Egypt. J. Chem. Environ. Health, 1 (1):1032-1048 (2015)

Detection of virulence genes in diarrhoeagenic *E.coli* isolated from different sources using Real Time PCR

Gad El-Said, W.A. .[1]; Sahar, T.M. |Tolba[2]; Soumaya, S.A. El-Shafii[3], Mohamed A. Abdelmonem[4]; Shaimaa, R.A.[3]; Mohamed A .Kelany[4]

[1] Microbiology department, Faculty of Veterinary Medicine, Cairo University

[2] Micribiology Department, Faculty of Science, Ain Shams University

[3] Bacteriology Department, Animal Health Research Institute (AHRI);

[4] Microbiology Department, Central laboratory of residues analysis of Pesticides and heavy metals in food (QCAP)

ABSTRACT

The present study aimed to investigate the diarrheagenic *E.coli* from different sources and study their genetic relationship and diversity. Different types of samples (cattle fecal samples and internal organs; different types of plants; conduit; environmental samples; drinking water and soil samples (1105 samples) were collected). The isolated (if you identified them, then why you subject them further identification?)serotypes were subjected for detection of different virulence genes. The isolated strains (97.5%) were serogrouped to O26 (3.1%), O111 (3.4%), O101 (1.2%), O55 (1.5%), O148 (0.45%), O158 (0.63%), O78 (1.9%), O1 (0.63%), O2 (0.45%) O157:H7 (1.08%) and O157: H- (1.62%) and 28 isolates of *E.coli* were untypable (2.5%) . *E.coli* O104:H4 2011 German strain was not detected in all DNAs extracted from all isolates.

Bovine internal organs (liver; intestine and spleen) of cattle showed the highest rate of O157: H- isolation (2%). While the rate of O157: H7 isolation was similar in calves and cattle fecal samples (2%). Internal organs of poultry (liver, intestine and spleen) showed the presence of *E.coli* O157:H7 (2%). The clinical mastitic milk showed absence of *E.coli* O157:H7, but it was isolated from market milk. In minced meat the rate of O157:H7 was 4%. , sewage and conduit in rates of 3.6%, 10% and 3.3% respectively.

Twenty six isolates express enterohemorrhagic virulence genes (stx_1, stx_2 and eae genes). The most predominant virulence gene detected was stx_1 gene in combination with eae gene (9 isolates) followed by 6 isolates express stx_2 alone, 6 isolates harbor both stx_1 and stx_2, 3 isolates express combination of stx_1, stx_2 and eae genes, only one isolate harbor eae gene alone. It was concluded, emphasizes the importance of safe water supply, good hygiene and sanitation practices in rural communities.

Keywords: Pathogenic *E. coli*, STEC, VTEC, EHEC, Real Time PCR.

INTRODUCTION

Escherichia coli is probably the most studied organism in microbiology. Since its first description this , the bacterium has become the model organism for much microbiological research, such that it is often forgotten that its main ecological niche is the alimentary tract of humans and most warm-blooded animals. **(Escherich, 1988 and Escherich, 1989)**

Diarrhea remains one of the main sources of morbidity and mortality in today's world and a large proportion is caused by diarrheagenic *Escherichia coli* **(Stuart, 2001).**

Recently, Diarrhoeagenic *E.coli* classified into seven classes, namely: enteropathogenic (EPEC); enterohaemorrhagic (EHEC); enterotoxigenic (ETEC);enterinvasive (EIEC); enteroaggregative (EAEC); Diarrhea-associated hemolytic (DHEC); or diffuse-adherent *E.coli* or cell detaching *E.coli* and Cytolethal distending toxin producing *E.coli* (CDTEC)**. (Stuart, 2001).**

The history of Shiga toxin-producing *E. coli* (STEC) dates back to late 1970, when Konowalchuk and colleagues showed that culture filtrates of some strains of *E. coli* produced a striking, irreversible cytopathic effect on cultured Vero cells, a cell line derived from African green monkey kidney cells **(Konowalchuk, et al. 1977).**

Many *E. coli* strains isolated from diarrheal illness produced a Shiga-like toxin (SLT), including one of the strains reported by **Konowalchuk et al. (1977)** to produce verotoxin (VT) **(O'Brien, et al. 1982)**. **O'Brien et al. (1983)** subsequently showed that SLT and the VT was the same toxin.

During the 1980s, most of the outbreaks of STEC O157 infection were foodborne and food vehicles implicated were mostly inadequately cooked hamburgers or other beef products, and unpasteurized milk **(Caprioli, et al. 2005)**.

There is no common biochemical characteristics associated with the great majority of STEC serotypes. The only way to identify all types of STEC in any kind of test sample is the detection of Stx produced by the bacteria. STEC can also be indirectly indirectly detected by examining *E. coli* strains or samples for the genes encoding stx.

