original article

Isolation of shiga toxin-producing *Escherichia coli* O157:H7/NM from hamburger and chicken nugget

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ABSTRACT

Aims: This study was conducted to determine the prevalence rate, virulence genes and antimicrobial resistance of *Escherichia coli* O157:H7/NM isolated from hamburger and chicken nugget in Isfahan, Iran.

Material and Methods: From June 2013 to July 2013, a total of 190 hamburger (120) and chicken nugget (70), were collected from four randomly selected factories in Isfahan, Iran. They were evaluated for the presence of *E. coli* O157:H7/NM using microbiological culture and polymerase chain reaction. Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago o, IL, USA).

Results: From a total of 190 samples analyzed four samples (2.1%) were contaminated with *E. coli* O157. All of the *E. coli* O157 were isolated from hamburger samples (3.3%) and chicken nugget samples were negative. Of four *E. coli* O157 isolated, only one sample was serotype *E. coli* O157:H7 and others were serotype *E. coli* O157:NM. Among four *E. coli* O157:H7/NM isolates, one strain was positive for all *stx1*, *stx2*, *eaeA* and *ehxA* genes. One strain was positive for *stx2* gene. The other two were negative for these genes. All isolates (100%) were resistant to one or more antimicrobial agents.

Conclusions: The results of this study showed that hamburger could be a significant source of *E. coli* O157:H7 and *E. coli* O157:NM serotypes in Iran and multi-resistance was found in 27% of *E. coli* O157 strains and this is a major public health concern.

Key words: Antimicrobial resistance, chicken nugget, *Escherichia coli* O157:H7/NM, hamburger, virulence genes

INTRODUCTION

Escherichia coli is a Gram-negative, facultative anaerobic and commensal bacterium which has been estimated for up to 1% of the bacterial flora of the gut.[1,2] Based on the specific virulence factor and their types of diseases, *E. coli* can be classified into six categories including enteropathogenic, enterotoxigenic, Shiga toxin-producing (STEC), enteroinvasive, enteroaggregative and diffuse-adhering *Escherichia coli*.[3]

Recently, it has been determined that strains of STEC, the well-studied strains of the *E. coli*, are the main global food safety concern.[4] Among these strains, O157:H7 and O157:NM are the most important due to their linked to severe diseases such as bloody diarrhea, hemorrhagic and hemolytic uremic syndrome (HUS).[5] Although *E. coli* O157:H7/NM was isolated for the first time in the 1970s but recognized as a food borne pathogen in the 1980s, however, since 1982 several outbreaks in all over the world established it as a food-borne pathogen.[6-8]

E. coli O157 can be found in a wide variety of foods such as meats, meat products, milk, dairy products and fresh products.[9-12] In addition, recently it has been investigated that *E. coli* O157 enable to survive in a wide range of conditions such as freezing temperature ($-20°C$) and acidic foods.[7,13] Several lines of investigations have shown that fewer than 100 bacterial cells are enough to cause an infection.[14-16] Based on literature reviews, there are some virulence factors for pathogenicity of this bacterium. These virulence factors are as follows: *stx1* and *stx2* Shiga toxins and correlated variants that encoded by *stx1* and *stx2* genes, intimin is the second virulence factor that is necessary for linked between bacteria and intestinal epithelium and it is encoded by *eaeA* gene. Last factor is enterohemolysin, which is encoded by *ehxA* gene.[17-19]

E. coli O157:H7 lives in the intestines of many animals including cattle, chickens, birds, pigs and sheep. Thus, when animals are slaughtered, bacteria from animal intestines may contaminate their meat.[20,21] It has been shown that about half of the diseases caused by *E. coli* O157:H7 in USA linked to consumption of undercooked ground beef. Therefore, meat and its products are the main sources of *E. coli* transmission.[22]

Previous studies indicated that there is a high-prevalence of *E. coli* O157:H7 on beef and chicken in the slaughterhouse of Isfahan.[23,24] Currently, there is limited information regarding the prevalence and antimicrobial susceptibility patterns of *E. coli* O157:H7/NM in meat products in Iran. The antimicrobial agents tested in this study are widely used to treat infections in people and in food animals in Iran. The aim of this study was to assay the prevalence, virulence factor and antibiotic resistance of *E. coli* O157:H7/NM in hamburger and chicken nugget. According to the authors information, this is the first study that investigated *E. coli* in chicken nugget in Iran.

MATERIALS AND METHODS

Sample collection
Hamburger ($n = 120$) and chicken nugget ($n = 70$) samples were collected from different product meat factories of Isfahan, Iran. Samples were taken under sterile conditions and immediately transported to the microbiology laboratory, from June 2013 to July 2013.

