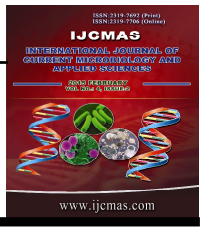
Int.J.Curr.Microbiol.App.Sci (2015) 4(2): 481-496

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 2 (2015) pp. 481-496**
http://www.ijcmas.com



Original Research Article

Shiga Toxin-Producing *E. coli* (STEC) Associated with Lebanese Fresh Produce

Khatib A[1], Olama Z[2]* and Khawaja G[1]

[1]Faculty of Science- Beirut Arab University, Lebanon
[2]Faculty of science- Alexandria University, Egypt
**Corresponding author*

A B S T R A C T

Shiga toxin-producing *E. coli* (STEC), the most common cause of food-borne illness, is associated with several human gastrointestinal and systemic diseases such as diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). In the present study, a total of 145 samples of Lebanese fresh produce were investigated for the occurrence of STEC, it was found that 39(26.8%) produce items were fecal *E.coli* contaminated, the highest contamination occurred in leafy green produce (purslane, thyme, parsley and lettuce) except peppermint items were fecal *E. coli* free. The isolates were subjected to PCR detection of STEC using primers specific for *stx1*, *stx2*, *eaeA*, *ehlyA*, and *fliC* virulence genes, it was showed that 7 produce samples were confirmed to contain STEC (two lettuce, two thyme, one tomato, one cucumber and one radish). Among seven STEC-positive isolates, six isolates were *stx1*-postive, three of which were *fliC*-postive and could be classified as O157:H7, on the other hand three isolates were *stx2*-postive and two isolates were *eaeA*-positive. However, *ehlyA* was not detected in any of the isolates. It was revealed that 18(46.2%) isolates were resistant to some of the antibiotics used in the present study, 9(23%) of which are considered to be multidrug resistant since they showed resistance to ≥ 3antibiotic classes (β-lactam, tetracycline and folate inhibitor). The antibiotics to which resistance was detected are: Amoxicillin /Clavulanic acid, Piperacillin / Tazobactam, Tetracycline and Sulphamethoxazole /Trimethoprim. Wild type *E.coli* J53 Azide^R acquired resistance to antibiotics mentioned above after conjugation with one of MDR isolates. This result indicates a high possibility of horizontal gene transfer between bacteria, via plasmids or transposons, and therefore contributing in the increase of the resistant genes in the environment.

Keywords

Lebanese fresh produce, *Escherichia coli* (STX-EC), Antimicrobial resistance, conjugation

Introduction

Recent studies showed that food, mainly ready-to-eat fresh fruits and vegetables are important contributors for the transmission of many diseases especially in the developing countries where hygienic standards are enforced and not followed (Berger et al., 2010). It has been also reported that *E. coli* can be detected in

vegetables if they are irrigated with contaminated water, fertilized with contaminated manure (Keskinen et al., 2009) or through cross-contamination , such as inadequate microbial safety precautions taken during food manipulation and person-to-person transmission (Franz &van Bruggen, 2008). Most *E. coli* strains are usually harmless members of human intestinal flora and other animals (Beutin et al., 2004), but some strains have acquired virulence factors that contribute to their pathogenicity associated to important intestinal and extra-intestinal diseases (Bielaszewska et al., 2011).These strains include enterotoxinogenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and enterohaemorrhagic *E. coli* (EHEC). The later includes Shiga Toxin-producing *E. coli* (STX-EC) (Torres et al., 2009 and Scavia et al., 2011).

Shiga toxin-producing *E. coli* (STEC) also known as Verotoxin-producing *E. coli* is the most common cause of food-borne diseases that have emerged within the past two decades (Beutin et al., 2007).This organism is associated with several human gastrointestinal and systemic diseases, such as non-bloody or bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), urinary tract infections (UTI), septicemia, and neonatal meningitis (Blanco et al., 2006 and Bielaszewska et al., 2011).

Most outbreaks cases of bloody diarrhea and HUS have been attributed to strains of STEC serotype O157:H7 (Pennington, 2010). However, the role of non-O157 STEC strains as causes of HUS, bloody diarrhea, and other gastrointestinal illnesses is being increasingly recently recognized in the United States and in Europe (Tzschoppe et al., 2012). The pathogenicity of STEC strains is mainly due to the production of shiga toxins encoded by the *stx1*and *stx2* genes, each of which exists as several variants (Zweifel et al. 2005). Human virulent STEC strains often may also contain other virulence factors such as intimin *eaeA*, a protein essential for the intimate attachment and the formation of attaching and effacing lesions on gastrointestinal epithelial cells, in addition to the plasmid-located enterohemolysin gene *ehly*, which has been suspected to play a role in pathogenicity of STEC infections (Ito et al.,2007). Moreover *f liC* genetic marker responsible for the production of the H7 *E.coli* flagellar protein (Zweifel et al. 2005).

Unfortunately, the wide application of antimicrobials has led to the emergence of drug-resistant bacteria in the environment (Duijkeren et al., 2003). Currently, multidrug resistance is commonly observed in *E. coli* isolates from human clinical cases world-wide, and this feature has an increasing impact on empirical treatment of community acquired infections by *E. coli*, leading to a high fatality rate especially among immuno-comprised individuals (Yüksel et al., 2006).

Previous studies showed that multi-drug-resistant *E. coli* isolates from animals and food products represent an important reservoir for transmissible resistance genes to opportunistic pathogenic bacteria (Slama et al., 2010).

The aim of the present study is to investigate the occurrence of Shiga toxin-producing *E. coli* (STEC) in Lebanese fresh produce. And the role of plasmids in conferring antimicrobial resistance to bacterial strains was studied by transforming plasmid from MDR *E. coli* isolate into azide resistant J53 *E. coli* by conjugation.

Materials and Methods

Sample collection

In the present study, a total of 145 vegetables samples were analyzed for the presence of *E.coli* and STEC. The samples were collected randomly from different 10 agricultural fields, 14 grocery stores and 7 market places in Lebanon from north to south areas (during June through December 2013). The target commodities included produce items that are mostly eaten raw (Table 1). The samples were placed into sterile sampling bags using sterile hand gloves and were immediately transported to the microbiology laboratory in a refrigerated container at 4°C until sample preparation and analysis (Lynette et al., 2005).