There are several non-O157 EHECs now described and there are sequence data available for ST/serotype: ST21/O26:H11, ST16/O111:H- and ST17/O103:H2. These non-O157 EHECs are from different lineages and yet contain a set of relatively conserved accessory genes **(Ogura et al. 2009).**

In May 2011, an unusually high number of haemolytic uraemic syndrome (HUS) cases were reported in Germany. The outbreak was caused by an enterohaemorrhagic *E. coli* (EHEC) which had characteristics of both a verotoxigenic *E. coli* (VTEC) and of the less well-known diarrhoeagenic *E. coli*, enteroaggregative *E. coli* (EAEC). The pathogen responsible for the outbreak proved challenging to characterize. Within most diagnostic laboratories the current methodology for VTEC detection is aimed at detecting sorbitol negative VTEC O157:H7 and for most European

countries, the sorbitol positive outbreak strain O104:H4 could not be detected. It is therefore important that we examine the methods used by diagnostic and public health microbiology laboratories to characterize VTEC isolates. A universal approach based on genomic features would be more generally applicable and transportable than current methods (**Scheutz, et al. 2011).**

The present study aimed to investigate the diarrheagenic *E.coli* from different sources and detection of shiga toxin virulence genes.

MATERIAL AND METHODS

Sources and types of samples: different types of samples were collected from different sources were illustrated in **Table (1).** Samples were collected separately in sterile plastic bags, labeled with clinical history, age and date of collection, ice packed and transferred to the laboratory promptly **(E1Sayed, 1995).**

- **Bacteriological examination**

Fecal samples were placed in cool boxes (4 to 8°C) and taken to the laboratory for immediate processing (usually within 24 h). Each sample of 1g of rectal stool was enriched in 19 ml of Trypticase soy broth at 37°C for 18 h. Ten microliters of the Trypticase soy broth culture were inoculated onto MacConkey (MAC) agar as well as on sorbitol MacConkey agar (SMAC). The MAC plates were incubated at 37°C for 18h.

Organs were directly inoculated on MAC agar and SMAC plates, and incubated at 37°C overnight overnight.

25g of meat or minced meat or 25ml of milk sample were inoculated in 225ml buffered peptone, incubated at 37ºC overnight and then cultured on different media as before.

Rose pink colonies on MacConkey and colorless colonies on sorbitol MacConkey plates were picked up and sub-cultured on eosin methylene blue agar and chromogenic media plates to observe for the development of characteristic metallic sheen and pigmentation respectively. Well separated colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological, biochemical tests as described by **Koneman *et al.* (1997)** and **Quinn *et al.* (2002)**. Serological identification of the isolates was carried out as described by **Sojka (1965)** using diagnostic *E. coli* antisera (Denka Seiken) and *E. coli* H7 antiserum (Wellcome).

Real time PCR:

1. **Extraction of DNA from isolated strains: (Mullis and Faloona, 1987 and CDC, 1993)**

   A small loopful of cells was suspended in 100µl of Prep Man Ultra sample preparation reagent (Life Technologies) in the appropriate micro centrifuge tube, then vigorously vortexed and heated in water bath at 100C for 10min. The tubes were cooled at room temperature for 2min., the tubes were spun in the micro

centrifuge at 15,000 rpm for 2 min. 50µl of the supernatant was transferred from the spun tubes into a second set of labeled micro centrifuge tubes and were stored at 4ºC for 1 month or were frozen at -20ºC. From each tube, 12 µl of supernatant was used per assay reaction.

2. **Amplification Protocol:** (**EU reference laboratory 2011)**

Steps	Replications	Time	Temp
Enzyme activation /template denaturation	1	10 mins	95°C
Amplification	45	15s	95°C
		45s	60°C

Table (1): Sources and types of collected samples

Source	Type	Number of sample
Bovine (450)	Calf fecal sample	100
	Cattle fecal sample	100
	Calf internal organs (intestines, liver, and spleen	100
	Cattle internal organs liver, intestine and spleen	50
	Clinical mastitic milk	100
Poultry (150)	Fecal swabs	100
	Internal organs liver, spleen and intestine	50
Mea t& Meat product (100)	Meat	50
	minced meat	50
Market Milk (55)	Bulk milk	55
Plants (266)	Lettuce	50
	Aragula	50
	Fenugreek	100
	Cucumber	66
Water (50)	Sewage	10
	Conduit	30
	sweage	10
Environmental samples (34)	Surface swabsfrom poultry farms	22
	Soil from dairy	12
Total No. of samples		1105

The sequences of the primers that were used to detect *E. coli* O157;stx_1; stx_2 Shiga toxin and eae encoding genes and probes are shown in **Table (2)**. **(Perelle et al 2004 and 20**

Table (2): The sequences of the primers The sequences of the primers *for E. coli* O157; Stx1; Stx2 Shiga toxin and eaeA encoding genes

Target gene	Forward primer, reverse primer and probe sequences (5`-3`)[a]	Amplicon size (bp)	Location within sequence	Genbank accession number
rfbE (O157)	TTT CAC ACT TAT TGG ATG GTC TCAA	88	348-372	AF163329
	CGA TGA GTT TAT CTG CAA GGC GAT		412-435	
Probe-	AGG ACC GCA GAG AAA GAG AGG AAT TAA AAGG		381-410	
stx_1	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	131	878-906	M16625
	CCC CAG TTC ARW GTR AGR TM ACR TC		983-1008	
Probe-	CTG GAT GAT CTC AGT GGG CGT TCT TAT GTAA		941-971	
stx_2	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	128	785-813	X07865
	CCC CAG TTC ARW GTR AGR TCM ACR TC		785-813	
Probe-	TCG TCA GGC ACT GTC TGA AAC TGC TCC		838-864	
eae	CAT TGA TCA GGA TTT TTC TGG TGA TA	102	899-924	Z11541
	CTC ATG CGG AAA TAG CCG TTA		1000-979	
Probe-	ATA GTC TCG CCA GTA TTG CCA CCA ATA CC		966-936	

[a] In the sequence Y is (C,T), S is (C,T), W is (A,T),R is (A,G), M is (A,C).

