table 2). *E. coli* O157:H7 strain Sakai (ATCC BAA-460) was used as a positive control for all PCR reactions.

EhxA subtyping

Six different genetic *ehxA* subtypes (A–F) were identified using PCR in conjunction with restriction fragment length polymorphism (RFLP) analysis. We performed PCR amplification of the entire *ehxA* gene (3 kbp) using primers described by Lorenz et al.[18]. For RFLP analysis, 9 µL aliquots of the *ehxA* PCR products were digested with 5 U of TaqI restriction enzyme (Thermo Fisher Scientific) and 1.1 µL of 10× restriction enzyme buffer, followed by incubation at 65 °C for 90 min[18]. The digestion mixture was then analyzed by DNA electrophoresis on a 2% agarose gel containing the safe stain dye Green Viewer. FUM collection strains and *E. coli* O157:H7 (295 EC-TMU) were used as the positive controls.

Plasmid replicon typing

The plasmid replicon types of the STEC isolates were determined using the method developed by Johnson et al.[19]. This method utilizes three multiplex PCR panels to identify 17 replicon types. The panels are as follows: panel 1: (B/O, FIC, A/C, P, T); panel 2: (K/B, W, FIIA, FIA, FIB, Y); and panel 3: (I1, Frep, X, HI1, N, HI2, L/M). We tested these panels on our new STEC isolates, while the collection strains were double-checked for their plasmid replicon types. FUM collection strains were used as positive controls. Figure 4 illustrates the workflow of the study.

Statistical analysis

The correlations between plasmid-encoded virulence genes, *ehxA* subtypes, and plasmid replicon types with studied animal hosts and *stx2*-positive isolates was illustrated using GraphPad Prism version 9.3.1 (https://ww

Primer name	Sequence (5'-3' direction)	Amplicon size (bp)	Annealing tm/time[a]	Reference
Virulence genes				
astA	CCATCAACACAGTATATCCGA	111	55 °C/60 sec	53
	GGTCGCGAGTGACGGCTTTGT			
cma	CCAGCAACCCTCTCACATTGCAG	556	67 °C/60 sec	54
	CCAGAAAACATCGCCCCGAGCC			
cba	TCGCTCCATCCATGCCTCCG	138	66 °C/60 sec	
	CCATCCCGACCAGTCTCCCTC			
T6-*rhsA*	CGCTATCTTTACGACCCGCT			26
T6-*rhsA1*	GGCAAGGGGAATGGTCTAGG	800	57 °C/60 sec	
T6-*rhsA2*	GATGTGGGGGTACCATGCC	875	57 °C/60 sec	
T6-*rhsC*	TAGGCGGTTTGTTGGGTCTC	832	57 °C/60 sec	
stcE	GGCTCCGGAGGTGGGGGAAT	399	58 °C/60 sec	55
	GAAGCCGGTGGAGGAACGGC			
katP	GCGGAAGAGAAGATGACTGG	277	55 °C/60 sec	56
	GCACCATGTGCTTTACCAAA			
toxB	ATACCTACCTGCTCTGGATTGA	602	51 °C/60 sec	57
	TTCTTACCTGATCTGATGCAGC			
espP	TTGCGAAAAATGGCGGAACTC	956	55 °C/60 sec	58
	CGGAGTCGTCAGTCAGTAGA			
subAB	GCAGATAAATACCCTTCACTTG	232	56 °C/60 sec	59
	ATCACCAGTCCACTCAGCC			
Iha^G	AACTGGCAGATCACCGAAGA	346	55 °C/60 sec	29
	GCGACATCCAGTAATTTCGCT			
LAA iha	TTTCAGCCAGCAGCATGGCA	172	55 °C/60 sec	
	ACATCCACACCCTCCACAGC			
saa	CGTGATGAACAGGCTATTGC	119	56 °C/60 sec	51
	ATGGACATGCCTGTGGCAAC			
T2-*etpD*	CGTCAGGAGGATGTTCAG	1061	53 °C/60 sec	38
	CGACTGCACCTGTTCCTGATTA			
cif	AACAGATGGCAACAGACTGG	383	54 °C/60 sec	59
	AGTCAATGCTTTATGCGTCAT			
epeA	CACCCTGTAGAATCTTA	1873	56 °C/60 sec	52
	CTGAATAAATCCAGCCC			

Table 2. Primers used for identification of plasmid-encoded virulence genes in the studied STEC isolates. [a] Annealing temperature and duration.