Bacterial isolation and bacteriological analysis

***E.coli* and coliforms detection:** Twenty five gram (25 g) of each produce sample were shaken for 2 min in 225 ml of lauryl sulfate tryptose (LST) broth as an enrichment medium , the coliform count was determined by the three tube most-probable-number (MPN) system using three ten-fold serial dilution in LST broth that were incubated for 48 h at 37°C. LST tubes showing growth and gas production were streaked on eosin methylene blue (EMB) agar plates for *E.coli* colonies isolation. Suspected *E.coli* colonies were confirmed by indol, methyl red, Voges Proskauer and citrate fermentation tests (Avik et al., 2003). Predominant coliforms in fresh produce were determined by identifying the isolated colonies from the highest dilution of the samples on EMB Plates using API stripes (Biomerieux, France).

E.coli O157:H7 Detection was performed by plating *E.coli* enrichment broth on sorbitol-MacConkey agar supplemented with potassium tellurite and cefixime (Lynette et al., 2005). The occurrence of *E. coli* O157:H7 in fresh produce was determined by the commercial kit VIDAS® ECO O157 (bioMérieux, France). Five colonies of each pure growth *E.coli* were picked up and inoculated in 1 ml brain heart infusion broth containing 25% glycerol and then stored at -70 for further investigation (Shereen & Asem, 2013).

Bacterial strains

Thirty nine *E. coli* isolates from Lebanese fresh produce were cultured directly on MacConkey's agar (Oxoid, England) and were identified as *E. coli* using the API 20E biochemical system (bioMerieux, France). *E. coli* A isolate was considered to be MDR organism since it showed resistance to several antibiotic classes and was used in the conjugation experiments as a donor strain while *E.coli* J53 Azide^{R}, that was kindly provided by Dr. Ghassan Matar, Department of Experimental Pathology, Immunology & Microbiology, Faculty of Medicine, American University of Beirut, was used as the recipient strain. *E.coli* O157:H7 DNA template kindly provided by Dr.Ghasan Matar and used as positive control in the PCR assays. All bacterial strains were maintained on nutrient agar slants at 4°C and with monthly transfer to fresh media, for long preservation 25% glycerol was added (Shereen & Asem, 2013).

DNA extraction

DNA was extracted from *E.coli* isolates grown on EMB agar plates following the manufacturer's protocol (GenElute^{TM} Bacterial Genomic DNA kits, SIGMA Aldrich, Germany), extracted DNA was stored at -20◦C. A 1µl DNA template was used per 25 µl Polymerase chain reaction (PCR) reaction (Ozpinar et al., 2013).

PCR assays

In order to detect STX-EC, primers were selected based on five genes specific for STX-EC strains. Primers to amplify Shiga toxins 1 and 2 (*stx1* and *stx2*) were selected as shown by Osek & Gallein (2002) (Table 4). STEC primers were used to amplify both *stx1* and *stx2* in combination, as described by Reischl et al. (2002). The three other genes that were targeted for amplification were *eaeA* gene, encodes for intimin gamma, *ehlyA*, encodes for enterohemolysin, and *fliC*, encodes for H7flagellar protein, as described by Reischl et al. (2002) and Osek & Gallein (2002), respectively (Table 4). Amplification of bacterial DNA was performed in 25µl volumes containing 12.5 µl (REDTaq , Ready Mix) DNA polymerase, 1 µl of primer mix, 10.5 µl of sterile water and 1 µl of extracted DNA. Amplification of *stx1*, *stx2*, *eaeA* and *ehlyA* genes were performed using a DNA thermal cycler (Bio-Rad) for 35 cycles for 1min at 93◦C, 30 s at 65 ◦C, and 2 min at 72 ◦C, and a final extension at 75 ◦C for 10 min. While *stx1*+ *stx2* and *fliC* genes was performed in similar conditions except for the annealing phase which was performed for 30 s at 55◦C(Osek & Gallein, 2002). After the PCR, 10- µl aliquots were analyzed through electrophoresis with 2% agarose gel containing 10 µl ethidium bromide. The DNA samples visualized through UV transmission, and photographed (Steve et al., 2005).

Detection of multi-drug resistant food-borne pathogens

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing and results interpretation were performed according to the recommendation of the Clinical Laboratory and Standards Institute (CLSI). The antibiotic (µg/disc) used in the present study were : gentamicin (CN - 10 µg), levofloxacin (LEV - 5 µg), norfloxacin (NOR - 10 µg), piperacillin /tazobactam (TZP - 100/10 µg), amoxicillin /clavulanic acid (AMC - 20/10 µg) , tetracycline (TE - 30 µg), cefotaxime (CTX - 30 µg), ceftriaxone (CRO - 30 µg), cefepime (FEP - 30 µg), sulphamethoxazole /trimethoprim (SXT-25 µg), cefpodoxime (CPD-10 µg), azetreonam (ATM-30 µg) and imipenem (IPM - 10 µg). Loaded Mueller Hinton agar plates were left for 30 min at room temperature for compound diffusion and then incubated for 24 h at 37◦C. Zones of inhibition were recorded in millimeters and the experiment was repeated twice.

Double-disk synergy test (DDST)

Disks containing cephalosporins (cefotaxime or ceftriaxone, ceftazidime, cefepime) are applied next to a disk with clavulanic acid (amoxicillin-clavulanic acid or ticarcillin-clavulanic acid). Positive result is indicated when the inhibition zones around any of the cephalosporin disks are augmented in the direction of the disk containing clavulanic acid. The distance between the disks is critical and 20mm centre-to-centre has been found to be optimal for cephalosporin 30µg disks (Drieux et al., 2008).

