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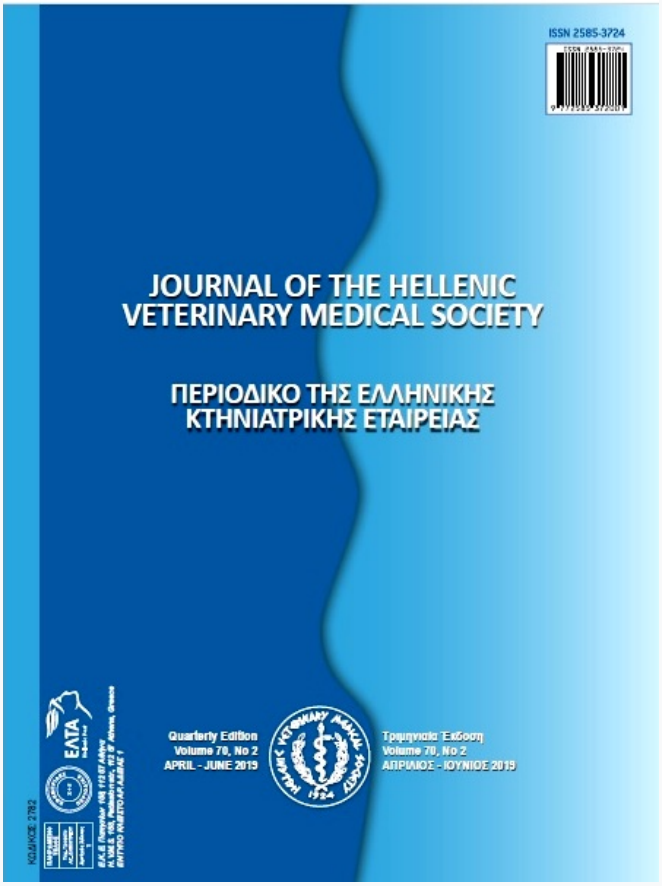

Remarkable characterization and determination of atypical E. coli O157 none producing shiga toxin which can produce cytopathic effect on Vero cell and diarrhea in mice

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Remarkable characterization and determination of atypical *E. coli* O157 none producing shiga toxin which can produce cytopathic effect on Vero cell and diarrhea in mice

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ABSTRACT. This study focused on the cytopathic effect of an unusual form of *E. coli* O157:H7 (atypical *E. coli* O157 (a-O157)) on Vero cell and infant mice. Multiplex PCR assay showed that they did not carry the gene for either shiga toxin 1 (stx1) or stx2 and other virulence factors. Increased changes in epithelial cell morphology, inter and intracellular gap junction and invading were assessed using Vero assay. In addition, a-O157infection causes disruptions of intercellular tight junctions, leading to clinical sequelae that include acute diarrhea in mice. Vero cell monolayers were exposed to a-O157influx and disintegrated tight junction of Vero cell line. Infected Vero cell enhance the number of attaching and effacing (A/E) lesions. Collectively, these findings provide in vitro evidence that Vero cell infected by a-O157was shown injury in epithelial cell barrier and induced A/E lesion the same as *E. coli* O157:H7 stx positive. These variants cannot be diagnosed by routine monitoring methods like biochemical assay for *E. coli* O157:H7 stx positive control. Finally, the data suggest that not only typical but also a-O157are an important cause of diarrhea in mice and produce cytopathic effect on Vero cell and may be harmful for food animal and also human.

Keywords: *E. coli* O157, Shiga toxin, Cytopathic effect, Vero cell, Diarrhea, Mice

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INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) O157 cause food-borne illness. It emerged in 1982 as a human pathogen that produces hemorrhagic colitis (HC) and the hemolytic-uremic syndrome (HUS) (Riley et al., 1983; Garcia-Aljaro et al., 2009). Most illness has been associated with eating undercooked contaminated ground beef, swimming in or drinking contaminated water, and eating contaminated vegetables (CCID, 2006). Although the primary reservoir and the main source of human infection are cattle, the bacterium O157 may be found in sheep, deer, goats naturally (Kudva et al., 2004), mice (Mohawk et al., 2010), rat (Zotta et al., 2008) and rabbit (García et al., 2006).

