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Survey frequency of verotoxigenic *Escherichia coli* (VTEC) in raw milk at Kerman city

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Abstract

Diarrhea is an important hygienic problem in under development countries. Among pathogenic agents *Escherichia coli* is the most etiologic agent of diarrhea in children below five years old. Some *E. coli* strains produce cytotoxins that have cytopathic effect on vero cell and identified as verotoxin. This study performs for resolve frequency of Vero Toxigenic *Escherichia coli* (VTEC) in Kerman city. Samples include 375 raw milk sample that obtained from city and 122 raw milk samples that collected from cattle farms**.** VTEC strains were serotype by Entro Pathogenic *Esherchia coli* (EPEC) antiserum. Verotoxin were measured by using vero cell. The results of this research show that from 375 raw milk samples (75.45 %) that prepared from Kerman city 7 samples (1.85 %) were contaminated and from 122 samples (24.55 %) that prepared from cow growth two samples have contamination with VTEC. Neutralization experiment show that from 9 VTEC strains that isolated from raw milk, 5 strain (55.5%) produce VT1 and 3 strains produce VT2 (33.3 %). Also one strain (11.11 %) produces VT1 and VT2. Degree of contamination within milks that sold in city than milk that prepare from cow growth have not any significant difference (P= 0.613). This study show VTEC have very important in Kerman city but O157:H7 serotype in the Kerman city have low frequency

Key words: Escherichia coli, Kerman City, Raw milk, Verotoxin

Introduction

Diarrhea is one of the most important health problems in developing countries and this disease in global scale is of the biggest causes of death after heart disease and cancer in many populations (Conedera et al., 2004). The highest rate of mortality due to diarrhea disease and intestinal infections are seen in infants and small children. As reported by the World Health Organization, each year five million children under 5 years in the world and 70 thousand children in Iran have died from diarrhea. Among infectious agents, *E. coli* are the most important etiological factors (etiology) of children under 5 years (Gillespie et al., 2005). By recognizing further virulence of pathogenic *E. coli*, a new classification formed which is based on the presence of specific genes that encodes virulence factors (Gillespie et al., 2005). EPEC and VTEC that the most strain is *E. coli* O157:H7 first time was isolated from the stools of patients with dysentery at 1982. This group of bacteria causes Hemorrhagic colitis, hemolytic uremic and acute renal failure,

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especially in young children. These bacteria produce verotoxin that anti *Shigella* antibody neutralized it. This toxin is cytotoxic for HeLa cells, Vero cells and lethal for mice. It has been shown that this toxin to be a virulence factor for pathogenic *E. coli* (Liptakova et al., 2004*).*

VTEC produce two cytotoxins includes: one of this neutralized by anti *Shigella* antibody that known as V1 toxin and other that not neutralized by anti *Shigella* antibody khown as V2 toxin (Beutin et al., 1999; Trung et al., 2005). Both toxin structural genes have 58% nucleotide sequence identity and 56% similarity in their amino acid sequence and both are equally toxin subunit structure (Liptakova et al., 2004). These toxins bind to Glycolipid Gb3 receptors and with a similar mechanism inhibit protein synthesis (Liptakova et al., 2004).

VTEC first time isolated from patients with hemorrhagic colitis and hemolytic uremic syndrome but later it was shown that it can cause diarrhea and dysentery. They causes disease sporadic or epidemic and in all countries where the research was done about them, have been found. Initially thought of uncooked beef meat source, but from person to person transmission has been observed. Now, most of the studies have focused on serotype O157: H7. Identification of VTEC as a causative agent of diarrhea, intestinal infections is the most important, and now the most exciting developments in this field (Zhang et al., 2003).

Since the animal is the reservoir of this bacterium and regards to close contact between animal and human in Kerman provenance and also contamination of environment with cattle manure totally it seems that Kerman Province These strains of *E. coli* are an important cause of gastroenteritis. The aims of this study are isolation and determination of distribution of VTEC in raw milk that sold in Kerman city in other hand the raw milks that consumes by people of Kerman city were investigated in this study. This study focus on raw milk that distributed on the Kerman city that located on south eastern of Iran in Kerman provenance.

Materials and Methods

Type of study, population and sampling method

This research is cross sectional study because in this study rate of contamination were evaluated. The population is milks that distributed in the Kerman city and also milks that collected from cattle farms. The collected samples were 497 that contain 375 milks that distributed in the city and 122 samples that collected from cattle farm. The method of sampling for milk that sold in the city was two stage cluster sampling and for cattle farms was random method.

Independent and dependent variables

Dependent variables include VTEC that assayed with specific antiserum for toxin. The year seasons and type of farm cattle are independent variable.

Culture method of samples

The collected milks samples were cultured by standards method of Edwards & Owing in Sorbitol Macconky broth and Macconky agar media (Vanduynhoven et al., 2002).

