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Prevalence of Shiga toxin genes and intimin genes in uropathogenic *Escherichia coli*Kobra Abbasi<sup>1</sup>, Elahe Tajbakhsh<sup>1\*</sup><sup>1</sup>Department of Microbiology, Faculty of Basic Science, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

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## ABSTRACT

**Objective:** To identify *stx1*, *stx2* and *eaeA* genes in *Escherichia coli* (*E. coli*) strains isolated from urine samples in Shahrekord, Iran.**Methods:** In this cross study a total of 147 middle urine samples from patients with symptoms of urinary tract infection (UTI), referred to clinical laboratories of Shahrekord were studied. Taken samples were cultured to detect Shigatoxin-producing strains and finally 76 *E. coli* isolates were identified using the standard biochemical tests as well as the selective and differential media. The multiplex PCR method was used to evaluate the presence of *stx1*, *stx2* and *eaeA* genes. DNA bacteria extraction was performed by boiling and then PCR was performed in the presence of specific primers.**Results:** A total of 147 urine samples were collected from patients with suspected UTI, and 76 samples (51.70%) were diagnosed with *E. coli*. Among 76 studied isolations of *E. coli*, 3 (3.94%) had a positive reaction to lactose and negative reaction to sorbitol. In the female gender, *stx1* gene that shown in the samples was related to 30–39 age group. In the other sample related to 20–29 age group, *stx1* and *eaeA* gene were shown. But in male gender *stx1* gene was reported in the sample related to 40–49 age group. *stx1*, *stx2* and *eaeA* genes were not observed together in any samples.**Conclusions:** Isolation of Shiga toxin-producing *E. coli* strains has great importance because of the possibility of clinical complications such as hemolytic-uremic syndrome.

## 1. Introduction

Shiga toxin-producing *Escherichia coli* (*E. coli*) strains (STEC) are most important food borne pathogenic bacteria. STEC are agents of serious illnesses such as food poisoning, dysentery, hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Most of the cases in hemorrhagic colitis, HUS and sudden death in all ages are related to O157:H7 serotypes that are considered as the most important serotypes of STEC[1,2].

In many cases, other Shiga toxin-producing serotypes such as non-O157:H7 serotypes were reported as outbreak or sporadic pathogens and also agents of HUS. Isolation of non-O157:H7 serotypes from pathogens has increased[3]. However, less reports

are for isolation of extraintestinal STEC[4]. Verotoxigenic *E. coli* discharge a toxin that is able to kill vero cells, verotoxin and it is similar to Shiga enterotoxin which is discharged by Shiga dysentery type 1, called a toxin similar to STEC[5]. Verotoxin-producing gene is set on the genome of bacteriophage and enter the *E. coli* by phage conversion[5]. Verotoxins are categorized in two groups: *stx2* and *stx1*. Verotoxin play a role by effecting on RNA ribosomal, preventing synthesis of the protein[6,7]. The pathogenicity of *E. coli* O157:H7 for sever illnesses of human is due to discharge of Shiga-like toxin I and varieties of such toxins[8].

The other pathogen is a protein named intimin that is a 94-kDa outer membrane protein and coded by *E. coli* attaching and effacing (*eae*) gene. The protein is responsible for close link of bacteria with intestinal epithelial cells and damage through special mechanisms called attaching and effacing[8]. Pollution with *E. coli* O157:H7 is often because of ingestion of infected food such as types of meat and mince, meat sandwiches, raw milk, and infected posturized milk, yogurt, chesses, hamburger, sausage, vegetables and fruit juices, water, contact person to person and animals[9].

Bovine is the main source of these bacteria. Also animals such

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as sheep, deer, goat, stag, pig, cat, dog, chicken and goose are main sources. No disease resulted by these bacteria in the mentioned animals has been reported till now. But it is possible to transmit the bacteria from animals to human directly by contacting with water, soil, and ruminant feces and consuming infected food[10-12].

