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Molecular characterization of Enteropathogenic *Echerichia coli* isolated from mollscus and studying their sensitivity to acetic acid.

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Abstract

The current study aimed to isolate, characterize, investigate the presence of various phylogenetic groups of EPEC in mollscus bivalve from different retail fish markets at Alexandria governorate and subsequently, investigate the antibacterial activity of acetic acid 5% on the recovered EPEC isolates as a natural and reliable substance. A total of 100 samples, of retailed live bivalve mollscus, 50 each of Donax Trunculus (Om El Kholoul) and Tapes de-Cussates (Gandofli), were randomly collected from different fish markets at Alexandria governorate. All samples were subjected to bacteriological analysis for isolation of EPEC. The results revealed that *E. coli* was isolated from 48% of the collected samples, distributed as 23 isolates (46%) recovered from Donax Trunculus (Om El Kholoul) and 25 isolates (50%) recovered from Tapes decussates (Gandofli). 27 (56.25%) out of 48 *E. coli* isolates were positive for hemolytic and 15 isolates with a percentage of 31.25% were positive for Congo red binding assay. Multiplex PCR was used for the determination of phylogenetic groups of the recovered EPEC which revealed that all the EPEC examined had *chuA* and *yjaA* genes with a molecular base 279 bp and 211bp respectively, while, *TspE4.C2* gene could not be detected in all examined isolates so, this result indicate that all EPEC isolates recovered belongs to phylogenetic group B ,thus, we proposed that it originated from food handlers or water contaminated with human faeces. Dipping mollscus samples contaminated with EPEC in acetic acid 5% for 10 minutes revealed 100% reduction for EPEC. This study confirmed that mollscus products is commonly contaminated by EPEC and acetic acid 5% is a powerful natural bactericidal substance, so the use of it and the routine examination of such products for EPEC would be advisable.

Keywords: *EPEC, mollscus, PCR, acetic acid.*

Introduction

Nowadays human nutrition has been reason of concern worldwide. The rapid increasing of world population leading to increasing demand for protein resources. Bivalves molluscus, consider as easily digested, rich in protein and trace minerals, free from additives. These features of bivalves make them a food product completely fulfilling the requirements of the consumers, so there is a global increase in the production of bivalves molluscs ***(*Avril, 2005)**. On the other hand, they may be known as vectors of infection agents because bivalve molluscs are eaten raw and / or partially cooked, They lived on bottom over surface of mud near shore or at estuarine which exposed to many types of pollutants specially sewage pollutions. They are filter feeders; can accumulate and concentrate bacteria, viruses, parasites, pesticides, biotoxins, heavy metals during their physiological activity **(Evison, 1985; Potasman *et al.,* 2002, Huss *et al*., 2003, Butt *et al*., 2004 and Topic *et al.*, 2010).** Bivalve mollsucs wedge shell Donax Trunculus and Tapes de-Cussates are locally named as Om Elkholoul and Gandofli, respectively. Consumption of raw and undercooked bivalves contaminated by pathogens may lead to serious and potentially fatal foodborne diseases where the most frequent clinical syndrome is gastroenteritis. So, there is a need

of thorough control of its bacteriological characteristics for ensuring consumer's health. Illnesses associated with these food sources originate principally from bacteria particularly *Escherichia coli* . It is one of the most common Gram-negative, facultative anaerobic bacterium which belonging to the family *Enterobacteriaceae* of the class Gamma *Proteobacteria* **(Williams *et al*., 2010)**. It is widely distributed in intestinal microbiota of humans and warm-blooded animals and in the environment **(Smith *et al*., 2004)**. All pathogenic *Escherichia coli* strains have already been directly related to foodborne disease outbreaks, including meat, vegetables, milk, and dairy products affecting mainly children and immunocom promised individuals in general. In year 2000, some researchers developed a method of triplex PCR based on the amplification of the *chuA* (the outer-membrane hemin receptor gene), *yjaA*, which encodes an uncharacterized protein and the DNA fragment of the *TspE4.C2* which has been recently identified as part of a putative lipase esterase gene, On the basis of amplification and the presence or absence of these genes, *Escherichia coli* is divided into four major phylogenetic groups A, B1, B2 and D **(Clermont *et al*., 2000)**. According to the combination between the genetic markers, *Escherichia coli* is classified into seven subgroups (A0, A1, B1, $B2_2$, $B2_3$, D1 and D2), which increasing the discrimination power of *Escherichia coli* population analyses **(Escobar -Paramo *et al*., 2006 and Tenaillon *et al*., 2010)**. Many researcher observed that phylogenetic groups are differ in characteristics such as virulence factors, ecologic niches, life history, carbohydrate fermentation, antibiotic resistance, growth rate and size of the genome. The overall aim of this work was to investigate the presence of various phylogenetic groups (A,B and D) of *EPEC* in bivalve mollscus samples sold in retail markets in Alexandria governorate using molecular markers and evaluate the efficacy of acetic acid as a natural and available substance to control human infection by EPEC from consumption of contaminated bivalve mollscus.

