

Identifying verotoxigenic *Escherishia.coli* isolated from cold water fishes by Multiplex PCR in Chaharmahal Va Bakhtiary province

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Abstract

Introduction: This study was to determine the prevalence of verotoxigenic *E.coli* and their virulence factors isolated from coldwater fishes (Rainbow trout) using multiplex PCR.

Materials and methods: A total of 100 samples of feces and skins were collected at the final step of production in the production pools. Bacteriological and biochemical examination were done for isolation and confirmation that the isolates belonged to the *E.coli* species. Multiplex PCR were used to identifying the *Stx*₁, *Stx*₂, *eae* and *hly* genes.

Results: The results showed that none of the isolates was *E.coli* O157: H7, but other non-O157 verotoxigenic strains were isolated. Totally 14% of the skin isolates and 4% of fecal isolates contain *Stx*₁, *Stx*₂, *eae* genes.

Discussion and conclusion: The results of this study revealed that the Rainbow trout meat may play a role as a vehicle to transmit the verotoxigenic *E.coli* to human. Water may contaminate with feces of animals, birds, contaminated fish foods and workers. Controlling the contaminating ones is recommended.

Key words: Cold water, Fish, Verotoxin, *E.coli*, Multiplex PCR

Introduction

Escherichia coli live commensally in the gastrointestinal tract of most mammals and incidence of this organism is very rare in fishes. However, some serotypes of *E.coli* are human pathogens and have been implicated in foodborne illness.

Verotoxigenic *Escherichia coli* (STEC) is an important group that may cause gastrointestinal disease in humans, particularly since these infections may result in life-threatening sequel such as the hemolytic- uremic syndrome (HUS) (1). STEC produces one or both of two major types of Shiga toxin, designated Stx1 and Stx2; production of the latter is associated with an increased risk of developing HUS (2). Other putative accessory virulence factors produced by subsets of STEC include the capacity to produce attaching and effacing lesions on intestinal mucosa, as well as megaplasmid- encoded factors such as the enterohemolysin (3 and 4). One of the most important serotypes with increasing frequency over the last 2 decades is O157: H7 which first time identified as a human pathogen in 1982 has emerged as a major cause of both sporadic cases and outbreaks of bloody diarrhea in North America. Most of *E.coli* O157: H7 infections are food- borne. Food of bovine origin have been identified as the principal vehicle in most outbreaks (3). Also, the non- O157: H7 serotypes that produce verotoxin, A/E lesions and enterohemolysin have been reported (5 and 6). DNA- based assays for identifying *Stx* genes, *eae* and enterohemolysin (*ehly*) genes do not seem to discriminate the non- O157 *E.coli* from

E.coli O157: H7 serotypes. This makes accurate diagnosis of non- O157 EHEC more difficult especially in developing countries where advance techniques are not available in most laboratory facilities. EHEC appears to be transmitted primarily through the ingestion of fecal contaminated foods, particularly undercooked beef (4). However, a large number of outbreaks of EHEC have also been associated with consumption of contaminated drinking water or contact with recreational water (7).

This study was carried out to identify the verotoxigenic *E.coli* and other virulence factors isolated from cold water fishes in Chaharmahal va Bakhtiari province, Iran.

Materials and methods

Collecting the samples

A total of 100 fecal samples and 100 skin and meat samples of the Rainbow trout fishes individually were collected. The samples were chosen randomly (cluster random sampling) from 10 producing pools located in the south of the Chaharmahal va Bakhtiari province at the final step of production in spring and summer 2012.

Isolation of *E.coli*

MacConkey and SMAC agar (Merck) were used to detection of the *E.coli* and EHEC colonies (8). A swab of fecal samples and suspension preparing from skin and meat (25 g of sample in 225 ml of LB broth) was cultured on MC and SMAC agar and incubated at 37 °C for 18 to 24 h. Five Non- Sorbitol fermenting (NSF) (colorless) colonies from SMAC agar and five red colonies from MC agar were transferred individually to fresh Lurian-

Bertani (LB) broth and incubated at 37°C for 18 h. Complete biochemical and differentiated tests were used to be certain that strains belonged to the *E. coli* species (8).