Usually, identifying the strains is not possible in laboratory and researchers try to find simple methods for screening such bacteria. One of the standard methods for identifying verotoxin is cell culture and effect of cytotoxic toxin on vero cells. This method is slow and it is difficult to make it standard and need special experience and skill. In addition, some facilities are necessary such as cell culture, vero antitoxin for neutralizing and identifying type of toxin. Several serotypes has been identified related to producing toxin and using serologic tests for serotype identification is time consuming and costly[13,14]. *E. coli* serotype O157:H7 is the most important serotype in enterohemorrhagic *E. coli*, and in most countries it is identified as the main agent of infection[15].

Isolation and detection of *E. coli* O157:H7 in laboratory based on two properties (absence of sorbitol fermentation and absence of beta-glucuronidase production) help to isolate phenotype of *E. coli* O157:H7 from other strains. So, we may use Sorbitol-MacConkey agar and CHROMagar O157 media for identifying bacteria[9]. For genotyping and simultaneously studying the pathogens of the bacteria, some scholars by using multiplex PCR method, suggested different primers for identifying genes[16-18]. Non-O157:H7 *E. coli* serotypes isolated from HUS cases has increased[19].

Studying the serotypes of non-O157:H7 *E. coli* is difficult as these serotypes are sorbitol-positive and there are no special media for serotype identification, so it is suggested to use molecular methods based on virulence genes like *stx*[19].

STEC strains produce two strong cytotoxins named Shiga toxins 1 & 2 (*stx2*, *stx1*) whose genes are coded by phage. Toxins with cytopathic effect on intestinal epithelial cells cause dysentery. The other pathogen is a protein named intimin which is responsible for attaching bacteria to intestine and special damages named attaching/effacing and cup like structures in epithelial intestine. So, coding gene of this protein is called *E. coli* attaching and effacing (*eae*)[11,12].

Although coding genes of *stx2* and *stx1* and *eaeA* genes are the most important identified pathogens of STEC strains, the separated evaluation of genes by PCR technique is time consuming and costly. Some researcher like Paton in 1997 in Australia[16], Pradel *et al.*, in 1997–1998 in France[17], Blanco *et al.*, in 2003 in Spain[18], Mohsin *et al.*, in 2005 in Pakistan[20] and García-Aljaro *et al.*, in 2005 in Mexico[21], by using multiplex PCR suggested different primers for identifying genes.

According to the above mentioned, and studies about these strains in Iran are limited and most of them are just using culture method for identifying, and unfortunately, till now studies are not enough and investigations has implemented for simultaneous evaluations of infected genes of this bacterial[22], the purpose of the present study is to investigate the outbreak of *stx2*, *stx1*, *eaeA* in STEC isolated from urinary tract infection (UTI) samples in Shahrekord.

## 2. Materials and methods

### 2.1. Collection, identification and storage of uropathogenic *E. coli*

Sampling was established at the laboratories of Shahrekord, Iran in 2014 following ethical approval from the Research Ethics Committee, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

In this cross study a total of 147 middle urine samples (98 samples from female and 49 samples from male) from non-hospitalized suspected cases of UTIs were collected from various clinical laboratories of Shahrekord region. For isolation of *E. coli*, these samples were directly inoculated on MacConkey agar (Merck, Germany) plates. After overnight incubation at 37 °C, lactose-fermenting colonies were identified as *E. coli* by characteristic morphology and inoculation on triple sugar iron agar (Merck, Germany) slants. The isolates were kept as stocks at 20 °C in 50% glycerol stocks for long term storage until PCR assays were performed. A total of 76 *E. coli* isolates were confirmed using standard biochemical tests for *E. coli* (IMViC reactions; *E. coli* isolates were identified by positive indole and methyl red tests, and negative Voges-Proskauer and citrate utilization tests)[23].

### 2.2. DNA extraction and PCR based confirmation of *E. coli* isolates

Each isolate was inoculated in 5 mL of trypticase soy broth. After overnight incubation at 37 °C, DNA bacteria extraction was performed by boiling method according to the previously described protocol with some modifications[24]. There are many virulence genes associated with uropathogenic *E. coli*. For confirmation of *E. coli*, PCR was performed by selected Shiga toxin genes (*stx1* and *stx2*) and intimin genes (*eaeA*). The primer sequences and expected sizes of amplicons for each PCR assay are described in Table 1.