Bacterial Conjugation

E.coli J53 Azide^{R} resistant to sodium azide was used as the recipient strain and the multi-drug resistant *E.coli* A was used as the donor strain. Both strains were incubated together in an LB broth tube at a ratio of 10:1 recipient to donor, and the mixtures were incubated at 37◦C for 18 h (Baroud et al., 2013). Transconjugants were selected on Muller-Hinton agar plates supplemented with 150 mg/L sodium azide (Sigma-

Aldrich) only or with 20 mg/L cefoxitin. Sodium azide was used to inhibit the growth of the donor strain and cefoxitin to inhibit the growth of the recipient strain (Ulises et al., 2012). To confirm that the transconjugant J53 cells had indeed taken up plasmids containing genes encoding for resistance and had thus acquired resistance to antimicrobials they were previously susceptible to, using the disk diffusion agar method, as previously described. Following an overnight incubation at 37˚C, the diameters of the zones of inhibition for the transconjugants were measured and compared to those of the parents and controls (Baroud et al., 2013).

Data Analysis

The average coliform counts were calculated and statistically significant differences between varieties of fruits and vegetables and between organic and conventional farms were determined using one sample test. The same statistical tool was used to compare the prevalence of *E.coli* among different produce varieties. The criteria for statistical significance was based on a (p<0.05).

Result and Discussion

A total of 145 conventional and organic produce samples were collected during a period of 7 months originating from different 10 agricultural fields, 14 grocery stores and 7 market places in Lebanon (Table 1) More than 50% of the produce items collected consisted of lettuce (17.2 %), tomato (13.1%), parsley (12.4 %) and cucumber (9.6 %) and the remainder included spinach, thyme, peppermint, purslane, arugula and radish.

In a trial to test the microbiological quality of different produce samples**,** coliform bacteria were detected in 71% of all the samples, and the overall average counts in the conventional produce from agricultural area, grocery and market place were 1.3 $\log_{10}$ MPN/g (±0.5 SD), 2.2 $\log_{10}$ MPN/g (±0.7 SD) and 2.0 $\log_{10}$ MPN/g (±0.4 SD) respectively (Table 2). Parsley, thyme and purslane collected from grocery and market place had slightly higher means counts of coliform, showing more than 1. $\log_{10}$ MPN/g greater counts than same types of fresh produce collected from agricultural area and these differences were statistically significant. In addition when the coliform counts were compared between ten different fresh produce types having the same origin, the differences were obvious and statistically significant (p<0.05).On the other hand when the predominant bacteria were determined in 145 samples, it was revealed that *E.coli* was the most common identified coliform bacteria (38%), besides *Enterobacter cloacae*, *Enterobacter sakazaki* and others were also found in some fresh produce. The coliform level on most of fresh produce reported in the present study were from 1 to 3 $\log_{10}$ CFU/g, these results consistent with those of Lynette et al. (2005) who examined the microbial levels on fresh produce items, but lower than those reported by Ruiz-Cruz et al. (2007) whereas levels on samples of tested fresh produce ranged from 10^4 to 10^6 $\log_{10}$ CFU/g.

As for *E.coli* occurrence in fresh produce, all samples were analyzed for *E.coli* O157:H7 and non O157 *E.coli*. Suspected isolates to be *E.coli* O157:H7,since it showed colorless colony on sorbitol-MacConkey, showed negative result using the commercial kit VIDAS® ECO O157 (bioMérieux, France). Data in Table 3 revealed that purslane was the most fecal *E.coli* contaminated produce, 5samples out of 10 was contaminated (50%), whereas peppermint was completely sterile, free produce fecal *E.coli* contamination (0%). On

the other hand thyme, parsley and lettuce showed slightly high contamination with fecal *E.coli* (46.1, 38.6 and 36% respectively). However tomato and radish showed moderate contamination (21and 23%) followed by spinach, cucumber and argula that showed a lower fecal *E.coli* contamination (14.2%), these differences were statically significant ($p<0.05$). For decades, *E. coli* has been used as the reference indicator for fecal contamination, and a number of surveys have reported its isolation from fresh fruits and vegetables (Jay, 2000). In a recent study that tested conventionally grown fresh produce at retail, only one sample tested positive for *E. coli* out of 50 samples that included alfalfa sprouts, broccoli, lettuce and celery (Thunberg et al., 2002). The percentage of *E. coli*–positive samples found in a survey of conventionally grown fresh vegetables in Japan (including cabbage, lettuce, onions, spinach, and celery) was 2% (Kaneko et al., 1999). However, in the present study *E. coli* was found in 26.8% of conventional fresh vegetables in Lebanon, this result is consistent with the prevalence of 25% this bacterium in ready-to-use lettuce (Soriano et al., 2000). A number of surveys have attempted to detect *E. coli* O157:H7 in fresh fruits and vegetables, in a study that included 3,200 vegetables, no O157:H7positive sample was detected, and in another survey of 890 fruits and vegetables, this pathogen could not be found either (Johannessen et al., 2000). Among fresh produce items, purslane appears to be more susceptible to bacterial contamination followed by thyme, parsley and lettuce showing 50, 46.1, 38.6 and 36% respectively of samples contaminated with fecal *E.coli* these results are similar to those showed by Avik et al.(2003), where leafy green and lettuce showed high level of fecal contamination, in addition recent evidence suggests that food-borne pathogens can be internalized into leafy green and lettuce leaves (Solomon et al., 2002), except for peppermint items that show no fecal *E.coli* contamination and this is maybe due to the strong antibacterial effect of this leafy green to wide spectrum of bacteria (Friedman et al., 2002).

Concerning the detection of shiga toxine (Stx)-producing *E. coli* (STEC), the fecal *E. coli* isolates were analyzed by PCR for the *stx1*, *stx2*, *stx1+stx2*, *eaeA* and *ehlyA* with the specific sets of primers (Table 4) . Those isolates were also tested for *fliC* genetic marker, which encodes for the production of *E. coli* O157:H flagellar protein *fliC*. Out of 39fecal *E. coli* contaminated samples, 7 were confirmed to contain STEC (two lettuce, two thyme, one tomato, one cucumber and one radish).

On the other hand it was found that total of 6, 3, 2, 2 and 3 of the tested isolates were *stx1*, *stx2*, *stx1+stx2*, *eaeA* and *fliC* positive respectively, while *ehlyA* was not detected in any of tested samples (Table 5; Fig 1,2,3,4 and 5).