Microbiological analyses
In order to enrichment, 25 g of each samples was added to a sterile plate containing 225 mL tryptone soya broth (TSB, Merck, Germany) that supplemented with novobiocin (20 mg/L) and then homogenized at least 2 min into a stomacher. The samples have been incubated at 37°C for 18-24 h. Subsequently, the enriched samples were streaked onto Levine eosin methylene blue agar and sorbitol McConkey agar plates supplemented with cefexime (0.5 mg/L) and potassium tellurite (2.5 mg/L). After incubation, non-sorbitol fermented colonies were confirmed by polymerase chain reaction (PCR).

Detection of E. coli O157 and virulence genes by PCR
All non-sorbitol fermenting colonies from the Sorbitol MacConkey Agar-Cefixime-Tellurite agar were assayed with PCR using the O-antigen encoding region of O157 gene and flagella H7 gene (fli C) and then *E. coli* O157 isolated were examined by PCR assay to determine the presence of virulence factors. The presence of virulence genes has been confirmed by following primers as described previously in Table 1.[25,26]

Deoxyribonucleic acid (DNA) was extracted by DNA isolation kit (Cinna Gen, Iran) according to the manufacturer's instruction.

Amplification of target DNA was conducted in a DNA thermal cycler (Master Cycler Gradiant, Eppendorf,

Gene	Primer	Oligonucleotide sequence (5'-3')	Fragment size (pb)	Reference
Table 1: Primer sequences and predicted lengths of PCR amplification products				
stx1	VT1-A	CGCTGAATGTCGCTCTGC	302	Blanco *et al.*[25]
	VT1-B	CGTGGTATAGCTACTGTCACC		
stx2	VT2-A	CTTCGGTATCCTATTCCCGG	516	Blanco *et al.*[25]
	VT2-B	CTGCTGTGACAGTGACAAAACGC		
ehxA	HlyAl	GGTGCAGCAGAAAAAGTTGTAG	1551	Alonso *et al.*[26]
	HlyA4	TCTCGCCTGATAGTGTTTGGTA		
eaeA	EAE-1	GAGAATGAAATAGAAGTCGT	775	Blanco *et al.*[25]
	EAE-2	GCGGTATCTTTCGCGTAATCGCC		
fliCh7	EAE-F	GCGCTGTCGAGTTCTATCGAGC	625	Alonso *et al.*[26]
	EAE-C1	CAACGGTGACTTTATCGCCATTCC		

PCR: Polymerase chain reaction

Germany). The amplification conditions and reagents for the PCR assays were those described by Rey *et al.*[27] The PCR products were separated by electrophoresis in 1.5% agarose gel and stained with ethidium bromide then were visualized under ultraviolet transilluminator.

Antimicrobial susceptibility testing

The isolated *E. coli* O157:H7/NM strains were tested for antibiotic resistance to eleven antimicrobial disks (Hi-Media Laboratories, Mumbai, India) including nalidixic acid (30 μg), cefuroxime (30 μg), erythromycin (15 μg), tetracycline (15 μg), streptomycin (30 μg), gentamicin (10 μg), amoxicillin (30 μg), ampicillin (10 μg), kanamycin (30 μg), doxycycline (30 μg) and chloramphenicol (30 μg). The test was carried out by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates (HiMedia Laboratories, Mumbai, India) supplemented with 5% defibrinated sheep blood, according to the Clinical Laboratory Standards Institute (CLSI, 2006).[28]

Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago o, IL, USA). Data were compared statistically between prevalence of STEC *E. coli* O157:H7/NM in hamburger and chicken nugget with Pearson's Chi-square test. The results were considered to be statistically significant with $P < 0.05$.

RESULTS

Table 2 shows the prevalence of *E. coli* O157:H7 and *E. coli* O157:NM isolated from hamburger and chicken nugget in Isfahan, Iran. From a total of 190 samples analyzed four samples (2.1%) were contaminated with *E. coli* O157. All the *E. coli* O157 was isolated from hamburger samples (3.3%) and no chicken nugget samples were positive. There was a significant difference in the level of contamination with *E. coli* O157 between hamburger and chicken nugget samples with $P = 0.045$.

Among four *E. coli* O157 isolates, only one of them was serotype O157:H7 and three others were serotype O157:NM. Among four *E. coli* O157:H7/NM isolates, one strain was positive for *all stx1*, *stx2*, *eaeA* and *ehxA* genes and one strain only was positive for *stx2* gene.

The resistance pattern of four *E. coli* O157:H7/NM isolates to eleven antimicrobial agents tested in this study is shown in Table 3. Overall, all of four *E. coli* O157:H7/NM isolates (100%) were resistant to one or more antimicrobial agent. Isolates were found to be more resistant to gentamycin, tetracycline and erythromycin, which have been extensively used in the livestock industry. All *E. coli* O157:H7/NM isolates were susceptible to cefuroxime and streptomycin. Multi-resistance which was defined as resistance to three or more of drug tested was found in 27% of *E. coli* O157 strains and this is a major public health concern.