Materials and Methods

Sampling:-

The alive mollscus bivalve samples including Om El Kholoul (Donax Trunculus) and Gandofli (Tapes decussates), 50 samples of each were collected from retail fish markets at Alexandria governorate. Each sample was collected in a sterile container and packed properly then transferred in a cool box with ice packs to the laboratory under complete aseptic conditions without any delay. All collected samples were subjected for bacteriological examinations as follows:

Preparation of samples: (West and Coleman, 1986)

The hands were thoroughly scrubbed with soap and water, then rinsed with 70% alcohol before preparation of samples, All samples were scrubbed with sterile brush, rinsed with cold , running tap water then Om Elkholoul and Gandofli samples were aseptically opened with a flame sterilized shucking knife and empty flesh and liquor into a sterile container.

Detection of *E. coli* by culture technique (Rivas *et al*., 2015):

Pre-enrichment stage:

25 g of seafood samples were homogenized in 225 ml brain heart infusion (BHI) broth in a stomacher for one minute. The samples were pre-enriched in BHI broth; incubated at 35 C for 3 h to facilitate resuscitation of sub lethally injured cells.

Selective Enrichment:

25 ml from the pre-enrichment homogenate was transferred to 225 ml of try tone phosphate (TP) broth and incubated at 44 °C for 20 h.

Plating on Solid Selective Media

Each selective enrichment broth was shaken and then a loopful from each of them was streaked onto plates of: EMB agar and Mac-Conkey agar plates. All plates incubated at 37 °C for 24 h. and then examined for typical *E. coli* colonies. Suspected isolates were inoculated on nutrient agar slants and incubated at 37°

C for 24 hours and then kept at 4°C for further identification.

Identification of *E. coli* (Quanin *et al*., 2002):

E. coli were identified morphologically by microscopic examination and biochemically. Suspected *E. coli* colonies; green metallic sheen on EMB or those appeared as brick red in color colonies on MacConkey agar plates .

Table (a). Biochemical reaction of identified *E. coli* isolates:

Indole	Methyle red	Vogas proskaeur	Citrate utilization	Urease	TSI	LDC	Oxidase	Motility
+	+	-	-	-	Y/Y	+	-	+

Determination of *E. coli* pathogensity:

- Hemolytic activity of the isolates (Vidotto *et al*., 1990):

E.coli isolates were streaked onto blood agar base supplemented with 5% sheep blood. Then incubated at 37^0C for 24 hours. Colonies produce clear zones of hemolysis recorded as positive.

- Congo red binding activity of the isolates (Berkhoff andVinal, 1986):

E. coli isolates were streaked on congo red agar and incubated for 72 hours at 37^0C. Reaction was recorded at 18, 24, 48 and 72 hours. Appearance of red colonies within 72 hours was recorded as a positive reaction. Negative colonies did not bind the dye and remained white or grey even after 72 hours were redorded as negative.

Detection of virulence genes of EPEC isolates (n=12)by multiplex PCR.

Positive *E. coli* isoltes for both hemolytic activity and congo red binding activity were subjected to multiplex PCR.

-DNA extraction: DNA extraction was performed from samples using The QIAamp DNA Mini Kit Catalogue no.51304. Briefly, 200 µl of the sample suspension was incubated with 20 µl of QIAGEN protease and 200 µl of AL buffer at 56˚C for 10 min. After incubation, 200 µl of ethanol (96%) was added to the sample. Then the sample was washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. Oligonucleotide primer. Primers used were supplied from Midl and Certified Reagent Company oilgos (USA) were listed in **table (b)**.

Table (b). Oligonucleotide primers sequences source (Midland Certified Reagent Company oilgos) (USA).

