

Full Length Research Paper

## Molecular detection of $\beta$ -lactamases and aminoglycoside resistance genes among *Escherichia coli* isolates recovered from medicinal plant

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Cross contamination from composting farm manure and surface run-off waters or the application of untreated sewage, or manure can be sources of pathogenic microorganisms that contaminate fruits and vegetables in the field. Therefore, the current study was aimed to detect the incidence of *Escherichia coli* isolates from herbs samples and the molecular detection of  $\beta$ -Lactamases and aminoglycoside resistance genes among *E. coli* isolates recovered from medicinal plant. 58 strains of *E. coli* (28.43%) were isolated and serotyping of the recovered *E.coli* isolates revealed 18 strains O125 (31.03%), 12 strains were (20.69%) O86, 10 strains were (17.24%) O112, 8 strains were (13.79%) O127, 6 strains were (10.34%) O128 and 4 strains were (6.90%) O44. The *E. coli* isolates were tested by PCR for detection of *aacC2* gene; all isolates showed amplification of 896 bp fragments specific for *aacC2* gene. Multiplex PCR showed amplification of 516 bp fragments specific for *blaTEM* gene in all isolates (100%). Moreover, 38 of the *E. coli* isolate (65.52%) had a band compatible with *blaSHV* gene (392 bp fragments) while, the presence of *blaOXA\_1* gene was not detected in all *E. coli* isolates.

**Key words:** Medicinal plant, *Escherichia coli*, resistance gene, molecular detection, multiplex PCR.

### INTRODUCTION

Bacterial food-borne pathogens are the most important food safety issue worldwide. During the past decade, outbreaks of human illness associated with the consumption of fresh fruits and vegetables have increased in the United States (Beuchat, 1996; Brackett, 1999; Blank et al., 2008; CDC, 2008; Berger et al., 2009). The risk of human illness associated with raw produce can be better predicted by monitoring microbial

contamination at points of potential contamination in the field during harvesting, during processing and distribution, or in retail markets (Beuchat and Ryu, 1997; Brackett et al., 1993). One major factor that can influence the microbial quality of produce is the type of irrigation used and the water source (Beuchat, 1996; Woodford et al., 2006). For example, cross-contamination from composting farm manure, flooding, and surface run-off

waters can be sources of pathogenic microorganisms that contaminate fruits and vegetables in the field (Beuchat and Ryu, 1997; Brackett, 1999, Bowen et al., 2006). The presence of enteric human pathogens in the soil may also be largely due to the application of faeces, untreated sewage, or manure either by chance or design. As a result, contamination of fresh produce by fecal coliforms is well documented and recognized as a potential public health problem (Backer et al., 2000; Campbell et al., 2001; Erickson et al., 2007). Several recent outbreaks of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* spp. have been associated with fresh produce (Backer et al., 2000; Brackett, 2001; Campbell et al., 2001; Itoh et al., 1998). The rapid and accurate identification of bacterial pathogens from food samples is important, both for food quality assurance and to trace outbreaks of bacterial pathogens within the food supply. Growing concerns regarding the safety of fresh produce warrant a greater emphasis on the development of more rapid, specific, and highly sensitive detection methods. Advances in biotechnology have permitted more reliable microbial identification and surveillance (Feng, 1997). Antimicrobial therapy is one of the measures taken to control the diseases caused by *E. coli* (Blanco et al., 1997), as a result, multi-resistant *E. coli* isolates may emerge and become a worldwide public health problem, including direct impact on food safety. Antimicrobial resistance determinants are carried mostly by mobile genetic elements such as plasmids, transposons, and integrons (Rowe-Magnus and Mael 2002).

Therefore, fresh or minimally processed vegetables which are often eaten raw or minimally processed, can compromise consumer health safety. The most common bacterial enteropathogens associated with fruits and vegetables are *Salmonella* spp. (Thunberg et al., 2002) and *E. coli* O157:H7 (Rangel et al., 2005; Friesema et al., 2008; Pakalniskiene et al., 2009). They are considered among the organisms that are most likely to cause an outbreak and therefore need to be studied for produce safety (Buck et al., 2003). *Salmonella* is responsible for a localized, self-limiting bacterial infection of the intestinal epithelium, known as “non-typhoid salmonellosis” or “gastroenteritis” and a systemic infection known as “typhoid” or “enteric fever” with severe consequences. The incidence of typhoid salmonellosis is stable, with very few cases in developed countries, but cases of non-typhoid salmonellosis are increasing worldwide (García del Portillo, 2000). *E. coli* O157:H7 is the most toxigenic serotype of the genus and is an important cause of diarrhoea, hemorrhagic colitis and haemolytic-uremic syndrome worldwide, associated with high mortality rates of between 10 and 40% (Palermo et al., 2009). Therefore, the main objective of this study was the molecular detection of the resistance caused by  $\beta$ -lactamases (blaTEM, blaSHV and blaOXA<sub>1</sub> genes) and the resistance caused by aminoglycoside resistance genes