Serology

Isolated *E.coli* was examined by O157 and H7 antisera (MAST), to identify O157 or O157: H7 serotypes.

Detection of virulence genes using PCR

PCR primers

The PCR primers were chosen from each of the verotoxins (*Stx1* and *Stx2*), *eae* and *hly* from published sequences (Table 1). The primers were selected to have similar melting temperatures and resulted in products of different sizes that could be easily distinguished in agarose gels (9).

PCR

Each test was performed in a volume of 25 ul containing the following PCR components: dNTP (200 umol/liter of each), PCR buffer 10x, MgCl₂ (final concentration 5 mmol/liter), various concentrations of each primer set (10 pmol/ul), 2 U of *Taq* DNA polymerase, and 2 ul of DNA template. A whole- cell preparation of isolates was tested as crude DNA templates and was prepared by resuspending 1 bacterial colony in 25 ul of sterile DNase- free, RNase- free deionized water. Before being added to the PCR mixture, to control purposes and isolated strain, DNA was isolated and purified from the reference *E.coli* O157: H7 using a genomic DNA isolation Kit (Fermentase). For the PCR amplifications, a total 50 ng of

the isolated DNAs was used. PCR amplifications were performed in a thermal cycle under the following conditions: an initial DNA denaturation step at 94 ° C for 5 min following by 35 cycles beginning with 1 min of denaturation at 94 ° C, 1 min of primer annealing at 50 ° C, and 1 min of extension at 72° C. The final extension step was performed at 72 ° C for 7 min. The amplified PCR products (10- ul aliquots) were analyzed by electrophoresis in 2% agarose gels in Tris acetate (TAE) buffer at 100 V for 45 min. The gels were stained with ethidium bromide and photographed under ultraviolet light using a commercial documentation system.

Optimization of PCR conditions.

To standardize the conditions for the multiplex PCR test, the concentration of each of the 4 primer pairs, the optimal annealing temperature, and Mg²⁺ concentration were determined using the DNA (50 ng) of isolated strains as templates. Initially, the same concentration (100 nmol/ liter) of each set of primers was used, but this approach resulted in uneven intensities or even lack of some of the amplified products. To overcome this problem, the concentration of each primer pair was adjusted without changing the optimal annealing temperature (50 °C) and the concentration of Mg²⁺ (5 mmol/liter). The best concentrations of each primer set that yielded 4 distinct PCR bands were 10 pmol.

Results

Identification of virulence genes

Out of the overall *E.coli* strains (18), 14 strains were isolated from skin and 4 strains were isolated from feces, but none of them reacted positively with O157 and H7

antiseras. The results of the multiplex PCR revealed that 8 (57%) of 14 strains isolated from skin and 2 (50%) of 4 strains isolated from feces contain virulence genes (*Stx1*, *Stx2* and *eae*) and none of the isolates contain *hly* gene (Table 2).

Table 1- Primers used in the multiplex PCR for *stxI*, *stxII*, *Intimin* genes

Virulence factor	Primer sequence (5'-3')	Size of product (bp)
<i>stxI</i> (F) <i>stxI</i> (R)	TTC GCT CTG CAA TAG GTA TTC CCC AGT TCA ATG TAA GAT	555
<i>stxII</i> (F) <i>stxII</i> (R)	GTG CCT GTT ACT GGG TTT TTC TTC AGG GGT CGA TAT CTC TGT CC	118
Intimin (F) Intimin (R)	ATA TCC GTT TTA ATG GCT ATC T AAT CTT CTG CGT ACT GTG TTC A	425
Hly (F) Hly (R)	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1551

Table 2- Virulence genes of *E.coli* isolated from feces, skin and meat of Rainbow trout fish