STEC strains have been shown to elaborate potent *phage-encoded* cytotoxins and other virulence factors as well. They divided into *two main groups*, shiga-like toxins (*stx1* and *stx2*) and several variants including three *stx1* subtypes (*stx1a*, *stx1c*, and *stx1d*) and seven *stx2* subtypes (*stx2a,* stx2b, *st x2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2g)* (Garcia-Aljaro et al., 2006; Melton-Celsa et al., 2015). The toxin requires highly specific receptors which bind covalently to sugar residues on *cell-surface* glycoproteins (Paton and Paton, 2010).

All the above mentioned research results demonstrate that 1) toxin and receptor are the main factors for pathogenicity, 2) clinical STEC- O157 isolates lost their toxin genes during sub cultivation and became non-toxigenic (Paton and Paton 2010) and 3) other virulence factors may influence pathogenesis (Whitworth et al., 2008).

Although many studies have shown that stx is necessary for host pathogenesis and cytopathic effect on Vero cell line (Garcia-Aljaro et al., 2009), a non taxonomic pathogroup of *E. coli* O157 strains is affiliated with diarrhea that has no explainable stx genes, but does have the capability of inducing cytopathic effect on the Vero cell. They have also absence of locus of enterocyte effacement (LEE). They so called atypical *E. coli* O157 (a-O157).

Many fecal samples from distress food animals with diarrhea send to our laboratory for bacteriological identification. They are including *culture* on SMAC-CT, *selective and differential media* for isolation of stx-producing *E. coli*. Finally, in contrast to *E. coli* O157 with pale colony, the designated strain had a primrose appearance. In this study, these novel atypical *E. coli* O157 were characterized.

In this study, the cytopathic effects of a-O157 strains that did not harbor stx genes belonging to serotypes isolated from different distress food animals including calves, kids and lamb were evaluated. In particular, the research determines whether stx and LEE negative STEC O157 isolates could be considered virulent and indicates cytopathic effect on Vero cell and is associated with diarrhea in mice.

MATERIAL AND METHODS

Samples

Recto-anal mucosal swap (RAMS) samples from cattle, sheep and goats (ill and healthy animal) were examined for the presence of STEC O157. The RAMS samples were directly streaked onto sorbitol Mac Conkey ager supplemented with appropriate 0.05 mg/L cefixime and 2.5 mg/L *potassium tellurite* (SMAC-CT) (Merck).

Serological test

Various O157 and H Latex agglutination Kit (Mast group, UK) was used for both sorbitol and non sorbitol fermenting colonies.

Bacteria

Strains included in table 1 were grown on 5% sheep blood agar plates (blood agar base, Merck) and incubated overnight (O/N) at 37°C. According to previous study with some modification multiplex variation polymerase chain reaction (MV-PCR) was applied for molecular analysis of *E. coli* isolates (Tahamtan and Namavari, 2014).

DNA extraction and PCR procedure

The isolates cells were grown O/N at 37°C in luria bernati (LB) (Hi-Media) broth with shaking and DNA was extracted using extraction kit (DNP extraction Cina-Gene Company- Iran) for use as a whole-cell template. The 25-µl reaction mixtures included 2.5 U/µl Taq polymerase, 2 mM MgCl2, 0.4 mM deoxynucleoside triphosphates, 5µl 10× buffer, and 2 µl of the DNA whole-cell template. Thermocycler (Eppendorf, Germany) parameters included 95°C (5 min) one cycle and 94°C (30 s), 58°C (45 s), and 72°C (90 s) for 35 cycles, followed by a final extension time for 72°C (10 min). The oligonucleotides used as primers in PCR to detect *E. coli*, *E. coli* O157 and Stx1, Stx2 with variants are shown in table 2. According to PCR analysis a-O157 was applied in the experiment.

Table 1. Bacterial strains isolated from cattle, sheep and goats during routine fecal examination for bacterial identification.

Source	*E. coli* strains	Serotype	PCR results		Vero assay	Mice assay
			Stx1	Stx2	CPE[4]	Diarrhea
1	EDL933	O157:H7	+	+	+++	+
Cattle	RCC-C1387[2]	a-O157:H7[3]	-	-	++	+
Goats	RCC-G1387	a-O157:H7	-	-	++	+
Sheep	RCC-S1387	a-O157:H7	-	-	++	+
TSB	Control	-	-	-	-	-

1: Reference strains kindly provided from Professor David Gally, University of Edinburgh, UK
2: RCC-C (Razi Culture Collection- Cattle), RCC G (goat), and RCC S (sheep)
3: atypical *E. coli* O157
4: Cytopathic effect

Table 2. Oligonucleotide used in this study.

