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Molecular and *in silico* analyses for detection of Shiga toxin-producing *Escherichia coli* (STEC) and highly pathogenic enterohemorrhagic *Escherichia coli* (EHEC) using genetic markers located on plasmid, O Island 57 and O Island 71

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Abstract

Background Due to the diversity of Shiga toxin-producing *Escherichia coli* (STEC) isolates, detecting highly pathogenic strains in foodstuffs is challenging. Currently, reference protocols for STEC rely on the molecular detection of *eae* and the *stx1* and/or *stx2* genes, followed by the detection of serogroup-specific *wzx* or *wzy* genes related to the top 7 serogroups. However, these screening methods do not distinguish between samples in which a STEC possessing both determinants are present and those containing two or more organisms, each containing one of these genes. This study aimed to evaluate *ecf1*, *Z2098*, *Z2099*, and *nleA* genes as single markers and their combinations (*ecf1*/*Z2098*, *ecf1*/*Z2099*, *ecf1*/*nleA*, *Z2098*/*Z2099*, *Z2098*/*nleA*, and *Z2099*/*nleA*) as genetic markers to detect potentially pathogenic STEC by the polymerase chain reaction (PCR) in 96 animal samples, as well as in 52 whole genome sequences of human samples via *in silico* PCR analyses.

Results In animal isolates, *Z2098* and *Z2098*/*Z2099* showed a strong association with the detected top 7 isolates, with 100% and 69.2% of them testing positive, respectively. In human isolates, *Z2099* was detected in 95% of the top 7 HUS isolates, while *Z2098*/*Z2099* and *ecf1*/*Z2099* were detected in 87.5% of the top 7 HUS isolates.

Conclusions Overall, using a single gene marker, *Z2098*, *Z2099*, and *ecf1* are sensitive targets for screening the top 7 STEC isolates, and the combination of *Z2098*/*Z2099* offers a more targeted initial screening method to detect the top 7 STEC isolates. Detecting non-top 7 STEC in both animal and human samples proved challenging due to inconsistent characteristics associated with the genetic markers studied.

Keywords Shiga toxin-producing *Escherichia coli*, STEC, Plasmid, HUS, Genetic markers

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Background

Enterohemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin-producing *E. coli* (STEC), cause severe human illnesses such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) [1]. The contamination of food products by EHEC remains a global concern, potentially leading to outbreaks of human disease [2–4]. STEC comprises more than 400 serotypes, many of which have been reported in foodstuffs and animals. However, in most cases, severe human illness is attributed to one of the seven well-defined EHEC serogroups, namely O26, O45, O103, O111, O121, O145, and O157 [5, 6]. These serogroups are considered adulterants in beef trim by the U.S. Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS), which routinely conducts verification testing for these strains in domestic and imported beef manufacturing trimmings [7].

The top 7 EHEC serogroups typically possess the *stx* and *eae* genes and are classified as having high virulence potential [8, 9]. The ISO/TS 13,136 (EU) and MLG5B.05 (US) reference methods include an initial screening for the presence of the *stx1*/*stx2* and *eae* genes. Presumptive-positive samples are then examined for genes associated with the top 5 (O157, O26, O103, O111, and O145) or top 7 serogroups, depending on whether the ISO/TS 13,136 or MLG5B.05 protocols are used, respectively [10, 11]. One of the main drawbacks of these reference methods is that many *eae*-negative STEC, enteropathogenic *E. coli* (EPEC), *E. albertii*, and free *stx*-converting phages can also react with these genetic targets, leading to false-positive results, especially in food, feces, and environmental samples [12–14]. Therefore, new genetic markers that specifically target EHEC (*stx*-positive and *eae*-positive strains of the top 7 *E. coli* serotypes) may improve the testing of food when this subpopulation of STEC must be detected in compliance with specific regulations, thereby reducing the rate of false positive results.

Various studies have reported potential genetic markers for EHEC. In 2014, Luedtke et al. at the U.S. Department of Agriculture evaluated the use of the EHEC-specific target *E. coli* attaching and effacing gene-positive conserved fragment 1 (*ecf1*) for the detection of EHEC directly from cattle feces [15]. The *ecf1* gene is located on a unique conserved 5.6-kb fragment on the enterohemolysin-encoding plasmid of *eae*- and *ehxA*-positive STEC [16]. This fragment is part of the *ecf* operon, which consists of four genes (*ecf1* to *ecf4*) and encodes four proteins involved in cell wall synthesis [17]. Other targets have been derived from several pathogenicity islands (PAIs) located on chromosomal regions. PAIs harbor genes that can serve as genetic signatures. The suitability of *Z2098* and *Z2099* genes, on the genomic O island 57 (OI-57), which may be associated with increased virulence of STEC strains in humans [18], has been tested for the identification of human-virulent STEC strains, particularly those of the top 7 EHEC serotypes [5]. Another chromosomally located gene, *nleA* (*espI*) on O island 71 (OI-71), has also been proposed as a candidate to distinguish EHEC from EPEC and STEC strains that may not be associated with severe and epidemic diseases [19].

The *ecf1*, *Z2098*, *Z2099*, and *nleA* genes may indeed have intrinsic value in identifying the top 7 EHEC [20]. The association of such genetic markers with highly pathogenic EHEC strains would be of interest to increase the specificity of the EHEC screening step in food, fecal, and environmental samples [21]. To clarify this relationship, we identified the presence of each of these genetic markers in STEC and EHEC strains belonging to different serogroups associated with animal hosts in Iran. Furthermore, we evaluated their combinations to find the best approach for a more specific and sensitive detection of the top 7 EHEC strains. Additionally, *in silico* PCR (polymerase chain reaction) analyses were conducted on whole-genome sequences of the top 7 and other emerging STEC/EHEC serotypes in human HUS patients retrieved from the NCBI GenBank database to explore the suitability of this approach for application to culture-independent NGS-based methods.