responsible for resistance to gentamicin (aacC2 gene) in resistant *E. coli* isolates recovered from medicinal plants.

## MATERIAL AND METHODS

### Bacterial strains

Eight L-lactamase-producing control strains were used to optimize the PCR assay. These included; *E. coli* pBR322 (TEM-1-producing), *E. coli* RP4 (TEM-2-encoding), *E. coli* CF804 (TEM-8-expressing), *E. coli* RHH-1 (TEM-9-producing), *E. coli* pUD18 (SHV-3-producing), *E. coli* (OXA-3-producing; donated by Prof. J. Vila) and *E. coli* (PSE-3 expressing). In addition, non-L-lactamase-producing *E. coli* strains, HB10, were included as negative controls.

### Samples

During the summer of 2010, a total of 204 herbs samples; 34 basil, 40 dry mints, 28 marjoram, 72 chamomile, 22 fennel and 8 calendula flower samples were collected and analyzed for detection and isolation of *E. coli*. Ten negative control field samples were collected from farms continuously monitored for *E. coli* by standard microbiological techniques.

All samples were transported to the laboratory under refrigerated conditions where they were processed and bacteriologically examined immediately.

### Isolation and identification of *E. coli*

Surface technique was used according to modified NMKL, 125-1996 and ISO/FDIS 7218-2007 and membrane filtration method ISO 9308-1, 2000). All samples were primarily cultured on MacConkey agar medium and incubated aerobically at 37°C. After overnight incubation, a part of single typical well isolated lactose fermenting colonies was tested for sorbitol fermentation by culturing on sorbitol MacConkey agar and sorbitol phenol red agar media and incubated at 37°C overnight. Morphological, cultural and biochemical examination were carried out according to methods described by Quinn et al. (2002).

### Serotyping of *E. coli*

Isolates that were primarily identified by biochemical tests were subjected to serological identification using diagnostic polyvalent and monovalent *E. coli* antisera (Welcome *E. coli* diagnostic antisera). Diagnostic *E. coli*-O157 antisera (Difco code 2970-47-7) and H7 antisera (Difco code 2159-47-0) were used for serological identification of *E. coli* O157: H7.

### Antimicrobial susceptibility test for non beta-lactam drugs

Antimicrobial susceptibility test to a range of antimicrobial agents was done using disks (ampicillin AMP10, ceftazidime CAZ30, ciprofloxacin CIP5, erythromycin E15, gentamicin CN10, nalidixic acid NA30, oxytetracycline OT30, penicillin G P10, amoxicillin-clavulanic acid AMC 30, norfloxacin NOR 10, chloramphenicol C 30, sulphamethoxazole-trimethoprim SXT 25, vancomycin VA 30) and adopting the Kirby-Bauer disk diffusion method using Muller-Hinton broth and agar and antibiotics disks (Oxoid Limited, Hampshire, England) according to the recommendations of Clinical

Laboratory Standards Institute (CSLI) formally National Committee for Clinical Laboratory Standards, (NCCLS), 2006.

### Resistance gene detection by PCR technique

PCR was conducted with primers described for the detection of *BlaTEM*, *BlaSHV*, *BlaOXA\_1* and *aacC2* genes. The primers sequences and the amplified fragments for the resistance genes are illustrated in Table 1.

### Extraction of DNA of *E. coli* samples by QIAamp DNA Mini Kit (Promega- catalogue number 51304)

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from tissues, swabs, CSF, blood, body fluids, or washed cells from urine. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time was only 20 min. Purification of DNA using the QIAamp DNA Mini Kit can be automated on the QIA cube.