### 2.3. PCR for virulence related genes

PCR was performed for each isolate for the identification of virulence related genes. Each reaction contained 2.5 µL buffer (10×), 1.5 µL MgCl<sub>2</sub> (50 mmol/L), 1 µL diethyl-nitrophenyl thiophosphate (10 mmol/L), 1 µL (50 pmol) for each of the forward and reverse primers (primers are listed in Table 1), 1 µL *Taq* DNA polymerase, (Fermentas, USA), 1 µL template DNA (200 ng), and 16 µL H<sub>2</sub>O in a final volume of 25 µL. All reaction mixtures were overlaid with 30 µL of mineral oil. The thermal cycler (MasterCycler; Eppendorf, Hamburg, Germany) conditions are as described in Table 1. The amplified PCR products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE [10.8 g of 89 mmol/L Tris-base, 5.5 g of 2 mmol/L boric acid, 4 mL of 0.5 mol/L ethylene diamine tetraacetic acid (pH 8.0)], with all components being combined in sufficient H<sub>2</sub>O, and aliquots 10 µL of PCR products

**Table 1**

Primers used for amplification in this study.

| Target genes | Primer sequences (5' to 3')                           | Amplicon size (bp) | Cycle condition   | References |
|--------------|---|--------------------|---|------------|
| <i>stx1</i>  | F-ACACTGGATGATCTCAGTGG<br>R-CTGAATCCCCCTCCATTATG      | 614                | 96 °C 5 min, 95 °C 1 min, 58 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min | [25]       |
| <i>stx2</i>  | F-ACCATGACAACGGACAGCAGTT<br>F-CCTGTCAACTGAGCAGCACTTTG | 779                | 96 °C 5 min, 95 °C 1 min, 58 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min | [25]       |
| <i>eaeA</i>  | F-GTGGCGAATACTGGCGAGACT<br>R-CCCCATCTTTTCACCGTCG      | 890                | 96 °C 5 min, 95 °C 1 min, 60 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 7 min | [26]       |
| 16s rRNA     | F-GCGGACGGGTGAGTAATGT<br>R-TCATCCTCTCAGACCAGCTA       | 200                | 94 °C 5 min, 94 °C 45 S, 55 °C 1 min, 72 °C 1 min for 30 cycles, 72 °C 5 min  | [27]       |

were loaded to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, images were obtained in UVIdoc gel documentation systems (UK).

#### 2.4. Statistical analysis

Analysis of data was performed by the SPSS version 18.0 computer software (SPSS, Chicago, IL) and the data were statistically analyzed using *Chi*-square and Fisher's exact tests. Analysis of variance was used to compare the prevalence of presence of different virulence related genes. Statistical significance was considered at  $P < 0.05$ .

### 3. Results

A total of 147 urine samples were collected from patients with suspected UTI, and 76 samples (51.70%) were diagnosed with *E. coli*. Among 76 studied isolations of *E. coli*, 3 (3.95%) had a positive reaction to lactose and negative reaction to sorbitol which were identified as STEC and 73 (96.05%) isolates were identified as non STEC strains.

From the 76 samples, 25 isolates were related to male (32.89%) and 51 isolates (67.11%) were related to female. Age levels of the study samples were divided to five groups, 40–49, 30–39, 20–29, 1–19 and up to 40, for female the highest infection level occurred in ages 20–29 and for male, in ages 40–49 (Table 2). Statistical analysis by Fisher exact test showed that there was no significant relationship between gender and the type of bacteria ( $P > 0.05$ ). Statistical analysis using *Chi*-square test showed that there was no significant relationship between age group and the type of bacteria ( $P > 0.05$ ).