Methods

STEC isolates investigated

A total of 50 STEC collection strains were utilized. These isolates were gathered from various provinces in Iran, including Tehran, Razavi Khorasan, Semnan, Mazandaran, and Khuzestan. They were obtained from different animal hosts (cattle, sheep, goats, and pigeons) during the period from 2018 to 2020, as documented in our previous studies (refer to Table S1 in the supplementary materials). Moreover, 46 new STEC strains were added to this study. These strains were collected from a total number of 75 fecal samples from cattle, 70 from sheep, and 15 from goats in Razavi Khorasan province during the period from April 2022 to June 2022 (Strains' features are provided in Table S1).

DNA extraction

All *E. coli* isolates were confirmed through culturing on MacConkey agar, Eosin-Methylene Blue (EMB) agar and subsequent biochemical tests. Afterward, a pure colony of each isolate was cultured on Luria Bertani (LB) agar and incubated for 24 h at 37 °C. After overnight culture on LB agar, total genomic DNA was extracted by the boiling method. In brief, a loopful from confluent growth area in LB agar culture was suspended in sterile microtubes containing 350 µL molecular grade water and boiled for 10 min. Then, samples were centrifuged at 5000×g for 5 min, and the supernatants were used as templates for end-point PCR assays. The quality and

quantity of the extracted total genomes were evaluated using the NanoDrop-1000 spectrophotometer (ThermoFisher). DNA templates were kept at −20 °C until the further analyses.

Strain characterization

To confirm the STEC genotype, a multiplex-PCR (Table 1) targeting *stx1*, *stx2*, *eae*, and *ehxA* was used as described previously [22]. For detection of *stx* in

Table 1 Primers used for identification of virulence/genetic markers and serogroups of studied STEC/EHEC isolates

Primer name	Sequence (5′-3′ direction)	Amplicon size (bp)	Reference
Virulence markers			
stx1	ATAAATCGCCATTCGTTGACTAC	180	[22]
	AGAACGCCCACTGAGATCATC		
stx2	GGCACTGTCTCTCTGAAACTGCTCC	255	
	TCGCCAGTTATCTGACATTCTG		
stx2f	AGATTGGGCGTCATTCACTGGTTG	428	[23]
	TACTTTAATGGCCGCCCTGTCTCC		
eae	GACCCGGCACAAGCATAAGC	384	[22]
	CCACCTGCAGCAACAAGAGG		
ehxA	GCATCATCAAGCGTACGTTCC	534	
	AATGAGCCAAGCTGGTTAAGCT		
ecf1	TATCAGCACCAAAGAGCGGGAACA	99	[30]
	CCCTTATGAAGAGCCAGTACTGAA		
Z2098	ACATCACAGGCTTCCTGAGC	423	[31]
	GGAACGTGCCTCCGAGATAG		
Z2099	TTCAACAGTAGCGCAGGCAA	266	
	CAAGCAGGGGCGGTTACTTT		
nleA	ATGAACATTCAACCGACCATACAATCTG	1326	[32]
	TTAGACTCTTGTTTCTTGGATTATATCA		
Serogroups			
O26	CAATGGGCGGAAATTTTAGA	155	[26]
	ATAATTTTCTCTGCCGTCGC		
O45	TGCAGTAACCTGCACGGGCG	238	
	AGCAGGCACAACAGCCACTACT		
O55	TCCTTATTTGTGTCGGGGG	207	[27]
	CCAGGAAAGCTGCCAATTATC		
O80	TGAGAGCCAAGATCCAAGCA	158	[29]
	TGGGCCATATTCGAAGTTTGAA		
O91	TTGCATCTGGCGCAATAAACACGG	616	[26]
	ACACCATCCCAAATACCTGCTTGC		
O103	TTGGAGCGTTAACTGGACCT	321	
	GCTCCCGAGCACGTATAAAG		
O104	TGAACTGATTTTTAGGATGG	351	
	AGAACCTCACTCAAATTATG		
O111	TGTTTCTTCGATGTTGCGAG	438	
	GCAAGGGACATAAGAAGCCA		
O113	TGCCATAATTCAGAGGGTGAC	514	
	AACAAAGCTAATTGTGGCCG		
O121	TCCAACAATTGGTCGTGAAA	628	
	AGAAAGTGTGAAATGCCCGT		
O128	ATGATTTCTTACGGAGTGC	782	
	CTCTAACCTAATCCCTCCC		
O145	TTCATTGTTTTGCTTGCTCG	750	
	GGCAAGCTTTGGAAATGAAA		
O146	ATTCGGGTAACGACCCTGTGTTGA	378	[28]
	AGACTGCTAATGCAAGGAACATGG		
O157	TCGAGGTACCTGAATCTTTCCTTCTGT	894	[26]
	ACCAGTCTTGGTGCTGCTCTGACA		

pigeon isolates, a single primers pair was used (Table 1) to amplify the *stx2f* [23]. *E. coli* O157:H7 strain (ATCC 35218) and *E. coli* O157:H7 strain Sakai (ATCC BAA-460) were used as positive controls. The strain PG90 from the Ferdowsi University of Mashhad (FUM) collection was included as a control for *stx2f*.