DISCUSSION

Based on evidence of outbreaks and recalls from market places, there has been an association between contaminated meat products and consumer health problems. For instance between 1992 and 1999, 16% of general outbreaks were related to consumption of contaminated red meat in England and Wales. On the other hand, due to contamination with *E. coli* O157:H7, more than 21 million pounds of ground beef were recorded in 2007.[29] Meat product samples have been examined in Iran and several countries for the presence of *E. coli* O157:H7/NM. The results of our study are comparable to some of the previous studies. Rahimi *et al.* detected *E. coli* O157:H7 in 13 (6.4%) of the 203 bovine carcass samples in Isfahan, Iran, but they did not check the presence of virulence genes in isolates.[30] Similarly, in another study in Iran, Hosseini *et al.* isolated bacteria in 8% to 18% of different types of hamburger samples. They detected 49 *E. coli* O157:H7 isolates.16 strains carried *stx1* gene and 41 strains possessed *stx2* gene.[31] In Italy, Stampi *et al.* detected the pathogen in one sample of hamburger (3.3%) and two samples of hamburger mixed with vegetables (8.3%). Two strains of *E. coli* O157 were positive for verocytotoxin production and one strain possessed the gene for *eae*A.[32] In Greece, 1.3% of 75 fresh sausage samples examined were contaminated with *E. coli* O157:H7 but no *E. coli* O157:H7 was isolated from 50 hamburger samples. The isolated strain carried both *stx1* and *stx2* genes.[6]

We isolated *E. coli* O157:H7/NM in 3.3% samples of hamburger, Out of a total 4 *E. coli* O157 isolates, one was

Table 2: Prevalence of *E. coli* O157:H7/NM isolated from hamburger and chicken nugget

Samples	No. of samples examined	No. of positive samples (%)	Confidence interval of the difference
Hamburger	120	4 (3.3)*	(0.001, 0.066)
Chicken nugget	70	0	
Total	190	4 (2.1)	

*One of the four *E. coli* O157 isolates, was serotype O157:H7. *E. coli*: *Escherichia coli*

Table 3: Antimicrobial resistance profiles of *E. coli* O157:H7/NM isolated from hamburger and chicken nugget

Antimicrobial agent	*E. coli* O157:H7 ($N = 1$) (%)	*E. coli* O157:NM ($N = 3$) (%)
Amoxicillin	0 (0.0)	0 (0.0)
Ampicillin	0 (0.0)	2 (66.6)
Cefuroxime	0 (0.0)	0 (0.0)
Chloramphenicol	0 (0.0)	1 (33.3)
Doxycycline	0 (0.0)	1 (33.3)
Erythromycin	1 (100)	2 (66.6)
Gentamicin	1 (100)	2 (66.6)
Kanamycin	1 (100)	1 (33.3)
Nalidixic acid	0 (0.0)	2 (66.6)
Streptomycin	0 (0.0)	0 (0.0)
Tetracycline	1 (100)	2 (66.6)

N: Number of isolates, *E. coli*: *Escherichia coli*

serotype O157:H7 and three were serotype O157:NM. Isolated *E. coli* O157:H7 was positive for all *stx1*, *stx2*, *eaeA* and *ehxA* genes. One strain of *E. coli* O157:NM carried *stx2* gene. Variation in the prevalence rate and virulence factors of *E. coli* O157 isolates in different studies may be raised from different samples, natural source, year, employed techniques, seasonal effects and or different employed laboratory methods.[33]

Although, antibiotics serve as the most important agent to save millions of human lives by improving human and animal health, but there are some concerns about their using in food-producing animals which affect public health and food safety. Excessive and irregular use of such antibiotics in animals in the recent years has caused to emerging antibiotic resistant bacteria.[34] Antimicrobial susceptibility testing in our study indicated that there is a high resistance of *E. coli* O157 to gentamycin, tetracycline and erythromycin but, intermediate resistance to amoxicillin, cefuroxime and streptomycin. On the other hand 50% of isolates were resistant to ampicillin, nalidixic acid and streptomycin as well as 25% to chloramphenicol and doxycycline, respectively.

The results of our study indicated that there is a correlation between antibiotics that are being used to treat infections in animals and antimicrobial resistance in Iran. Although antibiotic resistance is widespread, using antibiotics for managing human disease like diarrhea, HUS and etc. is still undeniable.[35]

CONCLUSION

The presence of *E. coli* O157 in hamburger showed the potential risk of infection with *E. coli* O157 in people who are consuming meat products. Our study showed that hygiene conditions improved in the meat industry in Isfahan. However, it is impossible to completely eliminate the pathogenic bacteria from all cattle. Hence it seems the establishment of techniques, screening and control programs such as hazard analysis and critical control points are necessary due to more reduction of microbial contamination during slaughter and meat processing.

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