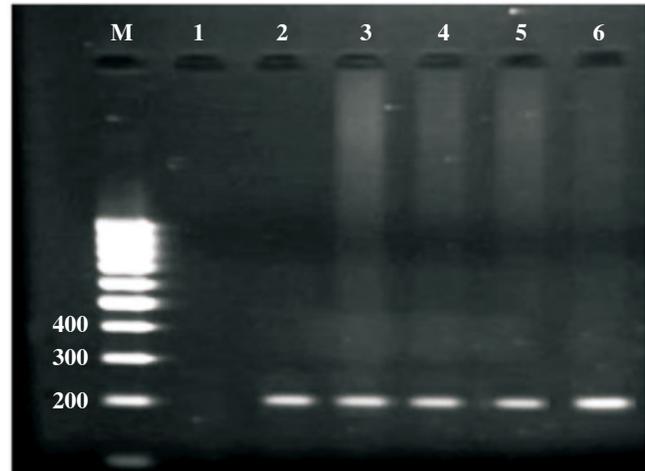
In the present study, PCR was used for the amplification of 16s rRNA genes for the detection of bacteria causing urinary tract infections. Results are shown in Figure 1.

**Table 2***E. coli* isolates in relation to sex and age. *n* (%).

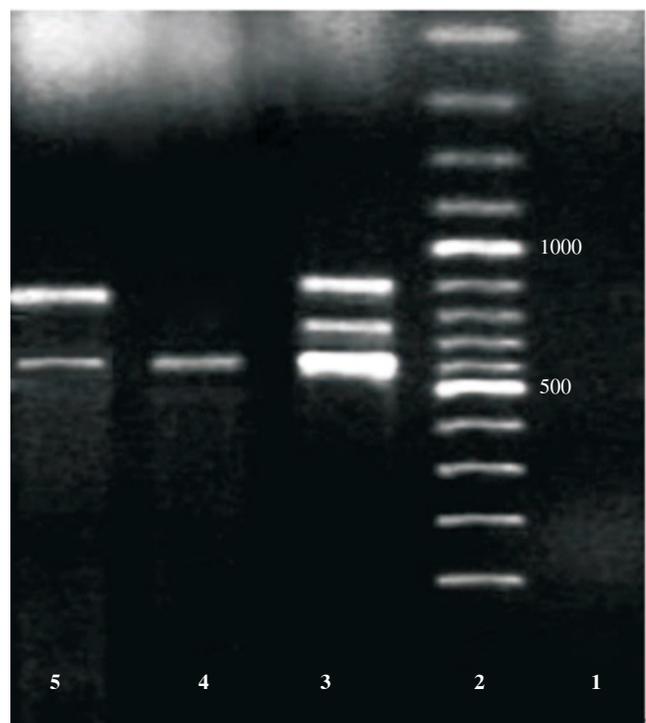
| Age groups | Gender      |             |
|------------|-------------|-------------|
|            | Male        | Female      |
| 1–19       | 3 (12.00)   | 10 (19.60)  |
| 20–29      | 5 (20.00)   | 20 (39.21)  |
| 30–39      | 7 (28.00)   | 8 (15.68)   |
| 40–49      | 8 (32.00)   | 8 (15.68)   |
| > 49       | 2 (8.00)    | 5 (9.80)    |
| Total      | 25 (100.00) | 51 (100.00) |

In this study, 3 STEC strains were isolated by multiplex PCR (1 STEC strains were isolated from male and 2 STEC strains were

isolated from female). Positive multiplex PCR results for the detection of *stx1* and *eaeA* genes in female are shown in Figure 2. And no band was observed to have *stx2* gene in any isolate.



**Figure 1.** Agarose gel electrophoresis of 16s rRNA gene in *E. coli* strains. Lane M: 100 bp DNA ladder; Line 1: Negative control; Line 2: Positive control; Lane 3-6: Positive samples (200 bp).



**Figure 2.** Result of the PCR assay for *stx1*, *stx2* and *eaeA* genes in *E. coli* isolates.

Lane 1: Negative control; Lane 2: DNA size ladder 100 bp (Fermentas); Lane 3: Positive control for *stx1* gene (614 bp), *stx2* gene (779 bp), and *eaeA* (890 bp) genes; Lane 4: Positive sample for *stx1* gene (614 bp); Lane 5: Positive samples for *stx1* (614 bp) and *eaeA* (890 bp) genes.

