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Screening of verotoxin-producing *Escherichia coli* (VETC) O104-2011 from Egyptian market in 2011

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***Escherichia coli* strains are important causes of diarrheal disease in the world and remain a major public health problem of animals and human. Sixty seven samples from different kind of food, water, soil and composit were screened for the detection of verotoxin-producing *E.coli* (VTEC) in the Egyptian markets from different location. Result showed that none of the samples gives positive results for VTEC (O104) (*vt1* and *vt2*) detection. All samples from the Egyptian markets are negative for Vero cyto toxin producing *E. coli* (O104) with percentage of 100.**

Key words: Verotoxin-producing *Escherichia coli*, Shiga toxins.

INTRODUCTION

Escherichia coli are bacterial population of the gastro-intestinal tract of humans and animals (Gross, 1994). They are commensals or pathogenic, enterotoxigenic, enteropathogenic, enteroinvasive, or enterohaemorrhagic according to the presence of specific virulence factors (Nataro and Kaper, 1998). Some isolates are shiga-like toxin producers which are zoonotic agents causing serious diseases like diarrhoea, haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS). The emergence of strains showing multi resistance to several antimicrobial drugs is a public health concern (White et al., 2002). Some strains of this species are agents of colibacillosis, an increasingly challenging disease in animal production, resulting in significant economic losses to the poultry industries (White, 2005). Shiga toxin–producing or verotoxin-producing *E. coli* (VETC) are the most important recently emerged groups of foodborne pathogens (Beutin et al., 2002). These *E. coli* produce either one or two cytotoxins called Shiga toxins *(stx1* and *stx2)* or verotoxins *(vt1 and vt2)* (Paton and Paton, 1998). Currently, available epidemiological information on this Shiga - toxin producing *E. coli* bacteria (STEC) outbreak in Germany suggests that STEC-contaminated food is the vehicle of infection.

A case control study carried out in Hamburg identified consumption of contaminated raw tomatoes, cucumbers and /or leafy salad as significant risk factors (Frank et al., 2011). The present study aims to screen the Egyptian

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Abbreviations: VETC, Verotoxin-producing *E. coli*; **HC,** haemorrhagic colitis; **HUS,** haemolytic-uraemic syndrome; **STEC,** Shiga-toxin producing *Escherichia coli* bacteria; **ECDC,** European Centre for Disease Prevention and Control; **EU,** European Union; **Rep-PCR,** repetative sequence based polymerase chain reaction; **PFGE,** pulsed-field gel electrophoresis.

Table 1. Dye settings for multiplex reaction.

Parameter	VT1 Probe	IPC
TaqMan® VT1 (stx1) Assay	Reporter = FAM™	Reporter = VIC®
TaqMan® VT1 (stx2) Assay	Reporter = FAM™	Reporter = VIC®
TaqMan® E. coli O104 Assay	Reporter = FAM™	Reporter = VIC®
TaqMan® E. coli O157 Assay	Reporter = FAM™	Reporter = VIC®

IPC, Internal positive control.

markets for detection of the main serotyping of VTEC (O104:H4).

On the 21st of May 2011, Germany reported an ongoing outbreak of Shiga-toxin producing *E. coli* (STEC45), serotype O104:H4 (Frank et al., 2011). In Germany, between the 1st of May and the 28th of June 2011, 838 Haemolytic Uremic Syndrome (HUS) cases and 3 091 STECcases with diarrhea was reported, of which 47 persons died (RKI, 2011). On Friday the 24th of June, France reported a cluster of patients with bloody diarrhoea, after having participated in an event in the Commune of Bègles near Bordeaux on the 8th of June. As of 28 June, eight cases of bloody diarrhoea and a further eight cases with HUS have been identified. Eleven (11) of these patients, seven women and four men, between 31 and 64 years of age, had attended the same event in Bègles.

Infection with *E. coli* O104:H4 was confirmed for four patients with HUS. Six of the cases reported having eaten sprouts at the event on the 8th of June, and leftovers were analysed. Outbreak investigation revealed that the suspected sprouts of fenugreek, rocket and mustard was privately produced in small quantities by the organiser of the event from seeds bought at an approved garden centre, and were not imported from the sprout producer implicated in the outbreak in Germany (INVS, 2011).