3. TaqMan® E.coli 2011 |O104:H4 assay: "AB applied biosystem part of life technologies"

The assay detects signature sequences unique to O104:H4-2011 strain from the 2011 outbreak, shown by life technologies top detect the German strain isolate of *E.coli* O104:H4, but not the 200 isolate. Sequence region not associated with any known gene. (**EU reference laboratory 2011),** extraction of DNA and amplification was applied as previously mentioned.

RESULTS AND DISCUSSION

Most *Escherichia coli* do not cause illness. Pathogenic *E. coli* strains are categorized into pathotypes on the basis of their virulence genes. A large proportion of diarrhea that can lead to morbidity and mortality is caused by diarrheagenic *Escherichia coli* **(Stuart, 2001)**

In the present study, 207 *E.coli* strains were isolated form 1105 samples (18.7%), of different sources (**Table 3**). The highest rate of isolation was obtained from environmental samples (50%) followed by water samples (40%) and plant samples obtained from street sellers. **Kaneko *et al*.,1999)** found in a survey of conventionally

grown fresh vegetables in Japan (including cabbage, lettuce, onions, spinach, and celery) the percentage of *E. coli* positive sampleswas 2%. However **Khatib et al. (2015)** detected *E.coli* in lettuce (36%, cucumber (14.2%) and argula (14.2) in Lebanon. In a previous study conducted in Egypt, from the over all prevalence of *E. coli* isolates, 17.3 % (49/283) were from stools of sheep, cattle and chicken, 17.3 % (49/283) were from fresh water, 6.02 % (17/283) from processed meat products and 1.1 % (3/283) from poultry products (liver) (**Selim et al. 2013**). The rate of isolation in another study was 20.5 % (152/740), 26.35 % (195/740) and 27.5 % (204/740) for fecal, water and vegetables samples respectively that have been isolated by (**Kabiru et al. 2015**) in Nigeria.

The rate of isolation of *E. coli* revealed a high rate in environmental and water samples comparing to other sources, these results were nearly agreed with **Naena, (2009)** that isolated *E.coli* from enivorenmental samples (35%) and from water samples (45%) in poultry house. These results also revealed the role of environment; plant and water in spread of different *E.coli* serogroups **(Seas et al. 2000 and Medema et al. 2003).** A review of published reports from over three decades found that non-O157 STEC were more prevalent in beef products compared with *E. coli* O157 **(Barkocy-Gallagher, et al. 2003)**.

Twenty eight isolates of *E.coli* were untypable (2.5%) and the rest were serogrouped into O26 (3.1%), O111 (3.4%), O101 (1.3%), O55 (1.5%), O148 (0.45%), O158 (0.63%), O78 (1.9%), O1 (0.63%), O2 (0.45%) O157:H7 (1.08%) and O157:H- (1.62%). The distribution of different serogroups were shown in **Table (4)**, where O26 was isolated from bovine samples, meat and meat products, plant, water and environmental samples (20%, 20.8%,15%, 40% and 23.5% respectively).in contrast, Bonardi *et al.* (2015) isolated O26 from cattle in a percent of3.8. O111 was isolated from bovine, market milk, plant and environmental samples in a rate of 26.6%, 29.4%, 40% and 29.4% respectively. The O111 serogroup is not common in slaughtered cattle and many studies reported negative findings (**Jenkins, *et al.* 2003, Pearce *et al.* 2004, 2006,Thomas *et al.* 2012).** In Belgium, STEC O111 was detected in male cattle faeces (0.5 per cent), but not in samples from female animals (Joris *et al.* 2011).O55 was isolated only from bovine, meat and meat products, and market milk in a rate of 16%, 12.5% and 11.7% respectively. 4 isolates of O148 were obtained fr om meat and meat products (16.6%) and from market milk (5.8%), while O158 was isolated from water and meat and meat products (15% and 16.6% respectively), O78 isolated from poultry, environment and bovine in a rate of 44.1%, 11.7% and 5.3 respectively, O1 isolated from water, milk and poultry in a rate of 15%, 11.7%and 5.8% respectively and O2 isolated from poultry and meat and meat products in a rate of 8.8% and 8.3% respectively **Table (5)**. These results revealed that rate of O26 were highly isolated from water and environmental samples. This first section of the discussion is poorly written and do not discuss anything but percentages?

In May 2011, a large outbreak of infections associated with Shiga toxin (Stx)–producing *Escherichia coli* (STEC) O104:H4 has occurred in Germany (**Frank, *et al.* 2011)**. The outbreak showed 3 unusual features: 1) a large proportion of case-patients with hemolytic uremic syndrome (HUS); 2) HUS in adults, although it usually affects children; and 3) frequent development of neurologic symptoms in patients when clinical and laboratory markers of HUS were improving (**Frank, *et al.* 2011 and Jansen and Kielstein, 2011**). A second point-source outbreak caused by the same STEC O104 strain was reported in June 2011 in France (**Gault, *et al.* 2011**). Both outbreaks were linked to eating fenugreek sprouts obtained from seeds produced in Egypt and distributed in Germany and other European countries **(European Food Safety Authority 2011)**.In the present study, *E.coli* O104:H4 2011 German strain was not isolated or detected in all DNAs extracted from all *E.coli* isolated from different sources including fenugreek sprouts. **Fig. (1).**