Samples	No	<i>E.coli</i>	<i>Stx1</i>	<i>Stx2</i>	<i>eae</i>	<i>Stx1, Stx2, eae</i>	<i>Stx2,eae</i>
Skin & meat	100	14 (14%)	3 (21.4%)	0	0	3 (21.4%)	2 (14.2)
Feces	100	4 (4%)	0	0	0	2 (50%)	0
Total	200	18 (9%)	3 (16.6%)	0	0	5 (27.7%)	2 (11.1%)

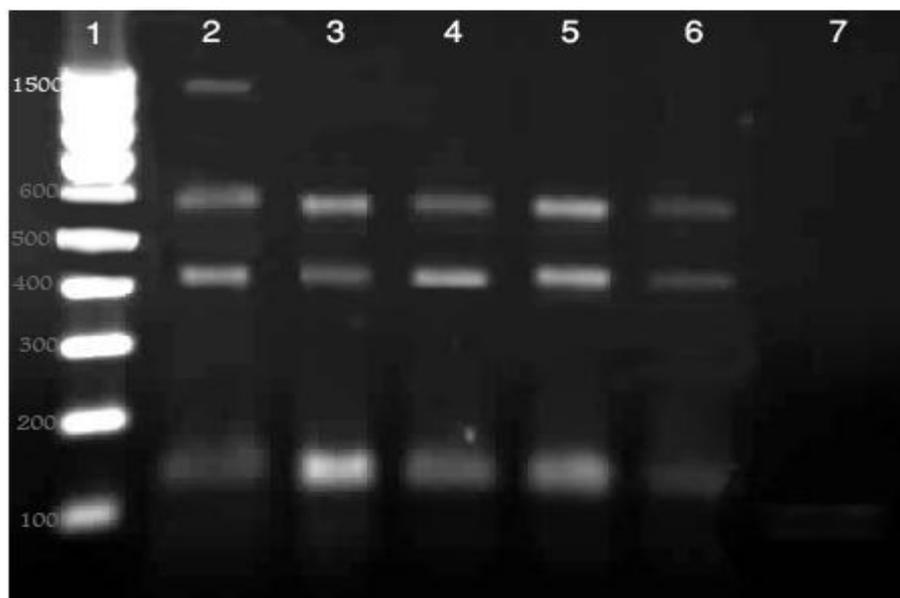


Fig 1- Multiplex PCR for detection of *Stx1* (555 bp), *Stx2* (118 bp) and *eae* (425 bp)
1: DNA Ladder, 2 Control positive, 7 Control negative. 3, 4, 5 and 6 positive samples

Discussion and conclusion

VTEC is a group of *E. coli* that produces one or more verocytotoxins (VT). Only a small fraction of all VTEC- types isolated from animals, food or the environment are associated with human illness. However, VTEC O157 is an important cause of bloody diarrhoea and kidney failure (haemolytic uraemic syndrome, HUS). VTEC O157 infections originate from ingestion of foods contaminated by ruminant or human fecal material and where the conditions in the food chain thereafter enable survival. Moreover, the issue of cross- contamination should not be ignored. The infectious dose for VTEC O157 is very low and an infection may result from consumption of contaminated foods in which the bacteria have survived, but not necessarily grown.

The multiplex PCR system described here is an efficient tool for detection of verotoxigenic genes of VTEC strains. Moreover, this assay allows simultaneous identification of pathogenic *E. coli* and differentiation of them from commensal isolates. The use of 3 sets of primers in a single PCR mixture allowed for successful amplification of *Stx1*, *Stx2* and *eae* genes.

Regarding to the results of present study 18 strains of *E. coli* were identified that 55% of them contain virulence genes (*Stx1*, *Stx2* and *eae*). In this case, most of the isolate related to skin and meat of the samples. Van den Broek showed that from 242 fishes, Gram negative pathogen was not detected in any samples (10). However, *E. coli* is not a normal flora of the intestine of the fishes but contamination of water using for culture is the main source of isolated strains.

Quines identified the presence of some coliforms like *E. coli* and *Klebsiella sp* in

some kind of fishes like *Oreochromis niloticus*, *Cyprinus carpio* and *Ophicephalus striatus*. (11). Akhondzade Basti recovered 65% *E. coli* from intestine of silver carp and common carp. He concluded that wide range of pathogenic microorganism in their intestine due to contamination of the water with sewage, land run- off, etc (12).