Based on the data collected annually by the European Centre for Disease Prevention and Control (ECDC), the 14 serogroups more frequently associated with the disease in humans in the EU include O26, O45, O55, O80, O91, O103, O104, O111, O113, O121, O128, O145, O146, and O157 [24]. Serogroups O26, O45, O55, O91, O103, O104, O111, O113, O121, O128, and O145 were determined [25] by PCR using the primers shown in Table 1 [26–28]. Additionally, the serogroups of O80 and O146 were tested as described elsewhere [29]. *E. coli* O157:H7 (295 EC-TMU) and FUM collection strains were used as positive controls.

PCR for *ecf1* gene

A PCR assay (Table 1), was used to amplify the *ecf1* gene as described previously [30]. Amplification was performed in a final volume of 20 µL containing 2 µL template DNA, 1 unit *Taq* DNA polymerase, 0.5 µM of each primer, 1.5 mM MgCl2, and 200 µM dNTP mix in 1× PCR buffer as follows: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 57 °C for 50 s and 72 °C for 30 s; and a final elongation step at 72 °C for 5 min. Amplicons were visualized after running at 100 V for 45 min on a 1.5% agarose gel containing green viewer safe stain. A 100 bp DNA ladder (CinnaGen, Iran) was used as a size marker. *E. coli* O157:H7 strain Sakai (ATCC BAA-460) was used as positive control in every PCR reaction (Table 1).

PCR for O Island 57 markers (*Z2098* and *Z2099* genes)

Amplification of *Z2098* and *Z2099* genes was carried out by a duplex-PCR assay (Table 1) according to our previous study [31]. Total DNA (2 µL) was used as template in a final volume of 25 µL mixture containing, 1 unit *Taq* DNA polymerase, 0.75 µM of each primer, 1.5 mM MgCl2, and 200 µM dNTP mix in 1× PCR buffer as follows: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 40 s, 56 °C for 30 s and 72 °C for 45 s; and a final elongation step at 72 °C for 7 min. Amplicons were visualized after running at 100 V for 1 h on a 1.5% agarose gel containing green viewer safe stain. A 100 bp DNA ladder (CinnaGen, Iran) was used as a size marker. The positive control was *E. coli* O157:H7 strain Sakai (ATCC BAA-460) that was used in every PCR reaction.

PCR for O island 71 marker (*nleA* gene)

The presence of *nleA* gene was evaluated using the uniplex-PCR (Table 1) designed by Mundy et al. [32]. PCR was carried out in 20 µL using 2 µL template DNA, 1 unit *Taq* DNA polymerase, 0.5 µM of each primer, 1.5 mM MgCl2, and 200 µM dNTP mix in 1× PCR buffer as follows: initial denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 52 °C for 55 s and 72 °C for 1 min; and a final elongation step at 72 °C for 5 min. Amplicons were visualized after running at 100 V for 45 min on a 1.5% agarose gel containing green viewer safe stain. A 100 bp DNA ladder (CinnaGen, Iran) was used as a size marker. *E. coli* O157:H7 strain Sakai (ATCC BAA-460) was used as positive control.

In silico PCR evaluation of genetic marker combinations in the top 7 and other important STEC/EHEC serotypes related to HUS patients

A total of 52 complete genome sequences of the top 7 ($n=40$) and other emerging ($n=12$) STEC/EHEC serotypes originating from human HUS patients were retrieved from NCBI GenBank database [33–36] (Accession numbers are provided in Table 2). Primer sequences of the genetic marker combinations, *ecf1*/*Z2098*, *ecf1*/*Z2099*, *ecf1*/*nleA*, *Z2098*/*Z2099*, *Z2098*/*nleA*, and *Z2099*/*nleA* were used to blast the genomes of the studied isolates to find the exact annealing sites and to extract the sequences corresponding the amplification products using CLC Genomics Workbench version 20.0 (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/). The complete genome sequence of strain Sakai (ATCC BAA-460) was used as a reference.

Results

STEC/EHEC serogroups of animal strains

Overall, the 96 animal STEC/EHEC isolates belonged to 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 defined based on the included serogroups surveyed.

STEC/EHEC serotypes of human genomes

The 52 whole genome sequences retrieved from the GenBank belonged to serotypes O26:H11 ($n=4$), O45:H2 ($n=2$), O45:H16 ($n=1$), O103:H2 ($n=1$), O103:H8 ($n=1$), O103:H11 ($n=1$), O103:H25 ($n=1$), O111:H8 ($n=2$), O121:H19 ($n=10$), O145:H25 ($n=2$), O145:H28 ($n=3$), O157:H7 ($n=12$), O10:H25 ($n=1$), O25:H4 ($n=1$), O55:H7 ($n=2$), O59:H19 ($n=1$), O78:H4 ($n=1$), O104:H4 ($n=1$), O109:H21 ($n=1$), O146:H28 ($n=1$), O165:H25 ($n=2$), and O182:H25 ($n=1$).