In one isolate from 20–29 year-old female, there were two genes of *stx1* and *eaeA* in this isolate and other isolates from 30–39 ages had just *stx1* gene. Only one isolate was positive for *stx1* gene in male aged 40–49. No band was observed to have three *stx1*, *stx2* and *eaeA* genes at the same time in any isolate. Among 20 isolates from female samples in 20–29 ages, one isolate had both *stx1* and *eaeA* genes, and in 8 isolates separated from female samples in 30–39 ages, *stx1* gene was observed.

#### 4. Discussion

Enterobacteriaceae family, especially *E. coli* is the main pathogen of UTI[28]. In the present study, among 76 *E. coli* isolates isolated from UTI cases only 3 isolates were identified as STEC strains. Bands related to *eaeA*, *stx1*, *stx2* genes were just observed in 3 isolates (3.94%). Also in another study, for 300 *E. coli* isolates separated from UTI in Tehran, high outbreak of *stx1*, *stx2* reported. As from 300 *E. coli* isolates of UTI, 30 samples (10%) with *stx1* gene, 18 samples (6%) with *stx2* gene and 3 samples simultaneously including *stx1* and *stx2* were reported[29]. It seems that the first report of high prevalence of *stx1* and *stx2* genes is in *E. coli* European pathogenic isolates[29]. Adeli *et al.*[11] reviewed the outbreak of Shiga-toxin genes and intimin genes in STEC strains isolated from UTI samples in Lorestan. From the study they found that from 100 *E. coli* isolates, 2 strains (2%) contained *stx2* and *eaeA*. It was determined that one isolate (1%) contained *stx1* and no isolate was observed to contain all three genes[11]. The results are in accordance with our study's results. In the study by Adeli *et al.*[11] the most pathogen of UTI was *E. coli* bacteria with 69.4 percent frequency and the other pathogens of UTI included *Klebsiella pneumoniae* (16.7%), coagulase-negative staphylococci (9.1%), *Enterobacter* (3.2%), *Proteus* spp. (0.8%) and *Pseudomonas aeruginosa* (0.8%)[11].

In a study by Navidinia *et al.*, among 12572 UTI samples in Mofid Children's Hospital, Tehran, Iran, 374 *E. coli* isolates were recognized and only 9 isolates were identified as enterohaemorrhagic *E. coli* and only 5 isolates are carriers of *vtx* gene[30].

In the study by Bonyadian *et al.*, to detect these serotypes specially *E. coli* O157:H7 in stool samples of patients with diarrhoea and to identify virulence genes (*stx1*, *stx2*, *hly* and *eae*) in Shahrekord-Iran area using PCR technique[31], it was showed that 27.6% contained *stx1* gene and 6.9% contained *stx2* gene and 13.8% contained both genes, so high outbreak of STEC strains was concluded in Shahrekord, Iran[31].

Kalantar *et al.* studied 438 samples of UTI in children by positive culture in 12 provinces of Iran[28]. The study reported *E. coli* as the highest pathogen (54.8%), followed by *Klebsiella pneumoniae* (16%), coagulase-negative staphylococci (11.6%), *Enterobacter* spp. (9.6%), *Proteus* spp. (1.4%) and *Pseudomonas aeruginosa* with frequency of 1.4%[28]. *E. coli* was more frequently isolated, which

was probably because the study implemented for children but present study is for all ages. Isolation of STEC from UTI is in very limited cases, but studies in different areas show outbreak of some dangerous syndromes such as HUS and hemorrhagic colitis following UTI[32]. Also, the cases of UTI outbreak resulted from STEC is increasing[30]. Data about frequency of outbreak of O157 *E. coli* is limited, because most of the medical diagnosis laboratories do not evaluate the organism daily and results of most of the tests show presence of sporadic of bacteria[11,12].

Diagnosis of STEC strains is very important, as it could result in outbreak of some dangerous syndromes such as HUS and hemorrhagic colitis. Therefore, adequate prevention against such infection depends on immediate diagnosis of pathogen by using immediate and exact method. In the present study, multiplex PCR method was used for study virulence genes of Shiga toxin and intimin for diagnosis of Shiga toxin-producing strains. In different studies, different methods were used for diagnosis of STEC strains in clinical samples. Johnson *et al.* in US studied 597 *E. coli* strains separated from UTI for their ability of producing Shiga toxins by immunoassay method with none of producing Shiga toxins *E. coli* strains found[4]. In study by Pulz *et al.* 295 samples of stool were used for study the *E. coli* stx producing strains by PCR and ELISA methods, and PCR was found as a good and useful method for immediate diagnosis of STEC strains in clinical samples[33]. Another study was implemented by Gilmour *et al.* in Canada to study the cytotoxic effect of *E. coli* strains from 876 children stool samples using methods of cell culture, immunoassay and PCR[34]. The results showed that molecular methods have higher ability for diagnosis of STEC strains in clinical samples[34].