In addition, it was showed that *stx1*was in association with the other virulence marker genes in 3 samples (Table 6), those seven isolates could be classified as enterohaemorrhagic *E. coli* (EHEC) or more specifically STX-EC, three of which could be classified as O157:H7 since they showed a very intense band of approximately 980 bp in PCR performed with *fliC* primer. On the other hand, H7-negative strains have recently been identified and it was demonstrated that they represent a distinct clone within the *E. coli* O157serogroup. However, it shares several virulence characteristics with other STX-EC of the O157:H7 serotype (Osek & Gallein, 2002). However, presence of the *eae* gene in STEC-positive samples was associated with

fliC genetic marker, Similarly, Fantelli and Stephan (2001) reported that all non-O157 STEC strains isolated from minced meat were negative for *eae* gene.

Conversely the present study revealed that 39 *E. coli*-positive samples, 32 (82%) were non-STEC, whereas only 7 (18%) were identified as STEC serotypes. The occurrence of STEC type carrying the virulence marker genes in some vegetables, such as lettuce, thyme, tomato, cucumber and radish which are consumed raw by the Lebanese consumers, represents a serious risk to public health as strains carrying these sequences are frequently associated with serious illness, such as HUS (Liua et al. 2007).

In an attempt to detect the antimicrobial resistance of fecal *E.coli* isolates, out of 39 fecal *E.coli* isolates, 18(46.2%) isolates showed resistance to some of the antibiotics used in the present study, 9(23%) isolates are considered to be multidrug resistant since they showed resistance to ≥ 3antibiotic classes (β-lactam, tetracycline and folate inhibitor). The antibiotics to which resistance was detected are: amoxicillin /clavulanic acid (AMC), piperacillin /tazobactam (TZP), tetracycline (TE) and sulphamethoxazole /trimethoprim (SXT). The remainder 21(53.8%) fecal *E.coli* isolates showed sensitivity to all used antibiotics in the present study (Table 7). ESBL *E.coli* strains were not detected, since no synergistic effect appears between amoxicillin /clavulanic acid (AMC), cefotaxime (CTX) and ceftriaxone (CRO) using Double-disk synergy test (DDST). The present study demonstrated that *E.coli* isolates from fresh produce were resistance to 30% of the antibiotics used in the treatment of urinary tract infection in Lebanon. A previous study carried out during 2000-2001, reported similar antimicrobial resistant patterns in uropathogenic *E.coli* isolates from patients (Shehabi et al., 2004). In addition to a recent study that found bacterial isolates from fresh vegetables exhibited higher resistance rates than our study to ampicillin, cephalothin, trimethoprime - sulfamethoxazole, aminoglycosides, tetracycline, fluoroquino-lones, amoxycillin-clavulanic acid, and chloramphenicol (Hassan et al., 2011).

Transfer of plasmid-encoded drug resistance genes between bacteria in natural habitats, represents a serious risk to public health (Steve et al., 2005).

The present study revealed that (46.2%) *E. coli* isolates were resistant to various antimicrobials (Table 7).Such resistance should be considered not only to its effect on human health, but also, it could be transferred to other important pathogenic serotypes (Dzidic & Bedekovic, 2003). In order to determine whether some antimicrobial resistance is plasmid-encoded, plasmids were transferred from MDR *E. coli* A into the wild type *E.coli* J53 Azide^{R} by bacterial conjugation procedure (Baroud et al., 2013). Interestingly, amoxicillin /clavulanic acid (AMC), piperacillin /tazobactam (TZP), tetracycline (TE) and sulphamethoxazole /trimethoprim (SXT) resistances were associated with all successful transformations (Fig 6-7). This result indicates a high possibility of horizontal gene transfer between bacteria, via plasmids or transposons, and therefore contributing in the increase of the resistant genes in the environment (Kruse & Sorum, 1994).

The present study provided valuable information regarding pathogenic *E. coli* contamination of fresh produce collected from several agricultural areas, grocery stores and market places in Lebanon. These

data will facilitate assessment of the risk of contamination by *E. coli*, including STEC serotypes, and characterization of STEC virulence genes in fresh produce consumed in Lebanon suggesting potential public health hazard.

Table.1 Distribution of conventional and organic samples, according to produce varieties

Produce varieties	% of samples (No. of samples)				
	Conventional			Organic	Total
	Agricultural Area	Grocery	Market Place		
Lettuce	11.4 (4)	16 (8)	26.4 (9)	15.3 (4)	17.2 (25)
Parsley	22.8 (8)	12 (6)	11.7 (4)	-	12.4 (18)
Spinach	8.5 (3)	8 (4)	-	-	4.8 (7)
Peppermint	11.4 (4)	10 (5)	8.8 (3)	-	8.2 (12)
Thyme	14.2 (5)	12 (6)	5.8 (2)	-	8.9 (13)
Purslane	8.5 (3)	8 (4)	8.8 (3)	-	6.8 (10)
Arugula	5.7 (2)	8 (4)	5.8 (2)	23 (6)	9.6 (14)
Radish	5.7 (2)	8 (4)	11.7 (4)	11.5 (3)	8.9 (13)
Tomato	8.5 (3)	10 (5)	11.7 (4)	26.9 (7)	13.1 (19)
Cucumber	2.8 (1)	8 (4)	8.8 (3)	23 (6)	9.6 (14)
Total	35	50	34	26	145

- : Not available samples

Table.2 Levels of coliform contamination in produce varieties

Produce varieties	Coliform count Mean log MPN/g ± SD				
	Conventional			organic	*p*-value
	Agricultural Area	Grocery	Market Place		
Lettuce	1.4±0.9	1.4±1.0	2.0±1.12	0.0	.066
Parsley	1.7±1.4	2.3±0.8	2.3±0.7	-	.009*
Spinach	1.8±1.2	2.9±0.3	-	-	.144
Peppermint	0.3±0.5	2.3±0.9	1.9±0.6	-	.126
Thyme	1.2±1.2	2.5±0.7	2.4±0.0	-	.037*
Purslane	1.2±1.0	2.6±0.9	2.6±0.8	-	.041*
Arugula	2.1±0.7	3.1±0.0	1.3±0.5	0.2±0.7	.066
Radish	1.5±0.1	2.3±1.3	1.5±1.1	0.0	.068
Tomato	0.5±0.3	1.4±1.3	2.2±1.0	0.0	.127
Cucumber	1.3±0.1	0.9±0.7	1.9±1.0	0.0	.080
overall	1.3±0.5x	2.2±0.7x	2.0±0.4x		