Table 2 Metadata of the 52 whole genomes of the top 7 ($n=40$) and other important ($n=12$) EHEC serotypes originating from HUS patients

Strain	Pathotype	Serotype	Collection date	Location	ecf1	Z2098	Z2099	nleA	Accession number
SEH1101	EHEC	O26:H11	2011	Sweden	+	+	+	+	JABWFR000000000
SEH0404	EHEC	O26:H11	2004	Sweden	+	+	+	+	JABWEW000000000
SEH1407	EHEC	O26:H11	2014	Sweden	+	+	+	+	JABWGD000000000
SEH1004	EHEC	O26:H11	2010	Sweden	-	-	-	-	JABWFP000000000
FWSEC0003	EHEC	O45:H2	2019	Canada	+	+	+	-	CP031916
2011 C-4251	EHEC	O45:H2	2018	USA	+	+	+	-	CP027388
SJ7	EHEC	O45:H16	2020	USA	-	+	+	-	CP044315
SEH1403	EHEC	O103:H2	2014	Sweden	+	+	+	-	JABWFZ000000000
SEH0702	EHEC	O103:H8	2007	Sweden	+	-	+	+	JABWFJ000000000
2013 C-4225	EHEC	O103:H11	2018	USA	+	+	+	+	CP027578
2013 C-3264	EHEC	O103:H25	2018	USA	+	+	+	-	CP027544
SEH1201	EHEC	O111:H8	2012	Sweden	-	-	-	+	JABWFT000000000
SEH0801	EHEC	O111:H8	2008	Sweden	+	+	+	-	JABWFK000000000
SEH0601	EHEC	O121:H19	2006	Sweden	+	+	+	+	JABWFF000000000
SEH1301	EHEC	O121:H19	2013	Sweden	+	+	+	+	JABWFW000000000
SEH0504	EHEC	O121:H19	2005	Sweden	+	+	+	+	JABWFA000000000
SEH0301	EHEC	O121:H19	2003	Sweden	+	+	+	+	JABWER000000000
SEH9705	EHEC	O121:H19	1997	Sweden	+	+	+	+	JABWEN000000000
SEH1002	EHEC	O121:H19	2010	Sweden	+	+	+	+	JABWFN000000000
SEH1402	EHEC	O121:H19	2014	Sweden	+	+	+	+	JABWFY000000000
SEH9401	EHEC	O121:H19	1994	Sweden	+	+	+	+	JABWEH000000000
SEH1404	EHEC	O121:H19	2014	Sweden	+	+	+	+	JABWGA000000000
SEH1202	EHEC	O121:H19	2012	Sweden	+	+	+	+	JABWFU000000000
CFSAN004176	EHEC	O145:H25	2003	USA	-	-	+	-	CP014583
CFSAN004177	EHEC	O145:H25	2004	USA	-	-	+	-	CP014670
RM13514	EHEC	O145:H28	2010	USA	+	+	+	-	NZ_CP006027.1
RM13516	EHEC	O145:H28	2007	Belgium	+	+	+	-	NZ_CP006262.1
SEH1003	EHEC	O145:H28	2010	Sweden	+	+	+	-	JABWFO000000000
TW14359	EHEC	O157:H7	2006	USA	+	+	+	+	NC_013008.1
Sakai	EHEC	O157:H7	1996	Japan	+	+	+	+	NC_002695.1
Xuzhou21	EHEC	O157:H7	1999	China	+	+	+	+	NC_017906.1
SEH9901	EHEC	O157:H7	1999	Sweden	+	+	+	+	JABWEO000000000
SEH9701	EHEC	O157:H7	1997	Sweden	+	+	+	+	JABWEJ000000000
SEH0602	EHEC	O157:H7	2006	Sweden	+	+	+	+	JABWFG000000000
SEH9501	EHEC	O157:H7	1995	Sweden	+	+	+	+	JABWEI000000000
SEH0302	EHEC	O157:H7	2003	Sweden	+	+	+	+	JABWES000000000
SEH0507	EHEC	O157:H7	2005	Sweden	+	+	+	+	JABWFD000000000
SEH0202	EHEC	O157:H7	2002	Sweden	+	+	+	+	JABWEQ000000000
SEH0402	EHEC	O157:H7	2004	Sweden	+	+	+	+	JABWEU000000000
SEH1802	EHEC	O157:H7	2018	Sweden	+	+	+	+	JABWGI000000000
115,237	STEC	O10:H25	2015	Finland	+	-	-	-	JAKYSD000000000
54,748	STEC	O25:H4	2000	Finland	-	-	-	-	JAKYQQ000000000
93,628	STEC	O55:H7	2009	Finland	-	-	-	-	JAKYOF000000000
118,916	STEC	O55:H7	2016	Finland	-	-	-	-	JAKYRB000000000
SEH1401	EHEC	O59:H19	2014	Sweden	-	-	-	-	JABWFX000000000
94,076	STEC	O78:H4	2009	Finland	-	-	-	-	JAKYOE000000000
2011C_3493	EAHEC	O104:H4	2011	USA	-	-	-	-	NC_018658.1
SEH1203	EHEC	O109:H21	2012	Sweden	-	-	-	-	JABWFV000000000
105,246	STEC	O146:H28	2013	Finland	-	-	-	-	JAKYVV000000000
SEH1405	EHEC	O165:H25	2014	Sweden	+	-	+	-	JABWGB000000000
SEH1501	EHEC	O165:H25	2015	Sweden	+	-	+	-	JABWGE000000000
96,308	STEC	O182:H25	2010	Finland	+	+	+	-	JAKYOB000000000

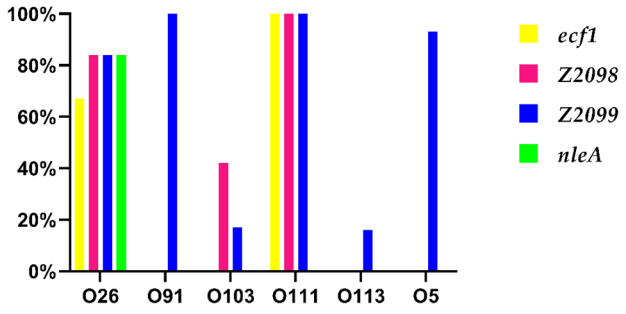

Fig. 1 Distribution of the genetic markers *ecf1*, *Z2098*, *Z2099*, and *nleA* in different STEC/EHEC serogroups. Isolates lacking defined serogroups were excluded