Primer set	Nucleotide sequence (5' – 3')	Size of amplified product (bp)	Gene specificity
Stx1-F Stx1-R	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	614	stx1 variants
Stx1OX3	GAA CGA AAT AAT TTA TAT GT CTC ATT AGG TAC AAT TCT	555	stx1OX3
Stx2-1 Stx2-2	CTT CGG TAT CCT ATT CCC GG GGA TGC ATC TCT GGT CAT TG	484	stx2 variants
Stx2-F Stx2-R	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA GCA CTT TG	779	stx2 variants [not stx2d
Stx2d-1 Stx2d-2	AAG AAG ATA TTT GTA GCG G TAA ACT GCA CTT CAG CAA AT	256	stx2d
Stx2v 1 Stx2v 2	CAT TCA CAG TAA AAG TGG CC GGG TGC CTC CCG GTG AGT TC	385	Stx2vha, stx2vhb, stx2d
3716F 3718R	GCCGCACAACAGCAGGATAAAC TCCGACCCGAAATTCCTTGC	500	T3SS
Stxe a Stxe b	CCT TAA CTA AAA GGA ATA TA CTG GTG GTG TAT GAT TAA TA	230	Stx2e
Intimin f Intimin r	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881	eae

Tissue culture

Vero cells were used as model epithelia to form monolayers to study the function of all *E. coli* strains. Vero cell lines were acquired from the Razi Institute Cell Bank (Tehran, Iran). They were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GiBco) and 2% penicillin-streptomycin (Biosera) in an atmosphere containing 5% CO2. Cells were grown in 25-cm2 flasks (Orange) until they were confluent. In addition, monolayers were grown on 6.5- or 12-mm-diameter plates (Corning) at 37°C in the presence of 5% CO2 until they were used.

Vero assay

Twenty-four hours prior to epithelial cells being infected by bacteria, the culture medium was replaced with antibiotic and serum-free medium. Prior to infecting epithelial cells, bacteria were inoculated into 10 ml trypticase soy broth (TSB, Merck) and grown O/N at 37°C with agitation and balanced at a final concentration of 5×10^7 colony forming unit (CFU) ml^{-1} [the minimum CFU of pathogen which has been

shown to have cytopathic effect on the Vero cell after 3 h incubation (Tahamtan et al 2011)]. Serial dilutions of a-O157 (cattle, goats and sheep isolates) were made 1:10 in DMEM and added to each well. *E. coli* O157 EDL 933 stx positive was applied as positive control. Just one well received TSB as negative control. Infected cells then were incubated for up to 72 h at 37°C in 5% CO_2. The viability of cell cultures was checked under the inverted microscope in 3, 6, 9, 12 and 24 h, and up to 72 h of incubation.

Mice assay

Five duplicate groups of germ free Balb/c weaned mice (15 in each group) were chosen. All mice groups were fed orally with serial fourfold concentration of a-O157 (cattle, goats and sheep isolates) and *E. coli* O157 EDL933 (table 1). The remaining one group of mice received TSB as negative control. The infantile mice were followed a week for diarrheal sign and probable mortality rate.

Analysis of data

The results are declared as means - standard *deviations* of the means. Analysis of variance (ANOVA) was utilized to distinguish statistical significant differences ($P<0.05$) among various groups. A two-tailed, paired Student's *t* test was carried out to characterize statistical variation between groups.

RESULTS

Serotype characterization

Serotyping confirmed that the isolates expressed O157 and H7 antigens.

PCR

MV-PCR analysis verified the references strains harbored the stxs genes, but most of the test strains, including the RCCs1387, did not possess any stxs genes (they so called a-O157) (table 1).

Vero cell assay

Supernatants of all lysogens were toxic to Vero cells culture. The incubation of a-O157:H7 (10^7 CFU ml^{-1}) for up to 72 h induced an 80-100% CPE on the Vero cells, the same as positive control. On the other hand, STEC O157 converted to a-O157 that has no stx gene but is able to cause lesions on the cell line (figure 1).