* Means of the bacterial counts in fresh produce indicate statistically significant differences (p <0.05) between locations. X indicates statistically high significant differences between overall counts of fresh produce from agricultural area, grocery and market place. Statistical analysis was only done on conventional produce types that supplied > 80% of the samples. - : Not available

Table.3 Incidence of fecal *E.coli* in collected fresh produce according to produce varieties

Varieties of the samples (No.)	No. (%) positive *E.coli* samples
Lettuce (25)	9 (36)
Parsley (18)	7 (38.8)
Spinach (7)	1 (14.2)
Peppermint (12)	- (0)
Thyme (13)	6 (46.1)
Purslane (10)	5 (50)
Arugula (14)	2 (14.2)
Radish (13)	3 (23)
Tomato (19)	4 (21)
Cucumber (14)	2 (14.2)
Total (145)	39 (26.8)
p-value	0.02

Table.4 Primers used in identifying STX-EC strains

Target gene	Primers	Sequence	Resource	Predicted size of the amplified product (bp)
stx1	stx1	F: CAGTTAATGTCGTGGCGAAGG R: CACCAGACAATGTAACCGCTG	Osek and Gallein (2002)	**348**
stx2	stx2	F: ATCCTATTCCCGGGAGTTTACG R: GCGTCATCGTATACACAGGAGC	Osek and Gallein (2002)	**584**
stx1+stx2	STEC	F: gA(Ag) C(Ag)A AAT AAT TTA TAT gTg R: TgA TgA Tg(Ag) CAA TTC AgT AT	Reischl et al. (2002)	**520**
eaeA	eaeA	F: gAC CCg gCA CAA gCA TAA gC R: CCA CCT gCA gCA ACA AgA gg	Reischl et al. (2002)	**383**
fliC	flicH7	F: GCTGCAACGGTAAGTGAT R: GGCAGCAAGCGGGTTGGT	Osek and Gallein (2002)	**984**
ehlyA	ehlyA	F: gCA TCA TCA AgC gTA CgT TCC R: AAT gAg CCA AgC Tgg TTA AgC T	Reischl et al. (2002)	**532**

Table.5 Isolates positive in PCR performed with different STX-EC genetic markers

E.coli	Total tested isolates	*stx1* positive	*stx2* positive	*STEC* positive	*eaeA* positive	*ehlyA* positive	*fliC* positive
Data	39	6	3	2	2	-	3

Table.6 Prevalence of virulence marker genes in *E.coli* isolates as tested by PCR

Isolate number	Produce type	Virulence marker gene					
		stx1	*stx2*	*Stx1+stx2*	*eaeA*	*ehlyA*	*fliC*
15	Lettuce	-	+	-	-	-	-
17	Tomato	+	+	+	+	-	+
18	Lettuce	+	-	-	-	-	-
19	Thyme	+	-	-	-	-	+
20	Radish	+	+	+	+	-	+
23	Thyme	+	-	-	-	-	-
25	Cucumber	+	-	-	-	-	-

+: presence of the gene ; - : absence of the gene

Table.7 Antimicrobial resistance patterns of *E.coli* isolates

Antibiotic used	Resistance of bacteria to antibiotics		
	Sensitive Count (%)	Resistant Count (%)	Total Count (%)
Trimethoprim–Sulfamethoxazole	30 (77%)	9 (23%)	39 (100%)
Norfloxacin	39 (100%)	-	39 (100%)
Piperacillin/Tazobactam	30 (77%)	9 (23%)	39 (100%)
Cefpodoxime	39 (100%)	-	39 (100%)
Amoxycillin/Clavulanic Acid	21 (53.8%)	18 (46.2%)	39 (100%)
Imipenem	39 (100%)	-	39 (100%)
Gentamicin	39 (100%)	-	39 (100%)
Cefepime	39 (100%)	-	39 (100%)
Ceftriaxone	39 (100%)	-	39 (100%)
Aztreonam	39 (100%)	-	39 (100%)
Cefotaxim	39 (100%)	-	39 (100%)
Levofloxacin	39 (100%)	-	39 (100%)
Tetracycline	30 (77%)	9 (23%)	39 (100%)

Fig.1 DNA fragments observed with specific primers for stx1 gene detection in E.coli isolates following PCR. L: Ladder; PC: Positive control; 16-26: isolates samples

Fig.2 DNA fragments observed with specific primers for *stx2* gene detection in *E.coli* isolates following PCR (A)-L: Ladder; PC: Positive control; 1-15: isolates samples. (B)-L: Ladder; PC: Positive control; 16-26: isolates samples.