Distribution of *ecf1*, *Z2098*, *Z2099*, and *nleA* in Animal STEC/EHEC strains

Overall, the genetic marker *ecf1* was detected in eight isolates: four O26 (4/6, 66.6%), three O111 (3/3, 100%), and one isolate for which the serogroup could not be identified (Fig. 1). All the eight *ecf1*-positive isolates were identified as EHEC strains (*stx* and *eae* positive) (8 of 32 EHEC, 25.0%) (Fig. 2). The genetic marker *Z2098* was present in 15 isolates: five strains of each of the O26 (5/6, 83.3%) and O103 (5/12, 41.6%) serogroups, three O111 (3/3, 100%), and two not defined serotypes (Fig. 1). Of the 15 *Z2098*-positive isolates, 9 strains were identified as EHEC (9/32, 28.1%) (Fig. 2). The *Z2099* genetic marker was detected in 38 isolates belonging to serogroups O5 (12 strains, 12/13, 92.3%), O26 (5 strains, 5/6, 83.3%), O111 (three strains, 3/3, 100%), O91 (three strains, 3/3, 100%), two isolates of each O113 (2/13, 15.3%) and O103 (2/12, 16.6%), and in 11 strains of not defined serogroup (Fig. 1). Among the 38 *Z2099*-positive isolates, 9 strains were identified as EHEC (9/32, 28.1%) (Fig. 2). The *nleA* genetic marker was detected in six isolates: five strains of O26 (5/6, 83.3%), and one isolate of a not defined serogroup (Fig. 1). All the *nleA*-positive isolates were identified as EHEC (6/32, 18.7%) (Fig. 2). More details are provided in Table 3.

The genetic markers were also explored to identify combinations possibly associated with the top 7 EHEC ensuring a better sensitivity and specificity. As shown in Table 4, we detected the combined presence of *Z2098*/*Z2099* with 100% frequency rate for O26, O111, O157 and 40.0% for O103; *ecf1*/*Z2098* and *ecf1*/*Z2099* presented a 100% distribution in the O111 and O157 strains and genomes tested, and 80% in O26; *Z2098*/*nleA* and *Z2099*/*nleA* combination showed 100% presence in O26 and O157; and *ecf1*/*nleA* a 100% distribution in O157 and 80% in O26. Interestingly, the non-top 7 isolates (O113, O5, and O91) were negative for all of these genetic marker combinations.

In silico PCR analyses for distribution of the single genetic markers and their combinations in the top 7 and emerging STEC/EHEC serotypes in HUS patients

In total, the *ecf1*, *Z2098*, *Z2099*, and *nleA* were present as single genetic markers in 75.0%, 69.2%, 78.8%, and 53.8% of all the 52 STEC/EHEC serotypes related to HUS patients. Among these, *Z2099* showed the highest presence (95%) in the top 7 EHEC serotypes and *nleA* had the lowest distribution (70%). For non-top 7 serotypes, *ecf1* was detected as the most abundant single genetic marker in 33.3%, while none of the non-top 7 isolates were positive for *nleA* marker (Table 2).

Distribution of the genetic marker combinations *ecf1*/*Z2098*, *ecf1*/*Z2099*, *ecf1*/*nleA*, *Z2098*/*Z2099*, *Z2098*/*nleA*, and *Z2099*/*nleA* among the different genomes of STEC/EHEC serotypes is shown in Table 5. Overall, the combinations of *Z2098*/*Z2099* (77.0%) and *ecf1*/*Z2099* (77.0%) had the highest frequency rate among the top 7 EHEC serotypes while *Z2098*/*nleA* (31.2%) were observed as the lowest. Interestingly, the genetic markers investigated were less commonly associated with non-top 7 serotypes; we only detected *ecf1*/*Z2099* in O165:H25 and *ecf1*/*Z2098*, *ecf1*/*Z2099*, and *Z2098*/*Z2099* combinations in O182:H25 (Tables 5 and 2). The protocol chart in Fig. 3 illustrates the summary of methods and results.

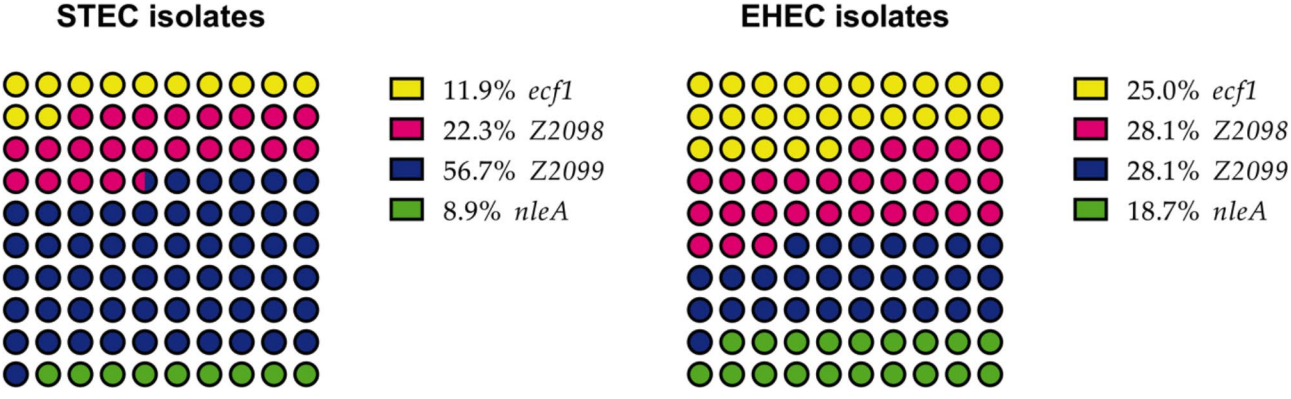

Fig. 2 Distribution of the genetic markers *ecf1*, *Z2098*, *Z2099*, and *nleA* in STEC and EHEC isolates