Gene	Primer Sequence 5'-3'	Amplified product	Reference
chuA	GAC GAA CCA ACG GTC AGG AT	279 bp	**Jeong *et al*., (2012)**
	TGC CGC CAG TAC CAA AGA CA		
yjaA	TGA AGT GTC AGG AGA YGC TG	211 bp	
	ATG RAG AAT GCG TTC CTC AAC		
*tsp*E4C2	GAG TAA TGT CGG GGC ATT CA	152 bp	
	CGC GYC AAC AAA GTA TTR CG		

-PCR amplification: For pathotypes genes (*chuA, yjaA* and *tspE4C2*) of EPEC multiplex PCR, Primers were utilized in a 25 µl reaction containing 12.5µl of Emerald Amp GT PCR Master Mix (2x premix) (Takara, Japan) kit , 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 5 µl of DNA template. The reaction was performed in a T3 Biometra thermal cycler as shown in **table (c)**

Table (c). Cycling conditions of different primers during cPCR:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
ChuA	95˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
YjaA	95˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
*tsp*E4 C2	95˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

- Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1-1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the multiplex PCR products and 15 µl of the uniplex PCR products were loaded in each gel slot. A gel pilot 100bp DNA Ladder (Qiagen, USA, cat. no. 239035) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (d). Criteria for assigning the phylogenetic groups:

Phylogroup/subgroup	Genes
A0	(chuA–, yjaA–, TspE4.C2–)
A1	(chuA–, yjaA+, TspE4.C2–)
B1	(chuA–, yjaA–, TspE4.C2+)
$B2_2$	(chuA+, yjaA+, TspE4.C2–)
$B2_3$	(chuA+, yjaA+, TspE4.C2+)
D1	(chuA+, yjaA–, TspE4.C2–)
D2	(chuA+, yjaA–, TspE4.C2+)
Untypable	(chuA–, yjaA+, TspE4.C2+)

According to (Escobar-Paramo *et al*., 2006 and Tenaillon *et al*.,2010).

Effect of acetic acid 5 %(vinegar of sugar cane) on EPEC in OmElkholoul samples:

EPEC positive Om-Elkholoul examined samples were selected (23 samples). Each sample was divided in aliquots into five subsamples. $1^{st,}$ 2^{nd}, 3^{rd} and 4^{th} were treated, by dipping, in 5% acetic acid for 1, 2, 5 and 10 minutes, respectively. The 5^{th} one was recorded as control positive (did not subjected for any treatment) after each treatment the subsamples were tested for EPEC on EMB agar, as the procedure mentioned before, and the results were recorded.

Results and Discussion

Table (1). Incidence of identified *E. coli* isolates among the examined mollscus bivalves:

Mollscus bivalves Type	No. of Examined samples	Positive samples	
		No.	%
Om El-Kholoul(Donax trunculus)	50	23	46
Gandofli(Tapes decussates)	50	25	50
Total	100	48	48

Table (2). Hemolytic and Congo red binding assay of identified *E. coli* isolates n= (48):