Strain O157: H- was found to be prevalent in many samples in contrast, to Jo *et al.* 2004 who investigated the prevalence of E. coli O157:H7 in fecal and meat samples in Korea, out of 86 *E. coli* O157 isolates only 13 were serotype as O157:H- (**Table 6)**. The highest rate was recorded in bovine internal organs. While the rate of O157: H7 isolation was similar in both calves and cattle fecal samples (2%). These results revealed the role of bovine in shedding *E.coli* O157 in feces. Cattle are considered the primary reservoir of *E. coli* O157:H7 that infect humans. Adult cattle and weaned calves that carry *E. coli* O157:H7 generally remain asymptomatic but shed the bacteria into the environment in their feces **(Wallace, 1999)**. *E.coli* O157 was absent in mastitic milk. In minced meat the rate of O157:H7 was 4%, while it was absent in meat.

In addition, *E.coli* O157:H7 was isolated from market milk, sewage, conduict and surface swabs in rates of 3.6%, 10%, 3.3% and Zero % respectively. While in plant O157:H- was only isolated from arugula in the rate of 2%, and absence of *E.coli* O157:H7 from all plant samples. O157:H7 /H- were isolated in the same rate in sewage and conduict (10% and 3.3% respectively).

Many of the foods implicated in human disease are of bovine origin (**Lisa and O'Brien 2000 and Mcgee *et al.* 2004)**. The prevalence of non-O157 STEC ranged from 1.7 to 58% in packing plants, from 3 to 62.5% in supermarkets, and an average of 3% in fast food restaurants and in a recent survey of retail ground beef products in the United States, 23 (1.9%) of 1,216 samples were contaminated with non-O157 STEC. **(Samadpour,et al. 2006).** In another study, researchers found a 10 to 30% prevalence of non-O157 STEC in imported and domestic boneless beef trim used for ground beef **(Bosilevac, et al. 2007).**

In our study the contamination of minced beef with the serotype O157 may be occurred during processing. **Elder *et al.* (2000)** reported the prevalence of *E. coli* O157 contamination in beef cattle and carcasses at four different meat processing plants. Their data revealed the prevalence of *E. coli* O157:H7 in cattle and on carcasses is much

higher than previously estimated, and the level of carcass contamination correlates with the level of *E. coli* O157:H7 in the cattle before processing. They also found evidence that current processing practices are reducing contamination levels. Internal organs of poultry showed the presence of *E.coli* O157:H7 (2%) which pointed to the role of poultry in spreading the infection with *E.coli* O157:H7. This result was contradicted with **Kalin *et al.* 2012** who isolated O157 in 0.1% (1/1000) and 0.4% (4/1000) of the liver and cecum samples of broiler chickens.

In the present study, no O157 were isolated from soil although the persistence of fecal bacteria in the soil has been reported elsewhere. **Jones (1999)** described that *E. coli* survived for at least 60 days in soil at 25 ºC and for at least 100 days at 4 ºC. **Bolton et al. (1999)** detected *E. coli* O157: H7 in soil 99 days after a fecal suspension containing this organism was applied to grassland. In a study by **Ingham et al. (2004),** when no manure was applied, bird and/ or mammal recontamination was the cause of the apparent persistence of indigenous *E. coli* in manure-fertilized soils.

Presence of O157:H- in aragula may be attributed to the use of water of sewage and conduict in irrigation process. These results agree with **Stephen and Uraih (2009)** who reported that *E. coli* 0157:H7 was not detected in any of the vegetable samples (cabbage and lettuce).

Only twenty six isolates express enterohemorrhagic virulence genes (stx_1, stx_2 and eae) from 207 *E.coli* isolates. The most predominant virulence gene detected was stx_1 gene in combination with eae gene (9 isolates) followed by 6 isolates express stx_2 alone, 6 isolates harbor both stx_1 and stx_2, 3 isolates express combination of stx_1, stx_2 and eae genes, only one isolate harbor eae gene alone **(Tables 7 and 8)**.

These results have revealed the presence of virulence markers in *E. coli* isolates from different sources (**Obi, et al. 2004)**, and indicated that ribotyping can be a useful tool for epidemiological investigation. However it was not able to discriminate between STEC isolates belonging to the same serotype. The STEC strains of the same serogroup showed high genetic diversity **(Varela, et al. 2008)**. A combination of serotyping and genotyping is the best way to identify an *E. coli* strain and is recommended in epidemiological studies (**Lipman, et al. 1995 and El-Jakee et al. 2012).**

Infections with EHEC in other serogroups, including members of O1, O111, O26, O78, O55, are increasingly recognized as causes of hemorrhagic colitis and HUS. Some of these organisms may be as significant in human disease as EHEC O157:H7; however, they are not recognized on the media used to isolate this organism and many laboratories do not routinely screen for other strains. Although many EHEC seem to be carried asymptomatically in animals, members of some non-O157 serogroups may cause enteric disease in animals. **Bettelheim (2007)** warning that we ignore these strains at our peril worldwide reports of human outbreaks of non-O157 EHEC.

It was concluded, emphasizes the importance of safe water supply, good hygiene and sanitation practices in rural communities. Also, the prevalence of VTEC serogroups

observed in examined samples underlines the need of amendment of regulations, should include the cited microorganisms in the microbiological criteria in different sources.