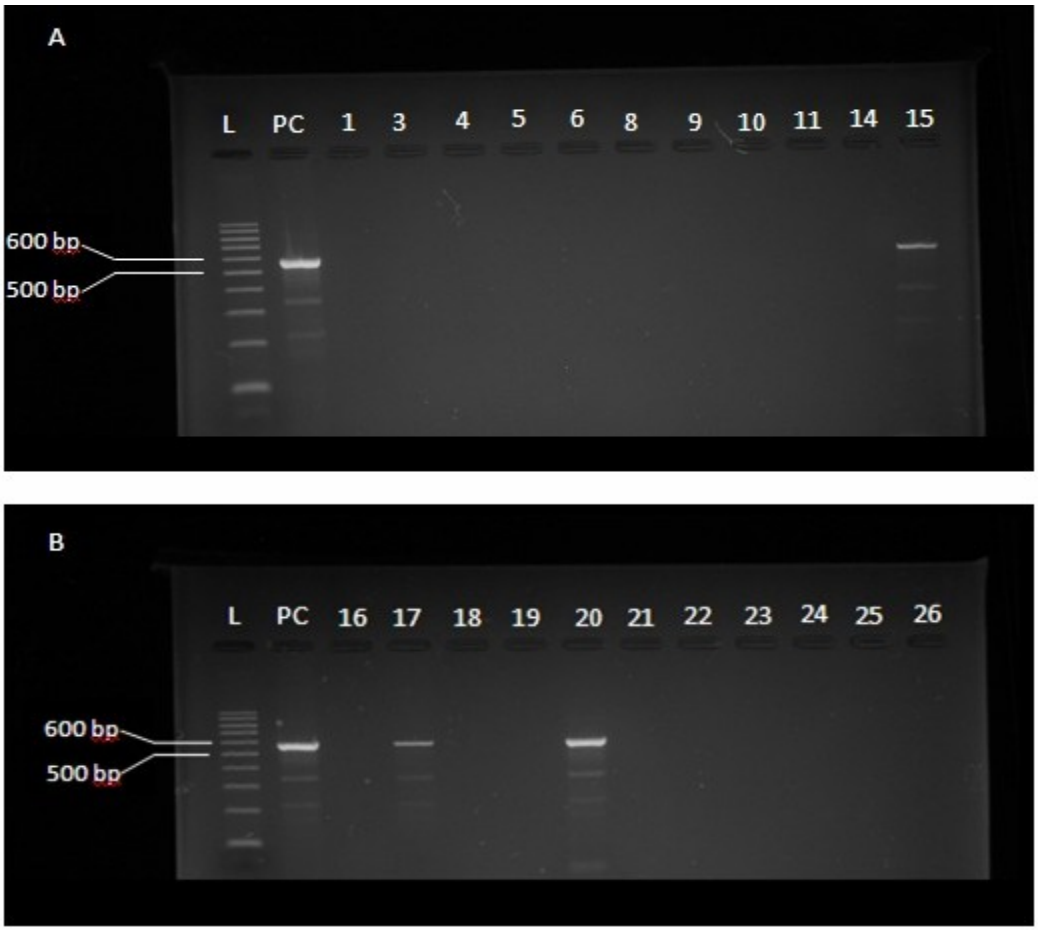


Fig.3 DNA fragments observed with specific primers for *stx1+stx2* gene detection in *E.coli* isolates following PCR. L: Ladder; PC: Positive control; 16-26: isolates samples

Fig.4 DNA fragments observed with specific primers for *eaeA* gene detection in *E.coli* isolates following PCR. L: Ladder; PC: Positive control; 16-26: isolates samples

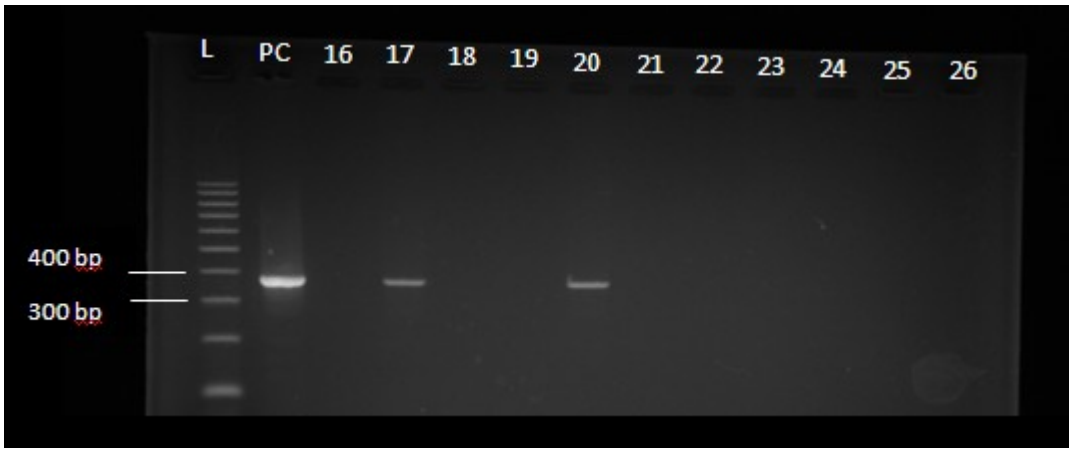


Fig.5 DNA fragments observed with specific primers for *ehlyA* gene detection in *E.coli* isolates following PCR. L: Ladder; PC: Positive control; 16-26: isolates samples

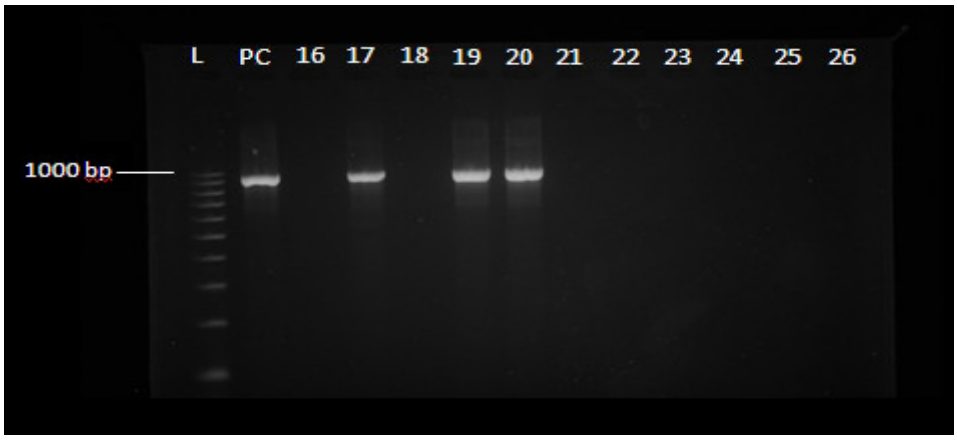


Fig.6 Sensitivity of wild type *E.coli* J53 Azide^{R} to antibiotics: (A):Tetracycline; (B): Amoxicillin /Clavulanic acid; (C): Sulphamethoxazole /Trimethoprim and (D): Piperacillin /Tazobactam