Table 3 Presence of genetic/virulence markers in 42/96 STEC and EHEC isolates included in this study

Serogroup	No. of isolates	Host	Genetic/Virulence markers						
			ecf1	Z2098	Z2099	nleA	stx[a]	eae	ehxA
Top 7									
O26	4	Cattle	+	+	+	+	+[1]	+	+
O26	1	Cattle	-	+	+	+	+[1]	+	-
O111	2	Cattle	+	+	+	-	+[1]	+	+
O111	1	Cattle	+	+	+	-	+[1]	+	-
O103	3	Sheep	-	+	-	-	+[1]	-	-
O103	1	Goat	-	+	+	-	+[1/2]	-	+
O103	1	Goat	-	+	+	-	+[1]	-	-
O157[b]	1	Human	+	+	+	+	+[1/2]	+	+
Others									
O113	1	Cattle	-	-	+	-	+[1/2]	-	+
O113	1	Goat	-	-	+	-	+[1/2]	-	+
O5	8	Sheep	-	-	+	-	+[1/2]	-	+
O5	3	Sheep	-	-	+	-	+[1]	-	+
O5	1	Goat	-	-	+	-	+[1/2]	-	+
O91	2	Sheep	-	-	+	-	+[1/2]	-	+
O91	1	Goat	-	-	+	-	+[1/2]	-	+
ND[c]	1	Cattle	+	+	+	+	+[1]	+	+
ND	4	Cattle	-	-	+	-	+[2]	-	-
ND	1	Goat	-	-	+	-	+[2]	-	-
ND	2	Cattle	-	-	+	-	+[1]	-	+
ND	2	Sheep	-	-	+	-	+[1]	-	+
ND	1	Sheep	-	+	+	-	+[1]	-	-

[a] +[1] = *stx1*, +[2] = *stx2*, and +[1/2] = *stx1* + *stx2*

[b] Positive control: *Escherichia coli* O157:H7 strain Sakai (ATCC BAA-460)

[c] Not Defined

Table 4 Frequencies of the genetic marker combinations according to the top 7 and other important serogroups in studied STEC/EHEC isolates

Serogroup[a]	STEC/EHEC	Host	ecf1/Z2098	ecf1/Z2099	ecf1/nleA	Z2098/Z2099	Z2098/nleA	Z2099/nleA
Top 7								
O26	EHEC	Cattle	80.0%	80.0%	80.0%	100.0%	100.0%	100.0%
O111	EHEC	Cattle	100.0%	100.0%	0.0%	100.0%	0.0%	0.0%
O103	STEC	Sheep/Goat	0.0%	0.0%	0.0%	40.0%	0.0%	0.0%
Others								
O113	STEC	Cattle/Goat	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O5	STEC	Sheep/Goat	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O91	STEC	Sheep/Goat	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

[a] No. of isolates: O26 ($n=5$), O111 ($n=3$), O103 ($n=5$), O157 ($n=1$), O113 ($n=2$), O5 ($n=12$), and O91 ($n=3$)

Discussion

Rapid and specific detection of EHEC strains is urgently needed by public health authorities to establish monitoring programs that track EHEC contamination in animals and foodstuffs. Generally, the ISO/TS 13,136 and MLG5B.05 reference methods rely on the presence of *eae* and the *stx1* and/or *stx2* genes, followed by the detection of O antigen genes (*wzx* or *wzy*) related to the top 5 and top 7 serogroups, respectively. These methods include screening for the detection of two genetic markers (*eae* and *stx*) in enrichment cultures, and presumptive positive results can be obtained from samples containing two or more organisms, each containing one of these genes [10, 11]. To this end, we examined the use of *ecf1*, *Z2098*, *Z2099*, and *nleA* genes as single markers, and *ecf1*/*Z2098*, *ecf1*/*Z2099*, *ecf1*/*nleA*, *Z2098*/*Z2099*, *Z2098*/*nleA*, and *Z2099*/*nleA* as genetic marker combinations to characterize a panel of STEC strains of animal origin and genomes from human cases of HUS to identify possible markers for the direct screening of food and animals for the presence of strains associated with severe human disease.

Table 5 Distribution of the genetic marker combinations in the top 7 and other important STEC/EHEC serotypes related to HUS patients based on the *in silico* PCR analyses

Serotype	STEC/EHEC	No. of isolates	ecf1/Z2098	ecf1/Z2099	ecf1/nleA	Z2098/Z2099	Z2098/nleA	Z2099/nleA
Top 7								
O26:H11	EHEC	4	75.0%	75.0%	75.0%	75.0%	75.0%	75.0%
O45:H2	EHEC	2	100.0%	100.0%	0.0%	100.0%	0.0%	0.0%
O45:H16	EHEC	1	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%
O103:H2	EHEC	1	100.0%	100.0%	0.0%	100.0%	0.0%	0.0%
O103:H8	EHEC	1	0.0%	100.0%	100.0%	0.0%	0.0%	100.0%
O103:H11	EHEC	1	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
O103:H25	EHEC	1	100.0%	100.0%	0.0%	100.0%	0.0%	0.0%
O111:H8	EHEC	2	50.0%	50.0%	0.0%	50.0%	0.0%	0.0%
O121:H19	EHEC	10	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
O145:H25	EHEC	2	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O145:H28	EHEC	3	100.0%	100.0%	0.0%	100.0%	0.0%	0.0%
O157:H7	EHEC	12	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Others								
O10:H25	STEC	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O25:H4	STEC	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O55:H7	STEC	2	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O59:H19	EHEC	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O78:H4	STEC	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O104:H4	EAHEC[a]	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O109:H21	EHEC	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O146:H28	STEC	1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
O165:H25	EHEC	2	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%
O182:H25	STEC	1	100.0%	100.0%	0.0%	100.0%	0.0%	0.0%