Table (3): Total No. of isolated *E.coli* from different sources

Source	Total No. of samples	Rate of isolated *E.coli*	
		No.	%
Bovine	450	75	16.6
Poultry	150	34	22.6
Meat and meat products	100	24	24
Market milk	55	17	30.9
Plant	266	20	7.5
Water	50	20	40
Environmental samples	34	17	50
Total	1105	207	18.7

Table (4): Total number of *E.coli* serogroups isolated from different samples

E.coli serogroups	No.	%*
O26	35	3.1
O111	38	3.4
O101	14	1.3
O55	17	1.5
O148	5	0.45
O158	7	0.63
O78	21	1.9
O1	7	0.63
O2	5	0.45
O157:H7	12	1.08
O157:H-	18	1.62
Untyped *E.coli*	28	2.5
Total	207	18.7

* Percentage was calculated according to the total number of sample

Table (5): *E.coli* serogroups isolated from different samples

Source	Total No. of samples	Total No. of isolated *E.coli*	*E.coli* Serogroup		
			Serogroup	No.	%*
Bovine	450	75	O26	15	20
			O111	20	26.6
			O55	12	16
			O78	4	5.3
			O157: H7	5	6.6
			O157: H-	5	6.6
			Untyped	14	18.6
Poultry	**150**	**34**	**O78**	**15**	**44.1**
			O2	**3**	**8.8**
			O1	**2**	**5.8**
			O157:H7	**1**	**2.9**
			O157:H-	**5**	**14.7**
			Untyped	**8**	**23.5**
Meat and meat products	100	24	O157:H-	2	8.3
			O157:H7	2	8.3
			O55	3	12.5
			O26	5	20.8
			O148	4	16.6
			O158	4	16.6
			O2	2	8.3
			Untyped	2	8.3
Market milk	**55**	**17**	**O101**	**3**	**17.6**
			O111	**5**	**29.4**
			O55	**2**	**11.7**
			O1	**2**	**11.7**
			O148	**1**	**5.8**
			O157:H7	**2**	**11.7**
			O157:H-	**2**	**11.7**
Plant	266	20	O111	8	40
			O101	6	30
			O26	3	15
			O157:H-	1	5
			Untyped	2	10
Water	**50**	**20**	**O157:H-**	**2**	**10**
			O157:H7	**2**	**10**
			O26	**8**	**40**
			O158	**3**	**15**
			O1	**3**	**15**
			Untyped	**2**	**10**
Environmental samples	34	17	O111	5	29.4
			O78	2	11.7
			O101	5	29.4
			O157:H-	1	5.8
			O26	4	23.5
Total (1105)				**207**	**18.7**

*Percentage calculated according to the total No, of isolates

Table (6): Distribution of *E.coli* O157 among different sources

Source	Type	Number of sample	Serogroups		
			Type	No.	%
Bovine (450)	Calf fecal sample	100	O157:H7 O157:H-	2 1	2 1
	Cattle fecal sample	100	O157:H7 O157:H-	2 1	2 1
	Calf internal organs	100	O157:H7 O157:H-	1 2	1 2
	Cattle internal organs	50	O157:H7 O157:H-	0 1	0 2
	Mastitic milk	100	O157:H7 O157:H-	0 0	0 0
Poultry (150)	Fecal swabs	100	O157:H7 O157:H-	0 3	0 3
	Internal organs	50	O157:H7 O157:H-	1 2	2 4
Meat & Meat product (100)	Meat	50	O157:H7 O157:H-	0 2	0 4
	minced meat	50	O157:H7 O157:H-	2 0	4 0
Market Milk (55)	Bulk milk	55	O157:H7 O157:H-	2 2	3.6 3.6
Plants (266)	Lettuce	50	O157:H7 O157:H-	0 0	0 0
	Aragula	50	O157:H7 O157:H-	0 1	0 2
	Fenugreek	100	O157:H7 O157:H-	0 0	0 0
	Cucumber	66	O157:H7 O157:H-	0 0	0 0
Water (50)	Sewage	10	O157:H7 O157:H-	1 1	10 10
	conduict	30	O157:H7 O157:H-	1 1	3.3 3.3
	Drinking	10	O157:H7 O157:H-	0 0	0 0
Environmental samples (34)	Surface swabs	22	O157:H7 O157:H-	0 1	0 0.54
	Soil	12	O157:H7 O157:H-	0 0	0 0
Total No. of samples		1105	O157:H7 O157:H-	12 18	1.9 1.6

Table (7): Distribution of verotoxin genes and entimin gene (eae) among different serogroups

Source	Serogroup	Stx1	Stx2	Stx1+stx2	eae	Stx1+eae	Stx1+stx2+eae	Total
Bovine samples	O157	-	-	1	-	-	-	1
	O78	-	-	2	-	-	-	2
	O111	1	-	-	-	-	-	1
	O111	-	-	-	1	-	-	1
	O157	-	-	-	-	1	-	1
	O111			1				1
	O55	-	-	-	-	2	-	2
Calves samples	O157					2	1	3
	O111					1		1
	O78			1				1
Minced meat	O157			1				1
	O157		1					1
Poultry samples	O157		1					1
Market milk	O157					2		2
Water	O157		1	-	-	1	-	2
	O26						1	1
	01		1				1	2
	O26		1					1
Environmental samples	O78		1					1
Total of virulence gene detected		1	6	6	1	9	3	26