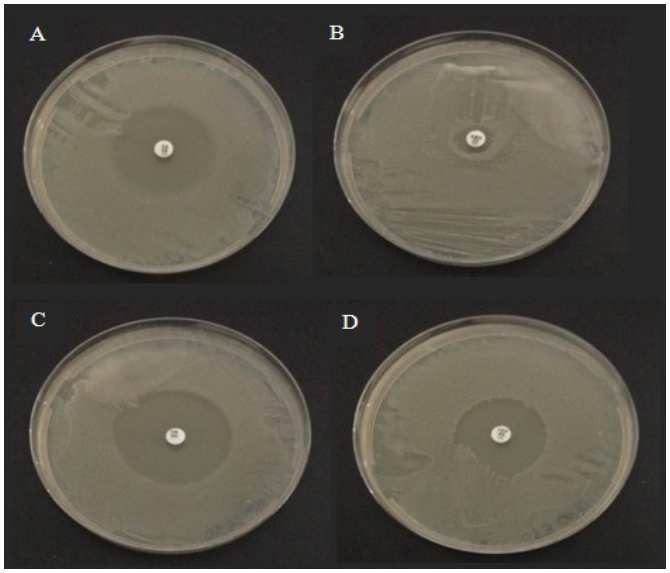
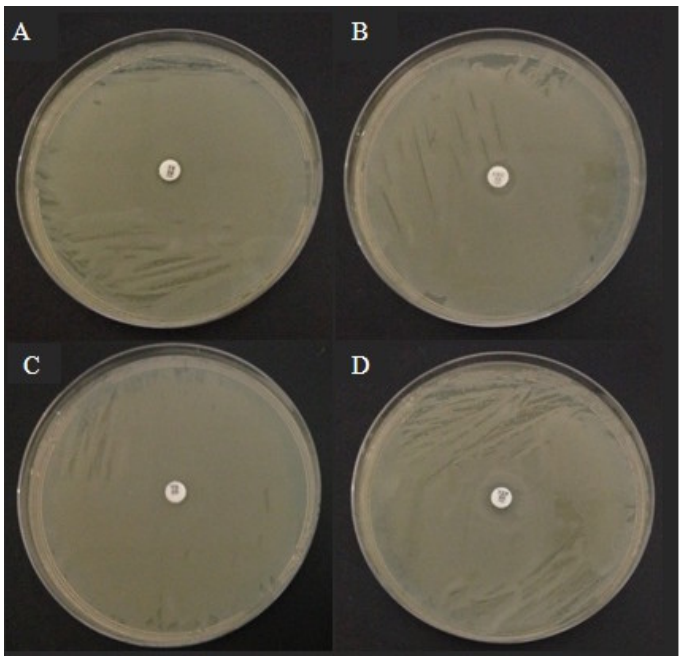


Fig.7 Sensitivity transconjugant J53 to antibiotics: (A):Tetracycline; (B): Amoxicillin /Clavulanic acid; (C): Sulphamethoxazole /Trimethoprim and (D): Piperacillin /Tazobactam



References

Avik, M.; Dorinda, S.; Elizabeth, D. and Francisco, D. (2003). Preharvest Evaluation of Coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157:H7 in Organic and Conventional Produce Grown by Minnesota Farmers. J. Food Prot. 67: 894-900.

Baroud, M.; Dandache, I.; Araj, G.; Wakim, R.; Kanj, S.; Kanafani, Z.; Khairallah, M.; Sabra, S.; Shehab, M.; Dbaibo,G.

and Matar, G. ((2013). Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. Int J Antimicrob Agents. 41: 75-79.

Berger, C.; Sodha, S.; Shaw, R.; Griffin, P.; Pink, D.; Hand , P. and Frankel , G.(2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ. Microbiol. 12: 2385- 2397 .

Beutin, L.; Krause, G.; Zimmermann, S.; Kaulfuss, S. and Gleier, K. (2004)

Characterization of Shiga toxin-producing *Escherichia coli* strains isolated

from human patients in Germany over a 3-year period. J Clin Microbiol 42:1099-1108.

Beutin, L.; Miko, A.; Krause, G.; Pries, K.; Haby, S. and Steege, K.(2007). Identification of human-pathogenic strains of Shiga toxin–producing *Escherichia coli* from food by a combination of serotyping and molecular typing of Shiga toxin genes . Appl Environ Microbiol. 73:4769-75.

Bielaszewska, M.; Mellmann, A.; Zhang, W.; Kck, R.; Fruth, A.; Bauwens, A.; Peters, G. and Karch, H. (2011) Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in

Germany: a microbiological study. Lancet Infect Dis. 11:671-676.

Blanco, M.; Blanco, J.; Dhabi, G.; Mora, A.; Alonso, M.; Varela, G.; Gadea, P.; Schelotto, F.; Gonzez, E. and Blanco, J.(2006). Typing of intimin (*eae*) genes from enteropathogenic *Escherichia coli* (EPEC) isolated from children with diarrhoea in Montevideo, Uruguay: identification of two novel intimin variants. J Med Microbiol. 55:1165-1174.

Drieux, L.; Brossier, F.; Sougakoff, W. and Jarlier, V. (2008). Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae : review and bench guide.Clin. Microbiol. Infect.14: 90-103.

Duijkeren, E.; Wannet, W.; Houwers, D. and Pelt, W.(2003). Antimicrobial

susceptibility of *Salmonella* strains isolated from humans, cattle, pigs, and chickens in the Netherlands from 1984 to 2001. J Clin Microbiol. 41: 3574-3578.

Dzidic, S. and Bedekovic, V. (2003). Horizontal gene transfer-emerging multidrug resistance in hospital bacteria . Acta Pharmacol Sin. 24:519-26.

Fantelli, K. and Stephan, R. (2001) Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* strains isolated from minced meat in Switzerland. Int J Food Microbiol.70:63-69.

Franz, E. and van Bruggen, A. (2008). Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. Crit Rev Microbiol. 34: 143-161.

Friedman , M.; Henika , P. and Mandrell, R. (2002). Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65: 1545-1560.

Hassan , S.; Altalhi, A.; Gherbawy , Y. and El-Deeb, B. (2011). Bacterial load of fresh vegetables and their resistance to the currently used antibiotics in Saudi

Arabia . Foodborne Pathog. Dis. 8: 1011-1018.