[a] Enteroaggregative haemorrhagic *Escherichia coli*

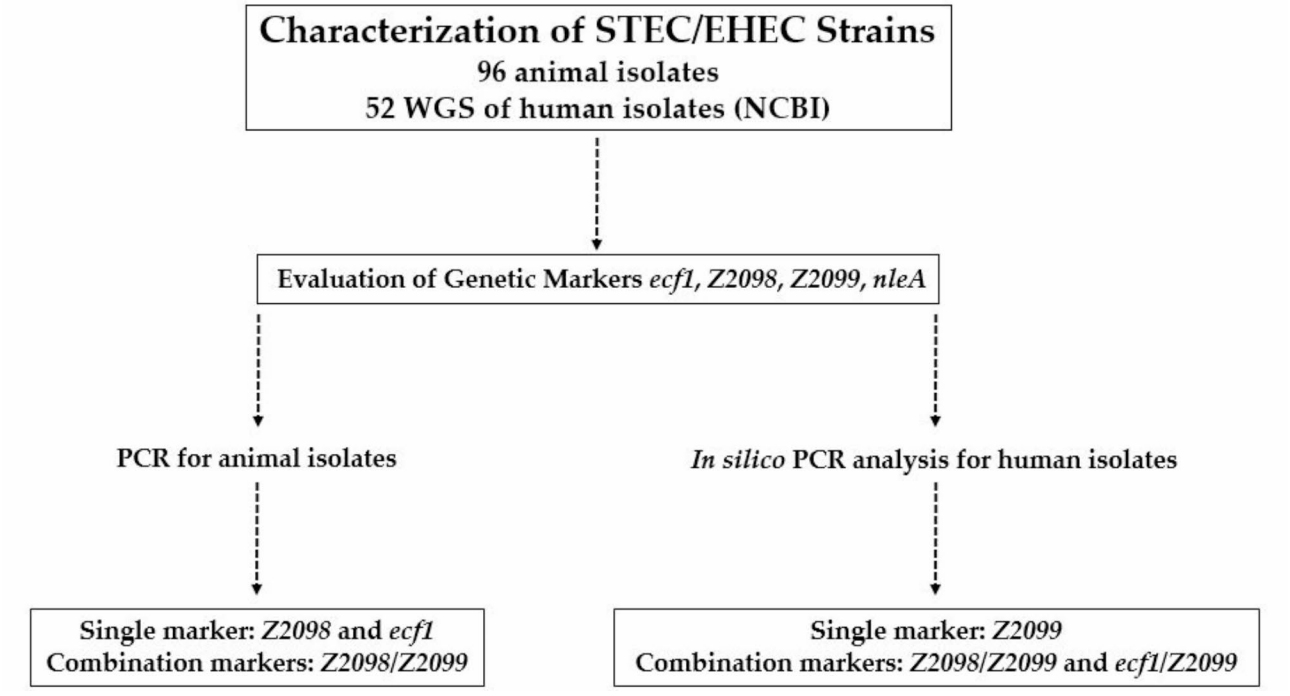

Fig. 3 The proposed single and combination of markers for searching the possible pathogenic strains in two categories of isolates (Animal STEC, human HUS) based on the findings of the current study

Z2098 and *ecf1* as the best single genetic marker for screening of the top 7 EHEC/STEC isolates in animal hosts

In this study, we sought to investigate the use of genetic markers *ecf1*, *Z2098*, *Z2099*, and *nleA* to screen the top 7 and other STEC/EHEC serotypes originating from animal hosts. In this regard, we found a strong linkage between *Z2098* and the top 7 STEC isolates, with an association of 100% (O26, O103, O111). Another gene marker, *Z2099*, showed significant potential to identify the top 7 isolates, as 76.9% of the O26, O103, and O111 serogroups were positive for the presence of this gene. Importantly, *Z2099* was also an excellent genetic marker for some emerging STEC isolates, as we detected the marker in 100% of the O113 STEC strains. Similar data were reported by Delannoy et al., who described that the *Z2098* and *Z2099* gene markers had a detection range of 89.6–95.5% for the top six serogroups and a range of 67.6–96.8% for emerging STEC from other serogroups [5]. Another gene marker, *ecf1*, was detected in 53.8% of the top 7 animal EHEC isolates (O26 and O111), while all of the non-top 7 serogroups were negative for the gene marker. The use of *ecf1* as a genetic marker to detect STEC isolates was examined by Livezey et al. (2015), who reported that 94.8% of the top 7 STEC strains were *ecf1* positive [30]. Although *ecf1* was more specific for the top 7 serotypes, the low incidence (53.8%) found in this work places this marker as the second choice for screening the top 7 STEC/EHEC serotypes after the *Z2098* gene marker. Moreover, *ecf1* is a plasmid gene marker that might be lost, leading to false negative results. Our animal samples were also screened for the presence of the *nleA* gene marker. Based on the results, this gene seems to have low potential for use as a marker, since only 38.4% of the top 7 serotypes (O26) were positive for *nleA*, and all of the non-top 7 serogroups were negative. However, a study conducted in the UK reported that 86% of the EHEC isolates from the patients with HUS and diarrhea were positive for the *nleA* gene marker [32]. These differences in the results obtained might be linked to the source of the samples studied, as the UK study was conducted on human samples instead of animal isolates. Hence, we also surveyed all the studied markers in highly pathogenic EHEC isolates originating from HUS patients via *in silico* PCR analyses to generate a comparison with animal isolates. Moreover, it has been reported that the *nleA* gene marker is associated with O26:H11, which confirms our results, as all of the *nleA*-positive isolates in our study were of the O26 serogroup [37, 38].