Table (8): Genetic profile of E.coli serogroups

Serotypes	Genetic profile	No.
O157:H7	stx_2	3
	stx_1+stx_2	2
	stx_1+eae	6
	stx_1+stx_2+eae	1
O26	stx_1	1
	stx_1+stx_2+eae	1
O111	stx_1	1
	stx_1+stx_2	1
	eae	1
	stx_1+eae	1
O78	stx_1+stx_2	3
	stx_2	1
O55	stx_1+eae	2
O1	stx_2	1
	stx_1+stx_2+eae	1

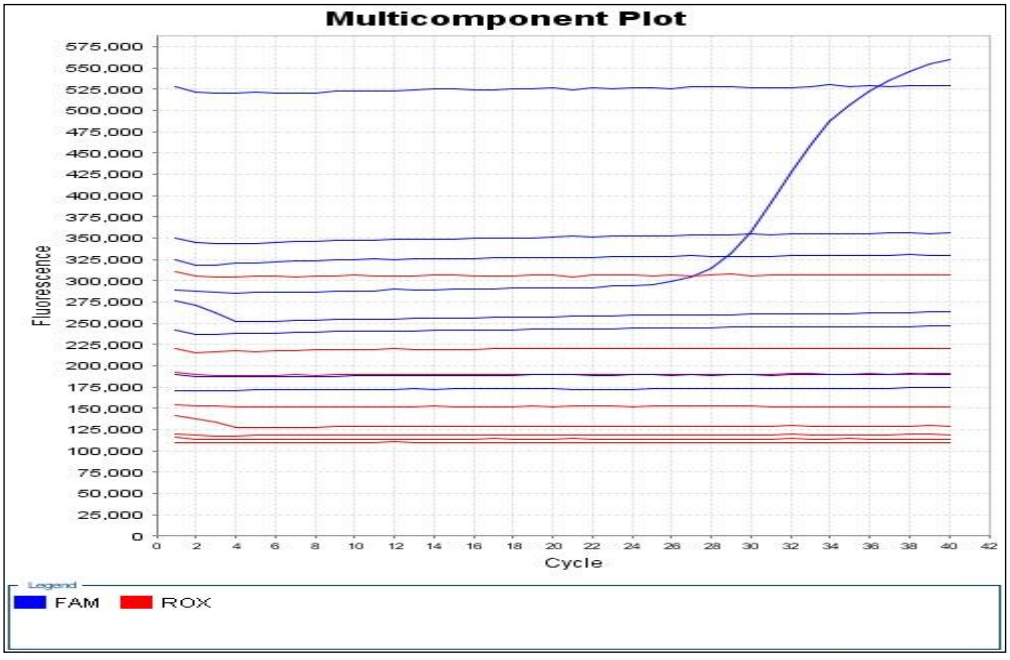

Fig. 1: Real time PCR for O104 H: 4, all samples were negative except control positive (blue lines)

References

Barkocy-Gallagher GA, Arthur TM, Rivera-Betancourt M, Nou X, Shackelford SD, Wheeler TL, and Koohmaraie M. (2003): Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. J Food Prot. 66(11):1978-86.

Bettelheim, KA. (2007): The non-O157 Shiga-toxigenic (Verocytotoxigenic) *Escherichia coli*; under-rated pathogens. Crit. Rev. Microbiol. 33: 67–87. http://dx.doi.org/10.1080/10408410601172172

Bolton, D. J. *et al.* (1999): The survival characteristics of a non-toxigenic strain of *Escherichia coli* O157:H7 Journal of Applied Microbiology, v. 86, p. 407-411.

Bonardi, S.; Alpigiani, I.; Tozzoli, R.; Vismarra, A.; Zecca, V.; Greppi, C.; Bacci, C.; Bruini, I.; Brindani, F. (2015): Shiga toxin-producing Escherichia coli O157, O26 and O111 in cattle faeces and hides in Italy. Vet Rec Open 2015;2:e000061. doi:10.1136/vetreco-2014-000061

Bosilevac JM, Guerini MN, Brichta-Harhay DM, Arthur TM, and Koohmaraie M. (2007): Microbiological characterization of imported and domestic boneless beef trim used for ground beef. J Food Prot. 70(2):440-9.

Caprioli, A., Morabito, S. Brugere, H. and Oswald,E. (2005):. Enterohaemorrhagic *Escherichia coli:* emerging issues on virulence and modes of transmission. Vet. Res. **36:**289-311.

CDC, (1993): Center for Dsease Control and Preention and National Institutes of Health, Biosafety in Microbiology and Biomedical Laboratories CDC03-8395.

EI-Sayed, N. E. (1995): Bacteriological studies on Colibacillosis in calves with special references to K99.Ph.D. theis (Microbiology) Cairo Univ.

Elder, R. O.; Keen, J. E.; Siragusa, G. R.; Barkocy-Gallagher, G. A.; Koohmaraie, M. and Laegreid,W. W. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. USA* 97:2999–3003.

El-Jakee, J.K.; Mahmoud, R.M.; Samy, A.A.; Mona A. El-Shabrawy; Effat, M.M. and Gad El-Said,W.A. (2012): Molecular Characterization of *E. coli* Isolated from Chicken, Cattle and Buffaloes. International Journal of Microbiological Research 3, (1): 64-74.

Escherich, T. (1988): The intestinal bacteria of the neonate and breast-fed infant. 1884. Rev. Infect. Dis. **10:**1220–1225.