Ito , K.; Iida , M.; Yamazaki, M.; Moriya, K.; Moroishi, S.; Yatsuyanagi, J.; Kurazono, T. and Hiruta, N. (2007). Intimin types determined by hetero duplex mobility assay of intimin gene (*eae*)-positive *Escherichia coli* strains. J Clin Microbiol. 45: 1038-1041.

Jay, J.(2000). Modern food microbiology, 6th. ed. Aspen Publishers, Gaithersburg, Md.

Johannessen, G.; Loncarevic, S. and Kruse, H.(2000). Bacteriological analysis of fresh produce in Norway. Int. J. Food Microbiol. 77:199-204.

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

Keskinen, L.; Burke, A. and Annous, B. (2009). Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves. Int. J. Food Microbiol. 132: 134-140.

Kruse,H. and Sorum, H. (1994).Transfer of multi-drug resistance plasmids between bacteria of diverse origins in the natural environment. Appl Environ Microbiol. 60:4015-21.

Liua, Y.; DebRoyb, C. and Fratamico, P. (2007) Sequencing and analysis of the *Escherichia coli* serogroup O117, O126, and O146 O-antigen gene clusters and development of PCR assays targeting serogroup O117-, O126-, and O146-specific DNA sequences. Mol Cell Probes. 21: 295-302.

Lynette, M.; Johnston, Lee-Ann, J.; Debora, M.; Martha, C.; Juan, A.; Brenda, M. and Christine, L.(2005). A Field Study of the Microbiological Quality of Fresh Produce . J. Food Prot. 68: 1840-1847.

Osek, J. and Gallein, P. (2002). Molecular analysis of *Escherichia coli* O157strains isolated from cattle and pigs by the use of PCR and pulsed-field gel electrophoresis methods. Vet Med-Czech.47:149-58.

Ozpinar, H.; Turan , B.; Tekiner , H.; Tezmen, G.; Gokce, I. and Akıneden, E. (2013). Evaluation of pathogenic *Escherichia coli* occurrence in vegetable samples from district bazaars in Istanbul using real-time PCR. Lett Appl Microbiol. 57:362-7.

Pennington, H. (2010). *Escherichia coli* O157. Lancet. 376: 1428-1435.

Reischl, U.; Youssef , M.; Kilwinski, J.; Lehn, N.; Zhang,W.and Karch, H. (2002). Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. J Clin Microbiol. 40:2555-65.

Ruiz-Cruz, S.; Acedo-Félix, E.; Díaz-Cinco, M.; Islas-Osuna, M.and González-Aguilar, G. (2007). Efficacy of sanitizers in reducing *Escherichia coli* O157:H7,*Salmonella* spp. and *Listeria monocytogenes* populations on fresh-cut carrots. Food Control.18: 1383-1390.

Scavia, G.; Morabito, S. and Tozzoli, R. (2011). Similarity of Shiga toxin–producing Escherichia coli O104:H4 strains from Italy and Germany. Emerg Infect Dis. 17:1957-1958.

Shehabi, A.; Mahafzah, A. and Al-Khalili, K. (2004). Antimicrobial resistance and plasmid profiles of urinary *Escherichia coli* isolates from Jordanian patients. EMHJ.6: 322-328.

Shereen, B. and Asem, S.(2013). Fresh leafy green vegetables associated with multidrug resistant *E.coli*. The International Arabic Journal of Antimicrobial Agents. 3: 2-3.

Slama, K.; Jouini, A.; Sallem, R.; Somalo, S.; Sáenz, Y.; Estepa, V.; Boudabous, A. and Torres , C. (2010). Prevalence of broad-spectrum cephalosporin-resistant *Escherichia coli* isolates in food samples in Tunisia, and characterization of integrons an antimicrobial resistance mechanisms implicated. Int J Food Microbiol.137: 281-286.

Solomon, E.; Yaron, S. and Mathews, K.(2002). Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68: 397-400.

Soriano, J.; Rico, M.; Molto, J. and M. J. (2000). Assessment of the microbiological quality and wash treatments of lettuce served in University restaurants. Int. J. Food Microbiol. 58:123-128.

Steve, H.; Hadi, Y.; Maya, G.; Elie, B.; Shadi, H.; Mutasem, F.; Imad, T.and Raja,T. Isolation, molecular characterization and antimicrobial resistance patterns of *Salmonella* and *Escherichia coli* isolates from meat-based fast food. Lebanon Science of the Total Environment . 341: 33-44.

Thunberg, R.; Tran, T.; Bennett, R.; Matthews, R. and Belay, N.(2002). Microbial evaluation of selected fresh produce obtained at retail markets. J. Food Prot. 65:677-682.

Torres, A.; Blanco, M. and Valenzuela, P. (2009). Genes related to long polar fimbriae of pathogenic *Escherichia coli* strains as reliable markers to identify virulent isolates. J Clin Microbiol. 47:2442-2451.

Tzschoppe, M.; Martin, A. and Beutin, L. (2012). A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to eat vegetables. Int J Food Microbiol. 152:19-30.

Ulises, G.; Humberto, B.; Maria, H.; Teresa, R.; Fernando, R.; Perla, T.; Victor, O.; Laurent, P.; Patrice, N.; Vincent, C.; Guillermo, R.; Jose, F.; Rosa, I.; Patricia, B.; Natividad, C. and Jesus, S. (2012)Transfer of quinolone resistance gene qnrA1 to *Escherichia coli* through a 50 kb conjugative plasmid resulting from the splitting of a 300 kb plasmid. J Antimicrob Chemother. 67:1627-34.

Yüksel, S.; Oztürk, B.; Kavaz, A.; Ozçakar, Z.; Acar, B.; Güriz, H.; Aysev, D.; Ekim, M. and Yalçinkaya, F.(2006).Antibiotic resistance of urinary tract pathogens and evaluation of empirical treatment in Turkish children with urinary tract infections. Int J Antimicrob Agents. 28: 413-6.

Zweifel , C.; Schumacher, S.; Blanco, M.; Blanco, J.; Tasara , T.; Blanco, J. and Stephan, R. (2005). Phenotypic and genotypic characteristics of non-O157 Shiga toxinproducing *Escherichia coli* (STEC) from Swiss cattle. Vet Microbiol. 105: 37-45.