An analytical epidemiological study went on with the persons that attended the event on 8th of June. Local trace back investigations in France suggested that the seeds for sprouting were distributed to the approved garden centre by a UK based company. European food safety authority (EFSA) was urgently requested by the Commission to initiate a comprehensive tracing back exercise (followed by tracing forward) to identify the source of the two outbreaks and identify appropriate risk mitigating measures regarding potential further outbreaks. These further investigations particularly aimed at determining whether the origin of the suspected sprout-seeds from the French cluster was linked to the large outbreak in northern Germany. This report documents the steps taken in the trace back process. Any activities already undertaken by the Task Force with regard to tracing forward are also described.

A trace back investigation is the method used to determine and document the distribution and production chain, and the source(s) of a product that has been implicated in a food-borne illness investigation. A trace forward investigation aims to find the distribution of the suspected food products along the food chain from the origin in the direction of the consumer. Using this approach for this investigation, at each step of the delivery/production chain identified in the trace back, further investigation was initiated to try and account for all seeds in any suspect lots.

The objective was to identify critical lots and their current location. To this end, detailed information on each lot of seeds was established for each step of the delivery/production chain back to the importation into the EU. The comparison of the back tracing information from the French and German outbreaks leads to the conclusion that a lot of fenugreek seeds imported by the Importer, from Egypt, is the most likely common link, although it cannot be excluded that other lots may be implicated.

MATERIALS AND METHODS

Samples

Sixty-seven (67) samples from different kinds of food, water, soil and composit was screened for the detection of VTEC in the Egyptian markets from different location.

Screening of VTEC

VTEC screening was carried after overnight incubation of the sample in Buffer peptone water at 37°C for 24 h, using Prep man ultra for extraction of DNA, TaqMan Environmental Master Mix, TaqMan *E. coli* 2011 O104:H4 Assay, Custom TaqMan *VT2 (stx2)* Assay, Custom TaqMan *VT1 (stx1)* Assay (Applied Bio system), Custom TaqMan O157 Assay and 7500 real time polymerase chain reaction (PCR) (Applied Bio system). The Master Mix Set-up was prepared as follow: 15 µL of 2X EMM 2.0 and 3 µL of 10X Target Assay Mix and 18 µL of total volume master mix per reaction. The dye settings for multiplex reaction were prepared as shown in Table 1 and the thermo cycler settings was carried out in 2 steps, enzyme activation and template denaturation step which occurred at 95°C /10 min and amplification step which is repeated 45 times, including stage 1 at 95°C /15 min and stage 2 at 60°C /45 min. Positive reference strains included within the run and purchased from reference laboratory of *E. coli* in Rome Italy incude *E. coli* O157 strain C210-03 genotype (*eae+, VTx2+, VTx1+*) and *E. coli* O104:H4 strain 11 2027 genotype (*eae-, VTx2+, VTx1-*).

Detection of VTEC from different types of food, water, soil and composit in the Egyptian markets (EU RL Method, 2011)

The procedure includes three main steps:

Enrichment

Food sample was enriched by adding 25 g sample to 225 ml of enrichment broth (buffer peptone water) then incubated at 37°C for 24 h. Water sample was enriched by filtration of 100 ml of water samples and the filterate added to 50 ml buffer peptone water.

Extraction of DNA

1 ml of enrichment was transfered in micro centrifuge tube, centrifuged at maximum speed (15000rpm/3 min) to spin down the contents, and the supernatant removed. The pellets were resuspended in 100 µl of PrepMan® Ultra Sample preparation reagent and vortex to mix the contents. The tube was heated in a heat block at 95 to 100°C/10 mins, then centrifuged at 15000rpm/3 min to spin down the contents. 10 µl of the supernatant (sample DNA) was transfered to a new tube containing 90 µl of water then vortexed to mix the contents, and then the sample DNA was ready for PCR.