Escherich, T. 1989. The intestinal bacteria of the neonate and breast-fed infant. 1885. Rev. Infect. Dis. **11:**352–356.

European Food Safety Authority (2011): Tracing seeds, in particular fenugreek (*Trigonella foenum-graecum*) seeds, in relation to the Shiga toxin–producing *E. coli* (STEC) O104:H4 2011 outbreaks in Germany and France [cited 5 Jul 2011]. http://www.efsa. europa.eu/en/supporting/doc/176e.pdf

Frank C, Werber D, Cramer JP, Askar M, Faber M, Heiden MA, et al. (2011): Epidemic profile of Shiga toxin–producing *Escherichia coli* O104:H4 outbreak in Germany—preliminary report. N Engl J Med. 2011 June 22; [Epub ahead of print].

Gault G, Weill FX, Mariani-Kurkdjian P, Jourdan-da Silva N, King L, Aldabe, **B, et al. (2011):** Outbreak of haemolytic uraemic syndrome and bloody diarrhoea due to *Escherichia coli* O104:H4, southwest France, June 2011. Euro Surveill. 2011;16: pii:19905.

Ingham, S. C. *et al.* (2004): *Escherichia coli* contamination of vegetables grown in soils fertilized with noncomposted bovine manure: garden-scale studies Applied and Environmental Microbiology, v. 70, p. 6420-6427.

Jansen A, Kielstein J. (2011): The new face of enterohaemorrhagic *Escherichia coli* infections. Euro Surveill. 2011;16:pii:19898.

Jenkins C., Pearce M. C., Smith A. W., Knight H. I., Shaw D. J., Cheasty T., Fosater G., Gunn G. J., Dougan G., Smith H. R., Frankel G. (2003): Detection of Escherichia coli serogroups O26, O103, O111 and O145 from bovine faeces using immunomagnetic separation and PCR/DNA probe techniques.

Jo, M.Y.; Kim, J.H.; Lim, J.H.; Kang, M.Y.; Koh, H.B.; Park, Y.H.; Yoon, D.Y.; Chae, J.S.; Eo, S.K. and Lee, J.H. (2004): Prevalence and characteristics of E. coli O157 from major food animals in Korea. Int. J. Food Microbiol. 95 (1):41-9.

Jones, D. L. (1999): Potential health risks associated with the persistence of *Escherichia coli* O157:H7 in agricultural environments. **Soil Use and Management**, v. 15, p. 76-83.

Joris M. A., Pierard D., De Zutter L. (2011): Occurrence and virulence patterns of E. coli O26, O103, O111 and O145 in slaughtered cattle. Veterinary Microbiology 151, 418–421.

Kabiru, L.M.; Bello, M.; Kabir, J.; Grande, L. and Morabito, S. (2015): Detection of Pathogenic *Escherichia coli* in samples collected at an abattoir in Zaria, Nigeria and at different points in the surrounding environment. Int. Journal of Environmental Research and Public Health 2015, 12, 679-691.

Kalin, R.; H.; Ongor, R.and Cetinkaya, B. (2012): Isolation and molecular characterization of *Escherichia coli* O157 from broiler and human samples. Foodborne Pathogens and Disease, 9; (4): 313-318.**Khatib A., Olama Z. and Khawaja G. (2015):** Shiga Toxin-Producing *E. coli* (STEC) Associated with Lebanese Fresh Produce. *Int.J.Curr.Microbiol.App.Sci* (2015) 4(2): 481-496.

Kaneko, K.; Hayashidani, H.; Ohtomo, Y.; Kosuge, J.; Kato, M.; Takahashi, K.; Shiraki, Y. and Ogawa, M. (1999): Bacterial contamination of ready-toeat foods and fresh products in retail shops and food factories. J. Food Prot. 62:644-649.

Koneman, E.W.; Hllen,S.D.; Janda, Schreckenberer, W. P. and Winner,J.R. (1997): Diagnostic Microbiolgy. Sth Ed. J.B. Lippincott Co. Newyork.

Konowalchuk, J.; Spiers,J. I. and Stavric, S. (1977): Vero response to a cytotoxin of Escherichia coli. Infect. Immun. 18:775-779.

Lipman, L.J.A., Nijsa, T.J. D.; Lam, G.M. and Gaastra, W. (1995): Identification of *E. coli* strains from cows with clinical mastitis by serotyping and DNA polymorphism patterns with Rep and Eric primers Vet. Microbiol., 43: 13-19.

Lisa J. G. and O'Brien A. D. (2000): Escherichia coli O157:H7 in beef cattle presented for slaughter in the U.S.: Higher prevalence rates than previously estimated PNAS, 97; (7) 2959–2961.

Mcgee, P.; Scott, L.; Sheridan, J. J.; Earley, B. and Leonard, N. (2004): Horizontal Transmission of Escherichia coli O157:H7 during Cattle Housing Journal of Food Protection, Vol. 67, No. 12, 2004, Pages 2651–2656.

Medema GJ, Payment P, Dufour A, Robertson W, Waite M, Hunter P, Kirby R, Anderson Y. (2003): Assessing Microbial Safety of Drinking Water Improving Approaches and Method. WHO & OECD, IWA Publishing; London, UK: 2003. Safe drinking water: an ongoing challenge. pp. 11–45.

Mullis,K.B. and Faloona,F.A. (1987): specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. 155:335-350.