There are a few reports from contamination of fishes to verotoxigenic *E. coli*. Adesiyun reported that 9% of the fish samples contaminated with *E. coli* but only 4.7% of the isolates produce verotoxins (13). Pao showed that 13.2% of cat fish were contaminated with *E. coli* but *E. coli* O157 were not found in any sample (14). In contrast, Tuyet reported that *E. coli* O157: H7 carriage by Zebu (*Bos indicus*) in the central Africa. He concluded that contamination of the field surface water appear to be important contributory factors (15). The results of present study are close to the results of Manna study in India. He showed that although *E. coli* O157: H7 was not detected from finfish and shellfish, a few samples were contaminated with non-O157 serotype of enterohaemolysin and shiga toxin- producing *E. coli* (16). Tavakoli reported the incidence of *E. coli* in Carp (8%) and Shad (2%) fishes in Iran (17). We have no report for incidence of *E. coli* in cold water fishes in Iran.

The contamination with verotoxigenic *E. coli* in fishes is very different from different parts of geographical conditions. Regarding to the results of present study most of the isolated strain were from skin and meat of the samples and contamination of feces of the sample to *E. coli* was low. It indicated that the source of the contamination is pool water using for cultivation.

Water may contaminate with feces of animals, birds, contaminated fish foods and workers.

The present study revealed that the high prevalence of verotoxigenic *E. coli* other than O157: H7 serotype in the cold water fishes and it may be the main concern for public health and it is needed to consider about the quality of water sources using in the cold water fishes farms.

The additional study to identify the main verotoxigenic serotypes of *E. coli* is recommended.

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شناسایی باکتری‌های *اشریشیا کلی* و روتوکسیژن جدا شده از ماهیان سرد آبی به وسیله واکنش زنجیره‌ای پلیمر از چند گانه در استان چهارمحال و بختیاری

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چکیده

مقدمه: این مطالعه به منظور شناسایی میزان شیوع سویه‌های وروتوکسیژن باکتری *اشریشیا کلی* در ماهی سرد آبی غزل آلاهی رنگین کمان توسط روش واکنش پلیمر از زنجیره ای چند گانه انجام شد.

مواد و روش‌ها: تعداد ۱۰۰ نمونه مدفوع، پوست و گوشت ماهی در مراحل آخر تولید از استخرهای پرورش اخذ و آزمون‌های میکروبی‌شناسی و بیوشیمیایی برای تشخیص باکتری *اشریشیا کلی* انجام شد. سپس، سویه‌های باکتری *اشریشیا کلی* جدا شده توسط روش واکنش پلیمر از زنجیره‌ای چند گانه برای شناسایی ژن‌های حدت *Stx1*، *Stx2*، *eae* و *hly* آزمون شدند.

نتایج: نتایج این مطالعه نشان داد که ماهیان سرد آبی به سویه‌های غیر از O157 که وروتوکسیژن هستند، آلوده بودند. در ۱۴ درصد موارد باکتری‌های جدا شده از پوست و در ۴ درصد موارد باکتری‌های جدا شده از مدفوع حاوی ژن‌های *Stx1*، *Stx2* و *eae* بودند.

بحث و نتیجه‌گیری: این مطالعه نشانگر این است که ماهیان سرد آبی پرورشی می‌توانند حامل سویه‌های وروتوکسیژن باکتری *اشریشیا کلی* بوده و موجب انتقال این سویه‌ها به انسان شوند. از علت‌های این مسأله ممکن است آلودگی آب استخرهای پرورش ماهی توسط مدفوع حیوانات، پرندگان، غذای ماهیان یا کارگران به باکتری را نام برد. توجه بیشتر برای محدودسازی عوامل آلوده کننده توصیه می‌شود.

واژه‌های کلیدی: ماهیان سرد آبی، *اشریشیا کلی*، وروتوکسین و پلیمر از زنجیره‌ای چند گانه

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