Preparing the sample for PCR

According to the number of samples, the premix solution of master mix and assay was calculated and added in external screw capped tube. Both the samples and negative controls require 15 µl of master mix and 3 µl assays. Premix solution (18 µl) was transfered into each well, gently pipetting at the bottom of the well. 12 µl of unknown sample was transfered into each well, gently pipetted to mix the solution. 12 µl of negative and positive control were transferred and the tubes closed.

Preparation of PCR run

The samples runned on real-time PCR System, plate were loaded into the instrument, and then the cycle was adjusted as follow holding stage where the temperature was gradually raised to 95.0°C/10min followed by cycling stages which include 40 cycles; 15 min at 95.0°C and 1 h at 60°C, then the run started.

Data analysis and documentation

Data analysis was carried out according to Flow-diagram of the screening procedure of VTEC according to EU RL (2011) method. 25 g of sample was added to 225 ml BPW and enriched at 37°C, 18 to 24 h followed by extraction of DNA for screening and detection of the presence of Verotoxin genes. Negative result to *vtx* gene will be reported as absence of VTEC. Positive samples to *vtx* genes will undergo test for O104 and 0157 gene, followed by isolation onto Maconkey agar or sorbitol Maconkey agar, then the isolated colonies were tested for vtx producing genes by real time PCR to confirm positive results by O104 and/or O157. Negative results indicate non VTEC producing O104 or O157 while positive VTEC, O104 and /or O157.

The interprition of results was carried out according to the analysis of verotxin where samples showing positive *VT1* and /or *VT2* are VTEC positive while samples showing negative *VT1* and /or *VT2* are VTEC negative.

RESULTS AND DISCUSSION

Sixty seven (67) samples from different kind of food, water, soil and composit were screened for the detection of VTEC in different places in the Egyptian markets. The result shows that none of the samples gave positive results for VTEC (vt1 and vt2) as shown in Table 2.

E. coli is a major pathogen of worldwide importance in commercially raised, contributing significantly to economic losses in both turkeys and chickens. *E. coli* has been associated with a variety of diseases in birds including enteritis, arthritis, omphalitis, coligranuloma, septicemia, salphingitis and complicated air sacculitis about 10 to 15% of intestinal coliform are pathogenic serotypes (Roy et al., 2006). Although normally commensal in nature, certain strains of *E. coli* are associated with a variety of infections in human and animals. *E. coli* are present on most uncooked foods and in the inanimate environment. The major route of *E. coli* transmission is through the consumption of contaminated food and water, person to person and animal to person contact (Heuvelink et al., 1995; Leyer et al., 1995; Reilly, 1998). Recent food borne outbreaks caused by *E. coli* had heighlighted the demand for rapid detection of this organism in food (Heuvelink et al., 1995; Takeda, 1995).

On the 21st of May 2011, Germany reported an ongoing outbreak of STEC, serotype O104:H4 (Frank et al., 2011). In the past, STEC O104:H4 had been isolated in humans twice in Germany in 2001 (Mellmann et al., 2008) and once in Korea in 2005 (Bae et al., 2006). In addition, according to the information reported to the European Centre for Disease Prevention and Control (ECDC), a total of 10 persons were infected with other STEC O104 types in the European Union (EU) Member States from 2004 to 2009 (EFSA 2011). In Germany, between the 1st of May and the 28th of June 2011, 838 HUS cases and 3,091 STEC cases with diarrhea were reported, of which 47 persons died (RKI, 2011). The last date of onset of disease reported from Germany was on the 23rd of June for all EHEC or HUS cases reported, while for confirmed STEC O104:H4 cases, the last date of disease onset was the 12th of June. Up to the 29th of June, 13 EU/EEA9 countries reported cases associated with the outbreak in Germany for a total of 885 HUS and 3,170 non-HUS STEC cases (ECDC 2011). Until a recent outbreak in the Bordeaux area in France, with a rare exception, these cases in other European countries had all been linked to travel to northern Germany, where the outbreak had occurred.