Serotyping of EPEC *E.coli*

The VTEC strains were stereotyped with EPEC antiserum that contains one unit of polyvalent antiserum, 4 unit of trivalent antiserum and 12 unit of monovalent antiserum. The strains that identified as *E. coli* were cultured on nutrient agar medium and then prepared suspension with serum physiology then by slide method agglutination assayed with polyvalent antiserum and in positive reaction agglutination performed by trivalent and monovalent antiserums (Beutin et al., 1999).

Agglutination with O157 and H7 antiserum

The sorbitol negative *E. coli* and VTEC were serotyped with O157:H7 (Difco) antiserum by tubular agglutination method for find O157:H7 serotype.

Production of verotoxin

For production of verotoxin the method of Katouli et al (1989) with slight modification were used. The procedure were is as follow, in the beginning sorbitol negative strains were cultured into 10 ml of Penassay Broth media then the medium were incubated in shaker (100 rpm) for 18-20 hours at 37 °C. the culture medium were centrifuged at 15000 g for 10 min and supernatant were discard and sediment were dissolved in 1ml of Phosphate Buffer Solution contain polymyxin (100 microgram) this solution were incubated for 30 min at 37 °C with shaking (100 rpm), finally his suspension transferred to 1.5ml tube and centrifuged at 12000 g for 10 min and after discarded the sediment, the supernatant (bacterial extract) were used for verotoxin assay against Vero cell (Trung et al., 2005).

Verotoxin assay

Vero cell were used for assay of verotoxin. Vero cell line was prepared from Iranian cell bank (Institute Pasteur). The methods of Konowalchuqk et al (1977) with slight modification was used for verotoxin assay (Konowalchuqk et al., 1977).

Verotoxin titration

Different dilutions of toxin were prepared with PBS and the diluted toxin was evaluated for Vero cell line. The highest concentration of toxin that show 50 percent toxicity was determined

Statistical analysis

Differences for individual parameters between control and treated groups were tested by analysis of variance (ANOVA) using SPSS Version 18.0 for Windows. Differences were considered significant if the P value was less than 0.01. All experiments were performed in triplicate and repeated three times.

Results

Distribution of collected samples in Kerman city

In Kerman city From April 2012 to April 2013 totally 497 samples were collected. The data from these samples were given in table (1). The distributions of these samples are as 375 samples from Kerman city and 122 samples from cattle farms.

Distribution of VTEC isolated from raw milk in Kerman city according to different seasons

From 375 collected samples (100 %) in Kerman city 70 samples (18.66 %) given in spring season one of these samples was contaminated to VTEC (1.43 %). But samples that collected in summer (110 samples, 29.33 %) two samples (1.82 %) were contaminated to VTEC, although from 130 samples (34.66 %) that

collected in autumn three samples (2.31 %) were contaminated to VTEC and finally from 65 samples (17.33 %) that collected in winter only one sample (1.54 %) was contaminated to VTEC. The result from this distribution was shown in table (2).

Distribution of VTEC isolated from crude milk in cattle farms according to different seasons

From 122 collected samples (100 %) in cattle farms 30 samples (24.59 %) given in spring season one of these samples was contaminated to VTEC (3.33 %). But samples that collected in summer (25 samples, 20.49 %) no samples observed for contaminated to VTEC although from 50 samples (40.99 %) that collected in autumn one sample (2 %) were contaminated to VTEC and finally from 65 samples (17.33 %) that collected in winter no samples observed for contamination to VTEC. the result from this distribution was shown in table (3).

Distribution of VTEC isolated from crude milk according to different seasons and source of isolation

From 375 samples (75.45 %) of crude milk that collected from Kerman city 7 samples were contaminated however from 122 samples (24.55 %) that collected from cattle farms only two samples (1.64 %) were contaminated to VTEC. The results were shown in table (2) and table (3) as shown in these tables the confidence distance (98 %) for evaluation of contamination to VTEC and comparison between the rate of contamination between different milk (from Kerman city and cattle farms) it can be concluded that there are not any significance ($P>0.05$). Also between seasons there are not any significance ($P<0.05$).

Production of Verotoxin by isolated VTEC strains

The neutralization experiment show that between 9 isolated strain of VTEC from milk the VT1 produce by 5 strains (55.5 %) and VT2 produce with 3 strains (33.3 %). One strain can produce VT1 and VT2 toxin.

Frequency of O157:H7 serotype between VTEC isolates

All strains of VTEC were serotype with antiserum of O157. The results show that between 9 isolated strains only one strain (11.11 %) had agglutination with antiserum. Then one strain between all isolates regards as O157:H7 strain.