### DNA amplification

Temperature and time conditions of primers during PCR were carried out according to the method of Palermo et al. (2009) and Friesema et al. (2008). PCR amplifications were performed in a final volume of 50  $\mu$ l in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5  $\mu$ l of the DNA template, 5  $\mu$ l 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 1  $\mu$ l dNTPs (40  $\mu$ M), 1  $\mu$ l 1U Ampli Taq DNA polymerase, 1  $\mu$ l (25 pmol) from the forward and reverse primers of both primer pairs and the volume of the reaction mixture was completed to 50  $\mu$ l using double distilled water (DDW). The thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of (denaturation at 94°C for 30 s, annealing at 56°C for s, and extension at 72°C for 1 min). Final extension was carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected.

### Agarose gel electrophoresis

The PCR products were tested for positive amplification by agarose gel electrophoresis as previously reported by Sambrook et al. (1989) using suitable molecular weight markers.

## RESULTS

### Standard bacteriological examination of the examined herbal samples

Fifty-eight (58) strains of *E. coli* (28.43%) out of 204 examined herbs samples were isolated and identified by standard microbiological techniques. 24 strains of *E. coli* (11.76%) were recovered from Chamomile herpes, while 16 (7.48%), 10 (4.90%), 4 (1.96%), 2 (0.98%) and 2 (0.98%) strains were recovered from Dry mint, Basil, Calendula flower, Marjoram and Fennel, respectively as shown in Table 2. Serotyping of the recovered *E. coli* isolates revealed 18 strains out of 58 recovered *E. coli* strains: O125 (31.03%), 12 strains were (20.69%) O86,

10 strains were (17.24%) O112, 8 strains were (13.79%) O127, 6 strains were (10.34%) O128 and 4 strains were (6.90%) O44 as shown in Table 2.

### Antimicrobial susceptibility testing

The 58 herbs strains has resistance pattern against erythromycin, penicillin, amoxicilline clauvenic and vancomycine with a percentage of 100% and gentamicin, ampicillin, chloramphenicol, oxytetracycline, norfloxacin, trimethoprim and nalidixic acid with percentages of 75.9, 65.5, 51.7, 48.3, 17.2, 6.9 and 6.9% respectively and non of the herbs strain had resistance against ciprofloxacin and ceftazidime.

### Results of Multiplex PCR for detection of $\beta$ -lactamases (*blaTEM*, *blaSHV* and *blaOXA\_1* genes) and single PCR detection of aminoglycoside resistance genes responsible for resistance to gentamicin (*aacC2* gene) in resistant *E. coli* isolates

Multiplex PCR was carried out to detect the resistance genes against ampicillin, amoxicillin clavulanic acid and gentamicine as shown in Table 3 and Figure 1.

A total of 58 *E. coli* isolates recovered by bacteriological examination were tested by PCR for detection of *aacC2* gene responsible for the gentamicin-resistant. All the isolates showed amplification of 896 bp fragments specific for *aacC2* gene as shown in Table 3 and Figure 2. A multiplex PCR was designed to detect *BlaTEM*, *BlaSHV* and *BlaOXA\_1* genes in amoxicillin-clavulanic acid and ampicillin resistant *E. coli* isolates. Among the 58 amoxicillin-clavulanic acid and ampicilline resistant isolates, multiplex PCR showed amplification of 516 bp fragments specific for *BlaTEM* gene in all isolates (100%). Moreover, 38 of the *E. coli* isolate (65.52%) had a band compatible with *BlaSHV* gene (392 bp fragments) while, the presence of *BlaOXA\_1* gene was not detected in all *E. coli* isolates as shown in Table 3 and Figure 1.

## DISCUSSION

In recent years, the growing consumption of fresh, minimally processed vegetables has lead to an increase in the number of outbreaks of food-borne disease linked to fresh produce (Anon, 2002; Blanck et al., 2008; Berger et al., 2009; Pakalniskiene et al., 2009; Palermo et al., 2009). Agricultural irrigation with wastewater that can be raw, treated and/or partially diluted, is a common practice worldwide and constitutes the main source of pathogen contamination (CDC, 2006; CDC, 2008; Friesema et al., 2008). In the present investigation, 204 herbs samples: 34 basil, 40 drymint, 28 marjoram, 72 chamomile, 22 fennel and 8 calendula flower samples were investigated