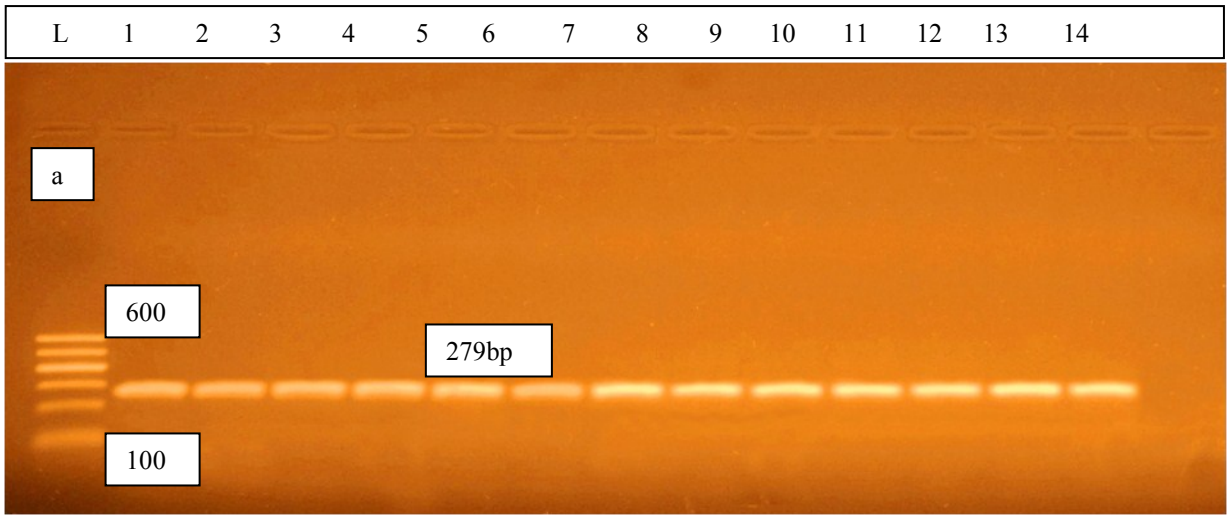
Mollscus bivalves Type	No. of *E. coli* isolates	Haemolytic assay				Congo red binding assay			
		Haemolytic		Non haemolytic		Positive		Negative	
		No	%*	No.	%*	No.	%*	No.	%*
Om El-Kholoul(Donax trunculus)	23	12	52.17	11	47.8	10	43.47	13	56.52
Gandofli (Tapes decussates)	25	15	60	10	40	5	20	20	80
Total	48	27	65.25	21	43.75	15	31.25	33	68.75

*Percentage according to total number of *E. coli* isolates.
NB: No. of positive *E. coli* isolates for both hemolytic and congo red binding assay together = 12 .

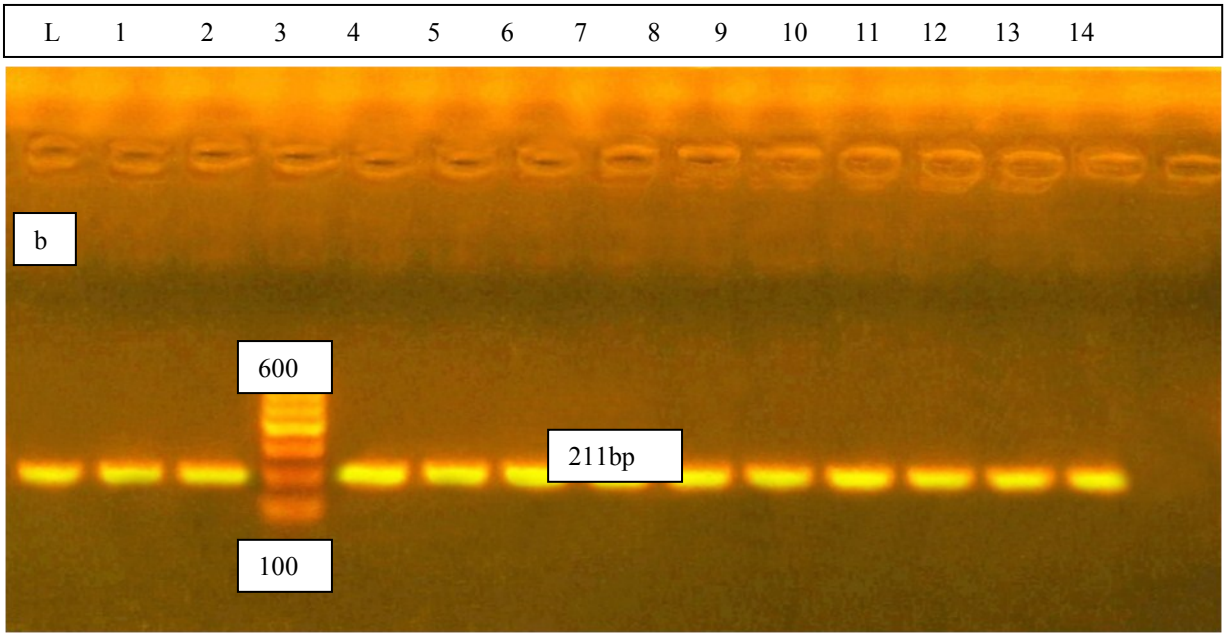
Table (3). Distribution of genetic markers and phylogenetic subgrouping of identified 12 EPEC isolates by multiplex PCR:

Mollscus bivalve type examined	No. of EPEC isolates	Genetic marker			Phylogenetic subgroups							
		ChuA	*yjaA*	*TspE4.C2*	A_0	A_1	B1	$B2_2$	$B2_3$	D1	D2	Untyable
Om El-Kholoul (Donax trunculus)	7	+	+	-	-	-	-	+	-	-	-	-
Gandoufli (Tapes decussates)	5	+	+	-	-	-	-	+	-	-	-	-

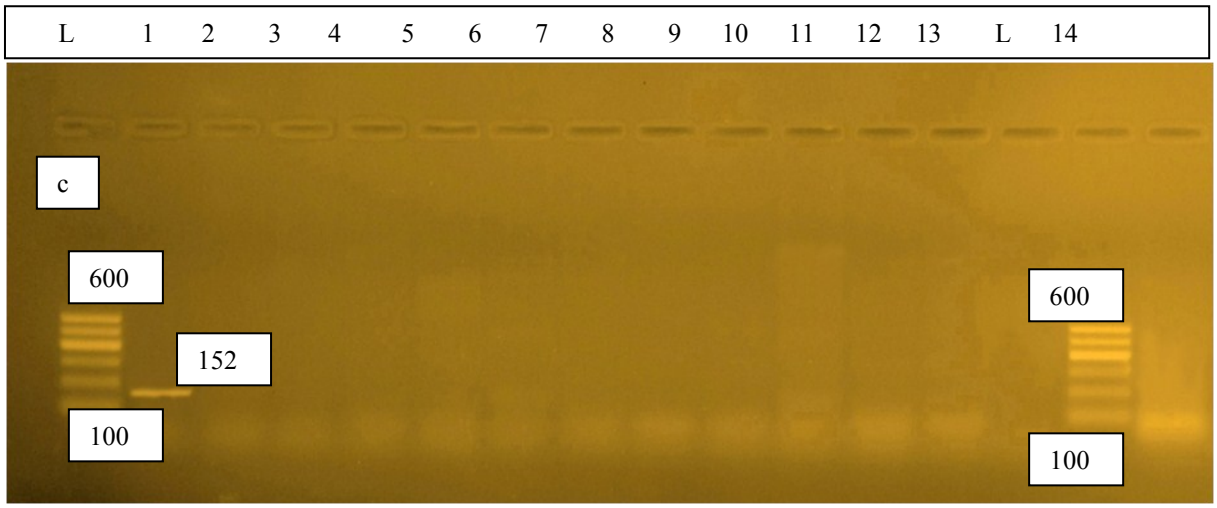
Figure (a & b & c). Agarose gel electrophoresis patterns showing PCR amplification:



Lane 1: Ladder (100-600bp); Lanes1-12 experiment samples;Lanes 13: positive control of *chuA* gene (279 bp) and Lane 14: Negative control.



L: Ladder (100-600bp); Lanes1-12 experiment samples; **Lanes 13:** positive control of *yjaA* gene (211bp) and Lane 14: Negative control.



L: Ladder (100-600bp); Lane 1 : positive control of *yjaA* gene (152bp), **Lanes: 2-13** experiment samples; and Lane 14: Negative control.

Table (4). Effect of acetic acid 5 % (vinegar of sugar cane) in Om El Kholoul (Donax Trunculus) positive samples for EPEC n= (23):

Subsample No.	Dipping time / Minute	Subsample after treatment	
		No.	Reduction %
1st	1	0	0
2nd	2	7	30.43
3rd	5	16	69.56
4th	10	23	100
5th	Control positive without any treatment		