Discussion

Diarrheal diseases are one of the major health problems in developing countries. Studies can support programs that are designed to control these diseases. The results as a guide to design procedures, and practices used to combat these diseases (Jayarao et al., 2006; Honish et al., 2005). There is little information about the worldwide prevalence of VTEC serotype O157: H7 and other serotypes in patients with diarrhea. Extensive efforts at the international level to coordinate the network of epidemiologists, laboratory and clinical scientists have taken up all the VTEC to be identified. The time duration of 1 to 6 years. Reports from Argentina, Australia, Belgium, Canada, Chile, Czechoslovakia, Denmark, France, Germany, Hungary, Italy, Japan, Lithuania, Mexico, Netherlands, Nigeria, Norway, Poland, the UK and the U.S. have shown that VTEC is prevalent Diarrhea pathogens than *Shigella* and *Yersinia* but less frequency than *Salmonella* and *Campylobacter* (Walker et al., 2007).

Studies have shown that in Madrid and Belgrade VTEC not considered as an important cause of diarrhea in Spain. In Iran, there is little information on the prevalence and significance of VTEC. There is only a

study by Katouli et al (1989) at Pasteur Institute of Iran on Verotoxin producing EPEC strains isolated from cases of diarrhea (Mc Kee et al., 2003; Katouli et al., 1989). Evaluation and measurement of verotoxin in VTEC strains of *E. coli* require cell culture facilities. These facilities are only a few areas in the Iran and seem to be one of the main causes of this field (Rey et al., 2006).

Therefore it was decided to evaluate the contamination of raw milk sold in the city of Kerman and the surrounding farms, and then we evaluated their role in causing diarrhea. During the study period of one year (2012-2013) some samples of raw milk in the traditional and industrial livestock sold was collected.

The main goal of the present study on VTEC. Although the scale of subjects in this study is very small compared to the whole country, however, data from this pilot study can be a guide for epidemiological studies for the whole country.

The amount of pollution from raw milk (unheated) in the Kerman city has come down to VTEC 7 cases (85/1%) and the amount of pollution that produced in the milk of industrial cattle farms in 2 (64/1 %). However, the overall contamination rate of the seals to a variety of strains of *E. coli* and other VTEC and non VTEC including 283 cases (57%).

In a study performed by Ralphexeal (1999) in the United States on a variety of animals and products of each of them these results were obtained: Contamination of Beef 7/3%, pork 5/1%, Poultry 5/1%, 2% in lambs and cows more than 5/1% contamination with VTEC strains O157: H7 have been reported the results of our study were consistent with this study (Ralphexeal et al., 1999).

In a study conducted in Denmark in 2002 VTEC infection rate of 3% was determined to cow's milk (Wood et al., 2002). Neutralization experiments showed that the most VTEC strains producing VT1. As of 9 VTEC strains isolated from raw milk, 5 strains (55/55%) produced VT1 and VT2 by the three strains (33/33%) were produced, and one strain (11/11%) from the same VT1 and VT2 was independent producers.

Some researchers extracted by using Polymyxin B, as it increases the release of toxins from the cells at different verotoxins understanding, postulate and high titers of all strains were produced toxin type VT1 made it. While some types of toxin producer VT2 had moderate or weak (Bouzari et al., 1994; Blanco et al., 1996)

In this study that performed over a period of one year in the city of Kerman the rate of isolation of VTEC were 9 strains (1.81 %). Prevalence of VTEC in Kerman city can be due to factors such as socio-economic and lower health status, high consumption of un pasteurized milk and in the communication is high between humans and animals in urban areas.

Conclusion

According to the results from this study and results from other researcher it can be concluded that for inhibition of contamination with VTEC animal reservoir must be controlled correctly. It can be suggested that similar research done for isolation of VTEC from cattle feces.

Acknowledgments

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Table 1. Distribution of collected milk samples from Kerman city and cattle farms

	Sampling source				Total	
Seasons	Kerman city		Cattle Farms		Number	Percent
Spring	70	14.08	30	6.03	100	20.12
Summer	110	22.13	25	5.03	135	27.16
autumn	130	26.15	50	10.06	180	36.22
Winter	65	13.07	17	3.42	82	16.5
T0tal	375	75.43	122	24.54	497	100

Table 2. Distribution of VTEC isolated from crude milk in Kerman city according to different seasons

Seasons	Total collected samples		Positive samples	
	Number	Percent	Number	Percent
Spring	70	18.66	1	1.43
Summer	110	29.33	2	1.82
Autumn	130	34.66	3	2.31
Winter	65	17.35	1	1.54
Total	375	100	7	1.87

Table 3. Distribution of VTEC isolated from crude milk in cattle farms according to different seasons

Seasons	Total collected samples		Positive samples	
	Number	Percent	Number	Percent
Spring	30	24.59	1	3.33
Summer	25	20.49	0	0
Autumn	50	40.98	1	2
Winter	17	13.94	0	0
Total	122	100	2	1.64