Z2098/*Z2099* as the best genetic marker combination for detecting of the top 7 EHEC/STEC isolates in animal hosts

Our study showed that the combinations of the genetic markers *ecf1*/*Z2098*, *ecf1*/*Z2099*, *ecf1*/*nleA*, *Z2098*/*Z2099*, *Z2098*/*nleA*, and *Z2099*/*nleA* were detected in 53.8%, 53.8%, 30.7%, 69.2%, 38.4%, and 38.4% of the top 7 EHEC/STEC isolates, respectively. To date, this is the first report on the evaluation of these genetic marker combinations as a tracking method for the top 7 EHEC/STEC isolates of animal origin. None of the combinations identified all the top 7 isolates, but *Z2098*/*Z2099* showed a percentage of 69.2% for the top 7 EHEC/STEC isolates. Nevertheless, *Z2098*/*Z2099* and other studied combinations were not capable of detecting non-top 7 isolates, especially the important serogroup O113, highlighting the need for complementary studies to investigate other genetic marker combinations for important non-top 7 O-groups.

Diagnostic application of single and combination of studied genetic markers in HUS isolates

In addition to animal hosts, the studied genetic markers were also investigated via *in silico* PCR analyses in the genomes of top 7 and emerging EHEC/STEC serotypes related to HUS patients. A comparison of the animal and human results revealed that among the studied markers, *Z2099* is more prevalent in the top 7 HUS isolates, with 95% of the strains testing positive, whereas *ecf1* and *Z2098* were detected in 87.5% and *nleA* in 70% of the top 7 serotypes. Such data are in accordance with a study by Delannoy et al., which showed a prevalence rate of 87% for *Z2098* and 91% for *Z2099* in EHEC and EHEC-like strains [5]. Combinations of the genetic markers also revealed that *Z2098*/*Z2099* and *ecf1*/*Z2099* are the most prevalent double markers for detecting the top 7 HUS isolates, with a positive rate of 87.5%. Considering these points, none of the single genetic markers were capable of detecting all EHEC isolates; thus, *Z2098*/*Z2099* or *ecf1*/*Z2099* offers a better choice for identifying the highly pathogenic EHEC with greater confidence. However, since we failed to detect the presence of these markers in 5 out of 40 strains, these combinations need to be investigated in a much wider panel of isolates to confirm their suitability as markers for highly pathogenic STEC. In contrast to the top 7 isolates, the other STEC/EHEC serotypes studied were not identified by the genetic marker combinations. Only one STEC strain (O182:H25) was positive for *ecf1*/*Z2098*, *Z2098*/*Z2099*, and *ecf1*/*Z2099*. Among the single markers, *ecf1* and *Z2099* had a very low frequency (33.3% and 25%, respectively) in the studied non-top 7 STEC/EHEC strains, with none of them positive for the *nleA* gene marker. Our study pointed out the need for additional markers to be tested in future research to find more sensitive and specific gene markers for non-top 7 HUS isolates.

We did not investigate these markers and their combinations in other pathogenic *E. coli* such as EPEC and other *E. coli* pathogroups. However, in Delannoy's report, the distribution of the studied markers was significantly

more prevalent in EHEC than in EPEC and apathogenic *E. coli* [7]. In the investigation conducted by Delannoy et al., it was demonstrated that 23.2% of the examined EPEC isolates exhibited positivity for the *Z2098* marker. This phenomenon is attributed to the presence of *stx*-negative variants of EHEC, particularly those belonging to the top 7 EHEC serotypes, as outlined by the authors. It is noteworthy that, to avoid presumptive positive results, we propose analyzing the single markers in enrichment cultures; if positive, testing for combination markers can be applied to pure isolates. This approach may be particularly suitable in low- and middle-income countries where NGS facilities to characterize isolated STEC strains are not widely available.

Conclusions

Our study identified alternative genetic markers (*Z2098*, *Z2099*, and *ecf1*) that are effective for screening the top 7 STEC/EHEC strains, providing a specific method for detection without relying on traditional *stx* and *eae* markers. However, these markers should be used on pure cultures to avoid false positives. Detecting emerging non-top 7 EHEC strains remains challenging, highlighting the need for further research to find additional markers for these strains.

Supplementary Information

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Supplementary Material 1

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Author contributions

A.N. collected samples, carried out the analysis of samples, data analysis, and wrote the manuscript. M.A., Gh.H. and S.M. designed the study, supervised the project, revised the data analysis, and critically revised all parts of the manuscript. A.D. formal analysis, writing—review and editing. All authors read and approved the final manuscript.

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Data availability

We confirm that all data and findings of this study are available within the Article/Supplementary material.

Declarations

Ethics approval and consent to participate

All procedures involving animals and their care in this study were approved (No. IR140257299) by Iran National Committee for Ethics in Biomedical Research. Moreover, a written or verbal informed consent was obtained from all participants for human experimentation and 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).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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