Molluscus are liable to contamination with various kinds of microorganisms from different sources. Such contaminants may render the food unsafe to consumer or impair its utility, especially in undeveloped countries, where the hygienic measures are still under way. Traditionally consumption of bivalve molluscus is raw, or only under-cooked so it represent a risk to the consumers if contaminated especially by pathogenic strains that may cause illness to the consumers. The presence of *Escherichia coli* in seafood is related to water contamination, unhygienic conditions during the handling process **(Huss, 1993)**, the quality of the ice used for storage (**Vieira *et al*., 1997 and Gerokomou *et al*., 2011)** and /or the contamination of food processing plants **(Bagge *et al*., 2003)**. The obtained results illustrated in **Table (1)** revealed that 46% and 50% of Om-Elkhloul and Gandofli examined samples respectively were positive for presence of *E. coli*. It is obvious that, relatively higher incidence of *E. coli* was found in Gandofli than Om-Elkhloul that considered high risk to consumers. These incidences nearly similar with the results recorded by **Andres *et al.*, (1994).** Higher incidence (100%) was recorded by **Abd-El-Massih (1994),** while **Parisi *et al.*, (2004) and Sagoo *et al*., (2007)** could detect *E. coli* in 4% of molluscus shellfish batches. *E. coli* was proved to be the commonest cause of urinary tract infection, supurative lesions, neonatal septicemia and meningitis in humans **(Collins *et al*., 1991)**. Also, it causes profuse watery diarrhea which is vary in its severity and persistence due to inflammation of intestinal mucosa. Hemolytic and Congo red binding assay of the recovered *E. coli* isolates as shown in **Table (2)** revealed that 56.25% of *E. coli* isolates showed hemolytic activity (52.17% and 60% from Om El Kholoul and Gandofli respectively). On the other hand, 31.25% of the recovered isolates showed positive reaction for Congo red binding assay (43.47% and 20% from Om El Kholoul and Gandofli respectively). EPEC isolates can be classified into four different major phylogenetic groups on the basis of combination of *chuA*, *yjaA* and *TspE4.C2* genetic biomarkers via multiplex PCR. Virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D **(Boyd et *al*., 1998; Picard et *al*., 1999and Johnson *et al*., 2000),** whereas most commensal strains belong to group A .The phylogenetic classification investigated in this study according to the presence or absence of these three genetic markers (*chuA*, *yjaA* and *TspE4.C2*) in each isolates, the phylogenetic groups were assigned as per criteria given in **Table (3)** According to this criteria, all the examined 12 EPEC isolates recovered from Om El Kholoul and Gandofli samples were having genetic markers for *chuA*, *yjaA* genes while, *TspE4.C2* genetic marker was absent. This results revealed that all EPEC isolates investigated were belonged to phylogentic group B2 (sub phylogenetic group $B2_2$) as shown in **Table (3). Johnson *et al* (2005) and Rúgeles *et al* (2010)** reported that approximately 90% of the foodborne isolates were included in phylogenetic groups A or B1, consistent with other results in which isolates obtained from foods belonged preferentially to these groups. Alternatively, strains belonging to subgroup B2 have a tendency to be isolated more in humans, particularly in patients **Branger *et al* (2005), Hannah *et al* (2009) .**

In the current study all isolates from Om El Kholoul and Gandofli samples belonged to phylogenetic group $B2_2$; thus, we proposed that it originated from food handlers or water contaminated with human faeces.

Om- El Kholoul is considered one of the most popular street food especially in coastal cities in Egypt. Because of it is eaten raw without any heat treatment may lead to sever public health hazard. So, natural antimicrobial preservative should be used without altered its characteristics. Acetic acid is a traditional, low cost and simply improving food safety and shelf-life **(kisla, 2007).** Results illustrated in **Table (4)** showed the efficiency of acetic acid 5% solution for controlling the growth of EPEC in Om- El Kholoul positive samples. Dipping for one minute could not reduce EPEC

from any examined positive samples, but dipping for 2 and 5minutes can eliminated 30.34, 69.56 % respectively, EPEC from positive examined samples. While, complete elimination of EPEC from positive examined samples could be getting by dipping the samples for 10 minutes in acetic acid 5% solution. Many efforts were done to produce food free from pathogens of public health hazards. The bactericidal action of this organic acid is induced by lowering the PH of the food and its solubility in the cell membrane of bacteria **(El-Shenaway and marth, 1989).**

In conclusion, occurrence of *E. coli* in Om El Kholoul and Gandofli are considered as a sanitary measures and may represent a risk to the consumers if related to pathogenic strains, especially diarrheagenic *E. coli*. However, the presence of non-pathogenic *E. coli* in bivalve mollscus is recognized as an indicator of fecal contamination, possibly indicating the presence of other enteric pathogens so its presence should also give an alert to public health. We recommended some sanitary measures to take in consideration to ensure that Om El Kholoul and Gandofli is not incriminated in transmission of EPEC to consumers;

- Firstly , bacteriological examination of water quality of capture area should be periodically performed .
- Secondly, application of hygienic care in handling after harvest . Besides that, it is extremely not recommended to consume raw or undercooked Om El Kholoul except after dipping in 5% acetic acid solution for not less than 10 minutes.

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