**Table 1.** The list of primers used in the present study.

Gene	Primer name	Sequence	Position	Size of PCR product (bp)	Reference
blaSHV	SHV-F	5'-AGGATTGACTGCCTTTTGG-3'	618-636	392	Colom et al. (2003)
	SHV-R	5'-ATTTGCTGATTCGCTCG-3'	993-1010		
blaTEM	TEM-C	5'-ATCAGCAATAAACCAGC-3'	385-401	516	Mabilat and Courvalin (1990)
	TEM-H	5'-CCCCGAAGAACGTTTTC-3'	885-901		
blaOXA_1	OXA-F	5'-ATATCTCTACTGTTGCATCTCC-3'	83-104	619	Colom et al. (2003)
	OXA-R	5'-AAACCCCTTCAAACCATCC-3'	685-702		
blaOXA_1	OXA-G	5'-TCAACTTCAAGATCGCA-3'	211-228	609	Speldooren et al. (1998)
	OXA-H	5'-GTGTGTTTGAATGGTGA-3'	803-820		
aacC2	aacC2-F	5'-TAG AGG AGA TAT CGC GAT GC-3'	75-94	896	Vanhoof et al. (1992); Vliegthart et al. (1990, 1991)
	aacC2-B	5'-ATT ATC ATT GTC GAC GGC CT-3'	971-952		

**Table 2.** Serotypes of *E. coli* isolates recovered from the examined herbal samples.

Kind of samples	Number	Poly 4 antiserum					Poly 3 antiserum			
		O86	O114	O125	O127	O128	O44	O112	O124	O142
Basil	10	0	0	0	0	0	4	6	0	0
Dry mint	16	12	0	0	0	0	0	4	0	0
Marjoram	2	0	0	0	2	0	0	0	0	0
Chamomile	24	0	0	18	6	0	0	0	0	0
Fennel	2	0	0	0	0	2	0	0	0	0
Calendula flower	4	0	0	0	0	4	0	0	0	0
Total	58	12	0	18	8	6	4	10	0	0

**Table 3.** Results of the resistance gene detection among the *E. coli* isolates

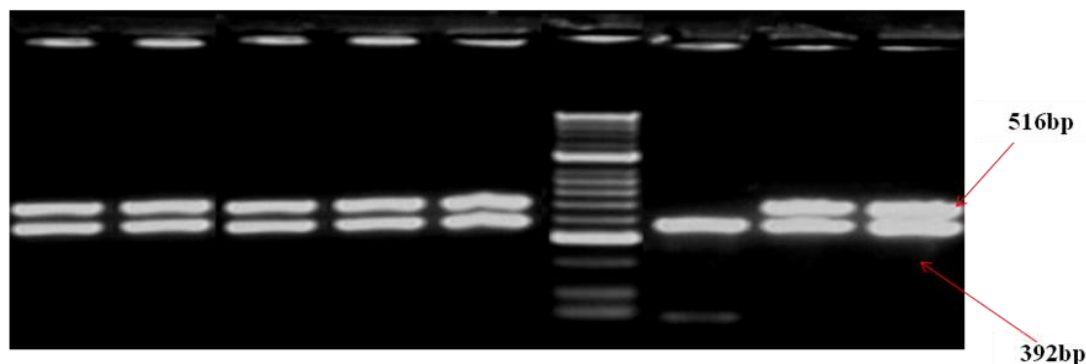
Samples	Number of isolates	Amoxicillin clavulenic acid and Ampicilline resistance gene				Aminoglycoside resistance genes (Gentamicin)			
		SHV gene		TEM gene		OXA_1 gene		aac2 genes	
		+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Basil	10	0	10	10	0	0	10	10	0
Dry mint	16	14	2	16	0	0	16	16	0
Marjoram	2	2	0	2	0	0	2	2	0
Chamomile	24	20	4	24	0	0	24	24	0
Fennel	2	2	0	2	0	0	2	2	0
Calendula flower	4	0	4	4	0	0	4	4	0
Total	58	38	20	58	0	0	58	58	0

bacteriologically to detect and isolate *E. coli*.