The German outbreak strain is a STEC that belongs to serotype O104:H4, and has been microbiologically characterised in detail (EFSA, 2011). Preliminary information on the microbiological characterisation of the isolates implicated in the French outbreak indicate that many characteristics (stx2 positive, eae negative, hlyA negative, multi-resistance pattern to antimicrobials) are common with the German outbreak strain. In addition, the two molecular techniques (repetative sequence based polymerase chain reaction (Rep-PCR) and pulsed-field gel electrophoresis (PFGE) used to fully characterise and compare the outbreak strains in France and Germany

Table 2. Result of screening of 67 samples from the Egyptian markets for the detection of VTEC.

Sample	Number of sample	Place	Results	
			VT1	VT2
Okra	1	Alexandria	Negative	Negative
Cheese	1	Cairo	Negative	Negative
Composite	1	Cairo	Negative	Negative
Corn	1	Cairo	Negative	Negative
Dehydrated leek	1	Cairo	Negative	Negative
Drinking Water	1	Cairo	Negative	Negative
Fenugreek	2	Cairo	Negative	Negative
Green beans	4	Cairo	Negative	Negative
Juice	1	Cairo	Negative	Negative
Nigella sativa	1	Cairo	Negative	Negative
Soil	1	Cairo	Negative	Negative
Strawberry	1	Cairo	Negative	Negative
Water irrigation	1	Cairo	Negative	Negative
Majoram	1	El fayoum	Negative	Negative
Carawy	1	El fayoum	Negative	Negative
Drinking water	1	El fayoum	Negative	Negative
Irrigation water	8	El fayoum	Negative	Negative
Fenugreek	1	El fayoum	Negative	Negative
Dry mint	2	El fayoum	Negative	Negative
Basil	1	El fayoum	Negative	Negative
Soil	3	El fayoum	Negative	Negative
Fym	1	El fayoum	Negative	Negative
Pepper	1	El fayoum	Negative	Negative
Water well	1	El fayoum	Negative	Negative
Fennel	3	El fayoum	Negative	Negative
Chamomile	3	El fayoum	Negative	Negative
Composit	1	El fayoum	Negative	Negative
Fenugreek	1	EL Minya	Negative	Negative
Compsite	2	EL Minya	Negative	Negative
Irrigation water	2	EL Minya	Negative	Negative
Waste water	1	El Minya	Negative	Negative
Soil	1	El Minya	Negative	Negative
Fenugreek	1	Ismailia	Negative	Negative
Water bore hole	3	Ismailia	Negative	Negative
Nigella sativa	1	Ismailia	Negative	Negative
Sweet potatoes	1	Ismailia	Negative	Negative
Canal water	2	Ismailia	Negative	Negative
Well water	1	Ismailia	Negative	Negative
Green beans	1	Minufia	Negative	Negative
Dehydrated beans	1	Minufia	Negative	Negative
Fenugreek	2	Minufia	Negative	Negative
Dry mint	1	Minufia	Negative	Negative

showed the genetic relatedness of the strains (Gault et al., 2011).

The analysis and discussion in EFSA (2011) focuses primarily on data obtained from the back tracing process to identify the source of the seeds suspected of causing the STEC O104:H4 outbreaks. The German EHEC Task Force trace back methodology was successfully extended to support the investigations involving five other European Member States. The comparison of the back tracing information from the French and German outbreaks leads to the conclusion that fenugreek seeds imported from Egypt are the common link for these two

outbreaks. The implication is that the seeds became contaminated with STEC O104:H4 at some point prior to leaving the importer. Such contamination typically reflects a production or distribution process which allowed contamination by faecal material of human and/or animal origin. The results show that from this importer, seeds were sold to many businesses in Germany and many other countries in Europe.

For these reasons, invistgation on 67 samples from different kinds of food, water, soil and composit was carried for the detection of VTEC in the Egyptian markets. The result show that all analyzed samples from the Egyptian markets were negative for Vero cyto toxin producing *E. coli* VTEC (vt1 and vt2) with 100% and there is no such serotype of VTEC (O104:H4) detected in any kind of food especially fenugreek and also water, soil and composite. Moreover, It is recommended that all laboratories for VTEC analysis use harmonised methods to define VTEC seropathotypes from human and non-human sources to allow more effective monitoring by comparison of isolates from food and animals with those from humans.

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