The incubation of Vero cells with a-O157 for 3 h up to 24 h reduced the viability of cell. A trend toward increased cell degeneration is observed with cell exposure duration. Complete syncytial degeneration and round shriveled cells occurred within 72 h after inoculation (figure 1).

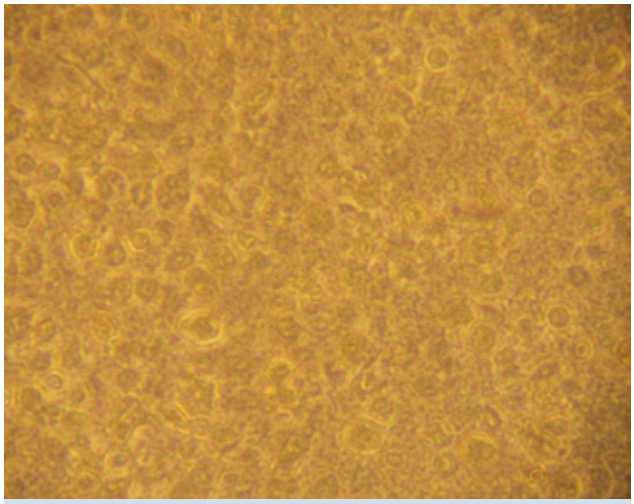

Figure 1. Photographs showing the infection by and the cytopathic effect on monolayer caused by minimum concentrations (10^4 CFU) of a-O157. Mild shrunken round cells are shown.

Logarithmic number of a-O157 demonstrated that viable cell lessened, but no significant differences was observed between lesions caused by various isolates (cattle, sheep and goats) ($p>0.05$). In contrast, a statistically significant increase in the cell degeneration among different exposure time was observed ($p<0.05$). The a-O157 at minimal concentrations (10^4 CFU ml^{-1}) produced only weak syncytial degeneration in the majority of infected Vero cultures when incubated for 3 to 6 h. No significant difference was observed between a-O157 and *E. coli* O157 EDL933 (positive control) ($p<0.05$).

Transmission microscopy of epithelial cells revealed normal cell morphology architecture with intact nuclei. In addition, phase contrast microscopy revealed that there were intact intercellular membrane appositions (Figure 2 a). In contrast, Vero cells infected with a-O157 had inter and intracellular vacuoles (Figure 2 b), and there were separate gaps in the conjunction of intercellular membrane contacts (Figure 2 c) and bacterium invaded cell (Figure 2 d).

Mice assay

a-O157 is able to induce generalized and persistent colonization in mice gastrointestinal (GI) tract the same as positive control. It is localized in the intestine, although it may preliminarily attach to payer's patches before colonization in colon. These strains cause a diversity of clinical complexities ranging from mild

diarrhea, bloody and severe diarrhea, to life threatening illness and finally the mice was dead.

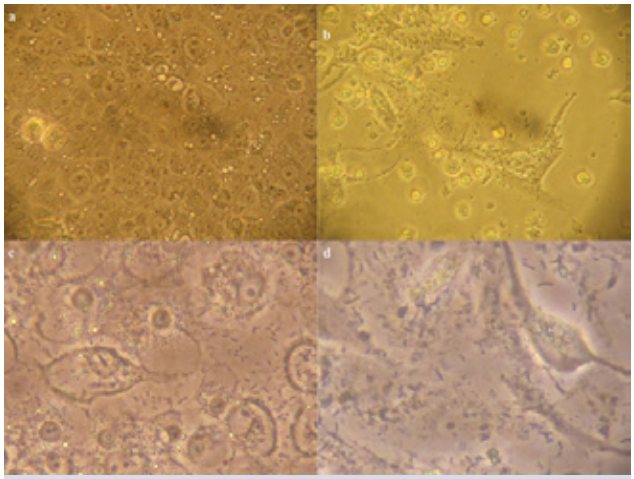

Figure 2. A-Bacterial strains adhere to the surfaces of Vero epithelial cells and demonstrated intact intercellular membrane appositions. B- Inter and intracellular vacuoles were observed after being infected by a-E. coli O157. C- After more exposure to a-E. coli O157 separate gaps in the conjunction of intercellular membrane were observed. D-Finally, the bacterium penetrating into the cells demonstrated cytopathic effect.