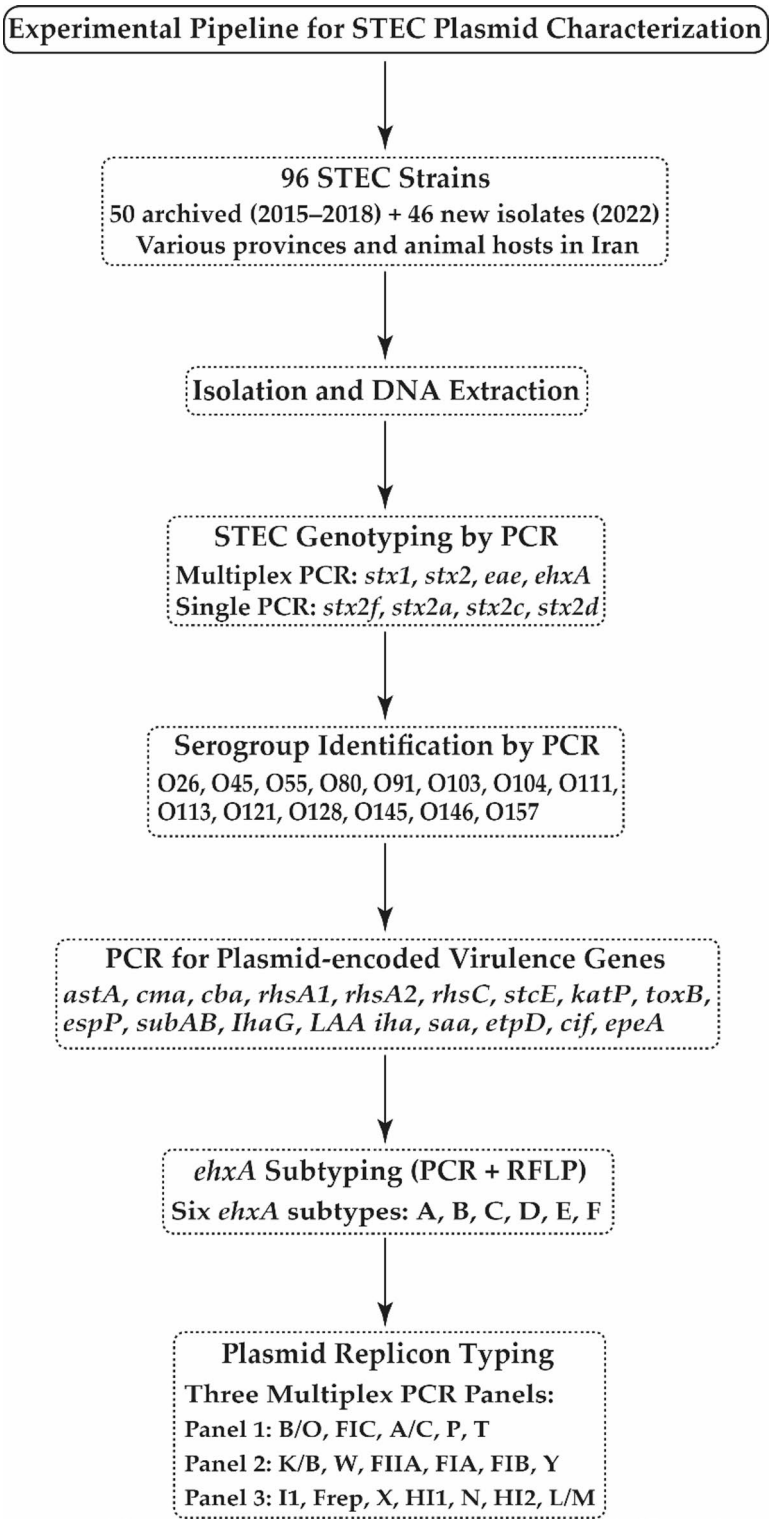

Fig. 4. This workflow outlines the plasmid profiling methodology for the 96 STEC strains, covering steps from sample collection to data interpretation.

w.graphpad.com/updates/prism-931-release-notes). The frequency comparisons between different groups were analyzed using Chi-square ($\chi 2$) and Fisher's exact tests in SPSS 26.0 software (https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-26). Results with a *p*-value of less than 0.05 were considered statistically significant and mentioned in the text.

Data availability

All data supporting the findings of this study are available within the manuscript and its supplementary information file. Raw data, are available from the corresponding author upon reasonable request.

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Author contributions
A.N. conducted the experiments, data analyses, and drafted the manuscript and draw the figures. M.A.B. designed the study, supervised the project, revised the data analyses, and edited the manuscript. G.H. assisted on data analysis and critically reviewed the manuscript. M.H. carried out some experiments, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
All procedures involving animals and their care in this study were approved (No. IR140357299) by Iran

National Committee for Ethics in Biomedical Research. Moreover, a verbal informed consent was obtained from the owners of the companion animals. The research committee of Ferdowsi University of Mashhad reviewed and approved that all the study protocols were conducted in accordance with the related guidelines and regulations (No. FUM57299). The study was carried out in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp? id=1357).

Additional information

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