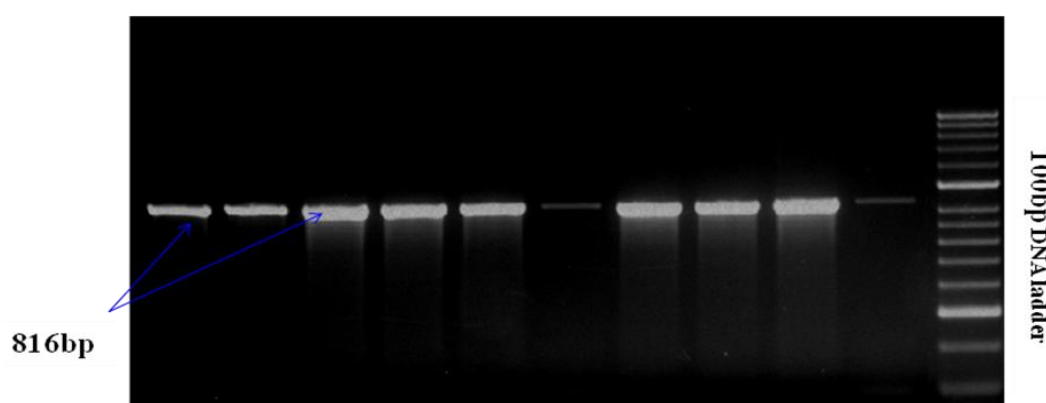
As shown in Table 2, 58 strains of *E. coli* (28.43%) out of 204 examined herbs samples were isolated and identified by standard microbiological techniques (Palermo et al., 2009). 24 strains of *E. coli* (11.76%) were recovered from Chamomile herpes, while 16 (7.48%), 10 (4.90%), 4 (1.96%), 2 (0.98%) and 2 (0.98%) strains were recovered from Dry mint, Basil, Calendula flower,

Marjoram and Fennel, respectively. These results confirm the results reported by Furlaneto and Mendes (2004) who isolated *E. coli* from herbs and the results indicate that the *E. coli* levels in basil (*Ocimum basilicum*) exceeded the regulation levels.

The *in vitro* sensitivity test of 58 *E. coli* strains isolated from herbs in this study against 13 antibiotics agents revealed that all of the tested strains were highly resistant



**Figure 1.** Multiplex PCR showing positive amplification of 516 base fragment specific for *BlaTEM* gene and 392 base pair fragments specific for *BlaTSHV* gene.



**Figure 2.** Agarose gel electrophoresis showing amplification of 896 *aacC2* gene responsible for the gentamicin resistant.

to penicillin, vancomycin, amoxicillin clavulanic acid and erythromycin with percentage 100% and this also had been reported by Nazir et al. (2005) who found that all the *E. coli* isolates were found resistant to penicillin G (94.45%).

These results agrees with those of Wani et al. (2004) who found that the *in vitro* sensitivity profiles revealed that all isolates were resistant to penicillin and erythromycin which indicated the need for judicious use of antibiotics.

Moreover, most of tested strains were resistance to gentamicin, ampicillin, chloramphenicol, oxytetracycline, norfloxacin, trimethoprim and nalidixic acid with percentage 75.9, 65.5, 51.7, 48.3, 17.2, 6.9 and 6.9% respectively.

Due to the emergence of large numbers of different *blaSHV* and *blaTEM*,  $\beta$ -lactamases isoelectrofocusing appears not to be the method of choice for establishing an ESBL phenotype (Hernandez et al., 2005). The solution to this problem has been to characterize ESBL-encoding genes using molecular biology techniques with specific primers for *blaSHV*, *blaTEM*, and *blaCTX-M*

genes, followed by DNA sequence analysis of the PCR amplicons (Boyd et al., 2004). Recently, multiplex PCR assays for detection of *bla-TEM*, *blaSHV* and *blaOXA-1* gene have been described (Woodford et al., 2006).

All the 58 *E. coli* isolates recovered by bacteriological examination were tested by PCR for detection of *aacC2* gene responsible for the gentamicin-resistant. All the isolates showed amplification of 896 bp fragments specific for *aacC2* gene as shown in Table 3 and Figure 2. The obtained results confirm the conclusion of Ho et al. (2010) who stated that the gentamicin-resistant isolates was PCR positive for the *aacC2* gene. Among the 58 amoxicillin-clavulanic acid and ampicillin resistant isolates, multiplex PCR showed amplification of 516 bp fragments specific for *BlaTEM* gene in all isolates (100%). Moreover, 38 of the *E. coli* isolate (65.52%) had a band compatible with *BlaSHV* gene (392 bp fragments). The predominant resistance genes were: ampicillin, *blaTEM1*-like and gentamicin *aacC2* genes (Guerra et al., 2003).

Therefore, in conclusion, this study show that multiplex PCR is a suitable tool for rapid screening of

plasmid-encoded L-lactamases which are known to confer amoxicillin-clavulanic acid resistance in *E. coli*.

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