DISCUSSION

Several STEC serogroups are important cause of human disease, and the most common of these is O157:H7 (Bielaszewska et al 2007). Although the actual *role* of shiga toxin of *E. coli* O157:H7 in human pathogenesis is well characterized, in addition to these *toxins,* several *other* factors or *toxins may also play* roles *in the pathogenesis.* Furthermore, several genes on the virulence plasmid and on the chromosome have been suggested to have a part in virulence (Gyles 2007).

a-O157 strains were indistinguishable as they exhibited identical biochemical profiles like typical O157:H7 including growth on SMAC agar with no sorbitol fermenting and positive reaction for O and H antiserum tests. According to the genetic profile, a-O157 strains do not harbor stx and other virulence genes. *It has been* suggested that a-*O157* arose from the STEC O157 and the *ancestral E. coli O157*:H7 *has been* maintained during its evolution (Karch et al 2005). Actually, one of the differences was the presence or absence of the genomic segment containing stx, suggesting that a-O157 strains were derived from the O157 strains by the loss of stx in these animals. These STEC strains lost stx gene, giving rise to the a-STEC O157 strains, but kept the toxigenic properties. In fact, STEC O157 converts to a-O157, likewise in microorganisms which are epidemiologically pertaining to or are derivatives of the similar isolate.

Karch and Bielaszewska (2001) also reported sorbitol fermenting (SF) *E. coli* strains serotype O157: H^- in their laboratory that did not contain stx genes. These isolates originated from unrelated patients who suffered from HUS and diarrhea. Random amplified polymorphic DNA PCR analysis showed all isolates belonged to the same genetic cluster.

Moreover, during a family outbreak in Austria, stx-negative SF *E. coli* O157: H^-strains were isolated from patients with negative obligatory bacterial enteric pathogens (Allerberger et al 2000). Before that in Germany, anti O157 serum positive was observed in patients with HUS due to stx-negative SF *E. coli* O157: H^- strains (Schmidt et al 1999).

All the above studies only reported the disease and none of them explained the bacterial strains were isolated. But we have isolated a-O157 strains, examined them, and despite having no toxin gene, they were found to have a detrimental effect on Vero cells and were virulent in mice.

The finding of these *a-O157* pathogens in Vero cell line indicate they may have originated from a *progenitor* of STEC O157. For example, in the transformation of a- O157, the prophage in an integrated phage transformed ca harmless ancestral bacterium into a lethal pathogen (Brussow et al 2004). Feng et al. (2001) findings suggest that isogenic strains (stx negative) are a progeny strain that arose from the parental strain by losing the stxs genes and therefore pathogenicity remained active after losing their stx genes. These may be useful in studying the pathogenesis of stx in O157:H7 infections in humans.

Indeed, whereas cattle have been well established as a major reservoir of STEC O157:H7 (Pierard et al 2012), the origin of stx-negative *E. coli* O157:H7 strains (a-O157), their role in disease, and their pathogenic mechanism are still *not* fully understood. This mention declares that the epidemiology of a-O157:H7 infections may differ from the epidemiology of infections caused by STEC O157:H7.

Pathogenicity of a-O157 isolates is primarily due to its ability to invade and destroy the tissue culture. These are detected by invasion assays using Vero tissue cell culture. However, regardless of these experimental conditions, Vero assay and mouse experiments are not

a good model for explanation of human disease.

a-O157 may internalize but not adhere to Vero cells, and then worsen the tissue culture. Some factors other than stx may exert cytopathic effect on monolayer. The mechanism by which the bacteria *lose* their *stx genes* and kept pathogenicity *has not been* explained. While *these findings suggest that cattle can* excrete very large amounts of *E. coli* O157, the rare isolation of these pathogens from *animals* which are considered as *reservoirs of* atypical O157 *led to the* hypothesis that atypical O157 might be compromised in the human GI tract. Even if this *hypothesis* is confirmed, works are in progress to purify and *further* characterize the influence of a-O157 in human disease. However, the a-O157 strains ability to influence cytopathic effect on Vero cell mediated by unknown process remains. Further study is needed for confirmation of isolates by genome sequencing to determine the phylogenetic relationship with other O157 to detect the heterogeneity within the isolates.

CONCLUSION

Cattle are the main reservoir of *E. coli* O157:H7 but a rare strain of this, a-O157 may not adhere to Vero cells and deteriorate the tissue culture with no Stx genes and might be challenge in human GI tract.

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CONFLICT OF INTEREST

There is no conflict of interest.

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