Naena, N. A. A. (2009): Diversity and Prevalence Of Escherichia Coli In Chickens, Environment And Related Persons. " A Thesis", MD, Faculty of Veterinary Medicine, Alexandria University, Microbiology Department.

O'Brien, A. D.; LaVeck,G. D.; Thompson,M. R. and S. B. Formal. (1982): Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. J. Infect. Dis. 146**:**763–769.

Obi, CL.; Green, E.; Bessong, PO.; de Villiers, B.; Hoosen, AA.; Igumbor, EO.; and Potgieter, N. (2004): Gene encoding virulence markers among *Escherichia coli* isolates from diarrhoeic stool samples and river sources in rural Venda communities of South Africa. Water SA Vol. 30 No. 1:37-42.

O'Brien, A. D.; Lively,T. A. and Chen,M. S. (1983): *Escherichia coli* 0157:H7 strains associated with hemorrhagic colitis in the United States produce a Shigella dysenteriae 1 (Shiga)-like cytotoxin. Lancet i:702.

Ogura Y, Ooka T, Iguchi A, Toh H, Asadulghani M, Oshima K, et al.(2009): Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic Escherichia coli. Proc Natl Acad Sci USA, 106:17939-17944.

Pearce M. C., Jenkins C., Vali L., Smith A. W., Knight H. I., Cheasty T., Smith H. R., Gunn G. J., Woolhouse M. E., Amyes S. G., Frankel G. (2004): Temporal shedding patterns and virulence factors of Escherichia coli serogroups O26, O103, O111, O145, and O157 in a cohort of beef calves and their dams. Applied and Environmental Microbiology 70, 1708–1716

Perelle S, Dilasser F, Grout J, Fach P (2004): Detection by 5'-nuclease PCR of Shiga-toxin producing Escherichia coli O26, O55, O91, O103, O111, O113, O145 and

O157:H7, associated with the world's most frequent clinical cases. Mol Cell Probes 18: 185-192.

Perelle, S., Dilasser, F., Grout, J. and Fach, P. (2005): "Detection of *Escherichia coli* serogroup O103 by real-time polymerase chain reaction." *Journal of Applied Microbiology* **98**: 1162-1168.

Quinn, P.J., B.K. Markey, M.E. Carter, Donnelly, W.J.C. and Leonard,F.C. (2002): Veterinary microbiology and microbial diseases. Black Well Scientific ublications, Oxford, London.

Samadpour, M., M. W. Barbour, T. Nguyen, T. M. Cao, F. Buck, G. A. Depavia, E. Mazengia, P. Yang, D. Alfi, M. Lopes, and J. D. Stopforth. 2006. Incidence of enterohemor;rhagic *Escherichia coli, Escherichia coli* O157, *Salmonella,* and *Listeria monocytogenes* in retail fresh ground beef, sprouts, and mushrooms. J. Food Prot. **69:** 441-443.

Scheutz F, Nielsen EM, Frobe I, Frimodt-Moller J, Boisen N, Morabito S, Tozzoli R, Nataro J, Caprioli A (2011): Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing Escherichia coli O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. Euro Surveill 16: 19889.

Seas, C.; Alarcon, M.; Aragon, JC.; Beneit, S.; Quiñonez, M.; Guerra, H. and Gotuzzo E. (2000): Surveillance of Bacterial Pathogens Associated with Acute Diarrhea in Lima, Peru. Int. J. Infect. Dis.;4: 96–99.

Selim, S.A.; Ahmed, S.F.; Abdel Aziz, M.H.; Zakaria, A.M.; Klena, J.D. and Pangallo, D. (2013): Prevalence and Characterization of Shiga-toxin O157:H7 and Non-O157:H7 Enterohemorrhagic Escherichia coli isolated from different sources. ISSN: 1310-2818 (Print) 1314-3530 (Online) Journal homepage http://www.tandfonline.com/loi/tbeq20 (Link of the article) http://dx.doi.org/10.5504/BBEQ.2013.0031

Sojka, W.J. (1965): *E. coli* in Domestic Animals and Poultry. 1st Ed. Commonwealth Agriculture.Bureau, Farnham, Royal Bucks, England.

Stuart C.Clarke (2001): Diarrhoeagenic Escherichia coli – an emerging problem? Diagnostic Microbiology and infectious diseases 41 (2001) 93-98.

Thomas K. M., McCann M. S., Collery M. M., Logan A., Whyte P., McDowell D. A., Duffy G. (2012): Tracking verocytotoxigenic Escherichia coli O157, O26, O111, O103 and O145 in Irish cattle. International Journal of Food Microbiology 153, 288–296

Varela, G.; Chinen, I.; Gadea, P.; Miliwebsky, E.; Mota, M.; vGonzález, S.; González, G.; Gugliada, M.J.; Carbonari, C.C.; Algorta, G.; Bernadá, M.; Sabelli, R.; Pardo, L.; Rivas, M. and Schelotto, F. (2008): Detection and characterization of shiga toxin-producing Enterohemorrhagic Escherichia coli (EHEC) infection *Escherichia coli* from clinical cases and food in Uruguay. Rev Argent Microbiol., 40: 93-1

Wallace, J. S. (1999): in *Escherichia coli O157 in Farm Animals*, eds. Stewart, C. S. & Flint, H. J. (CABI Publishing, Wallingford, U.K.), pp. 195–223.