Mohsin *et al.*[20] carried out a study among 200 children by multiplex PCR. Results showed 22 persons (11%) with one or two Shiga toxin genes, as frequency of *stx1* and *stx2* genes was reported as 1.57%, and *eae* gene was 8.42%.

In a study by García-Aljaro *et al.*[21], from 22 STEC strains isolated from patients with diarrhea, most of them had *stx2* gene or a mix of *stx1* and *eaeA* genes, as 9 strains with *stx2* gene, 9 strains with *eaeA-stx1* genes, 3 strains with *stx1* gene and 1 strain with *hlyA-eaeA-stx1* genes.

Isolation of the stx toxin-producing *E. coli* from urine rarely happens. Beotin *et al.*, first reported about it, that they could separate a STEC strain from urine of a patient with no signs of HUS[35].

Our results showed that 3 isolates were STEC strains (1 isolate from male and 2 isolates from female), and *stx1*, *stx2* or *eaeA* genes were found in STEC using multiplex PCR method. According to the previous study, presence of *stx1* and *stx2* genes with *eaeA* showed in O157:H7 serotype[16]. O157:H7 serotype and non-O157:H7 serotype are pathogens of most of the illness[36]. Estimating outbreak of illness from strains like non-O157:H7 STEC is very complex and it need

molecular check for presence of genes like *stx1* and *stx2*, because these serotypes are usually sorbitol positive, on the other hand, there is not media such as sorbitol-MacConkey agar for determining their nature[36]. It is estimated that in North America between 20 to 25 percent of HUS cases are resulted from non-O157:H7 serotypes[36]. In a study in Belgium, 62% of STEC were related to non-O157:H7 serotypes[37]. In a study in Tehran on UTI samples by PCR method, results showed that among 180 strains of *E. coli*, 24% of the samples were related to O157 serotype and 76% related to non-O157 serotype[30]. In another study in Spain, outbreak of O157:H7 serotypes separated from trap was higher than O157:H7 serotypes[36]. In a study in Japan, pretty much STEC strains separated from cows with O157:H7 serotypes and non-O157:H7 serotypes had *stx* gene. Also, most of them were recognized as carriers of *eaeA* gene, which showed that O157 serotypes and non-O157 serotypes separated from cattle can be pathogens for human[38]. In a study in Spain, outbreak of *stx1* (9.7%) and outbreak of *stx2* gene (48.4%) were found and 41.9% of strains had *stx1* and *stx2* genes. Also, *stx2* gene was found in all O157:H7 *E. coli* strains and in 88% of non-O157:H7 strains, except that two strains were carriers of *eaeA* genes[36]. Isolation of *eaeA* STEC strains from patients with severe illness were reported[39]. Therefore, isolation of *eaeA* gene specially with *stx* gene is important in the present study.

Generally, isolation of STEC *E. coli* strains of UTI samples in Shahrekord show the presence of O157:H7 serotypes and non-O157:H7 serotypes in this area. Isolation of the above strains is important due to possibility of dangerous clinical side effects, for example HUS. More studies by using exact molecular methods in different areas in Iran has increased our knowledge about outbreak of important serotypes and it is useful for management of illness and preventing clinical dangerous syndromes. Also exact physiologic evaluation, metabolism and bacterial genetic help to better understand its pathogenicity. Because of severe pathogenicity, low dose of infecting and non-routine review in clinical laboratories, it is suggested to have more extent studies on genotyping bacteria *E. coli* O157:H7 in other areas of the country. The prevention and control measures of infections resulted from the bacteria should be scheduled.

### Conflict of interest statement

We declare that we have no conflict of interest.

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