Bacteriological and molecular studies of garlic effect on some virulence genes of *Escherichia coli* of chicken origin.

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A B S T R A C T

Avian colibacillosis is the most significant infectious bacterial disease of poultry worldwide. Many antimicrobial-resistant *Escherichia coli* pathogens are increased due to drug overusing in veterinary medicine and animals feed. The objective of this study was to observe the *in vitro* inhibitory effect of garlic (*Allium sativum*) as alternative natural agent against *Escherichia coli* isolates and their virulence genes expression. The antimicrobial effects of aqueous and ethanolic garlic extracts against multidrug-resistant (MDR) *E. coli* isolates were studied using agar well-diffusion method as well as the minimum inhibitory concentration (MIC) of garlic and consequently their subinhibitory concentration (SIC) (the concentration of garlic that did not inhibit *E. coli* growth after 24 hr. incubation at 37ºC) were calculated by a modification of broth macrodilution method. The *E. coli* virulence was assessed via mRNA expression of their genes such as increased serum survival (*iss*), verotoxine *(vt2*e) and intimin (*eae*A) before and after garlic treatment using reverse transcriptase real time PCR. One hundred and thirteen (46.12%) of 245 isolates were identified as *E. coli* that were highly recorded in intestinal samples as 64.60% followed by liver 24.77% then heart 10.6%. The most chemotherapeutic resistant 14 *E. coli* isolates showed high garlic susceptibility rates mainly for aqueous extract and had nine different serotypes, the most predominant one was O146 of 21.42%. The complete inhibition of and downregulating of *eaeA* and *vt*2e genes expression were proved respectively in the *E. coli* treated isolates with garlic SIC (1%). In conclusion, the garlic supplementation through feed can reduce infection by *E. coli* via decrescent their toxin production and may be the adhesion ability to the intestinal mucosa of the host either animal or human.

KEY WORDS: *Garlic* (*Allium sativum*)*-MIC– E. coli- virulence gene*

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1. INTRODUCTION

Avian colibacillosis signs are nonspecific and vary with age, organs involved, and concurrent disease. Young birds dying of acute septicemia have few lesions except for an enlarged, hyperemic liver and spleen with increased fluid in body cavities. Birds that survive septicemia develop subacute fibrinopurulent airsacculitis, pericarditis, perihepatitis, and lymphocytic depletion of the bursa and thymus (unusually pathogenic Salmonellae produce similar lesions in chicks). Although airsacculitis is a classic lesion of colibacillosis. In addition, their sporadic lesions include pneumonia, arthritis, osteomyelitis, peritonitis, and salpingitis (Lutful Kabir, 2010**).** Diarrhea is a clinical sign of a disease that may have many causes, and *E. coli* has been frequently implicated as the primary bacterial cause **(**Yamamoto and Nakazawa, 1997**).** Diarrhea is an extraordinary common disease with worldwide distribution and diarrhoeagenic *E. coli* is an important bacterium to cause it, the pathogenic strategies of these diarrhoeagenic strains exhibit remarkable variety in causing it **(**Clarke, 2001**).** Virulence is the measure of the pathogenicity of an organism. The degree of virulence is related directly to the ability of the organism to cause disease, despite host resistance mechanisms. Virulence factors are produced by a microorganism and evoke disease such as toxins, surface coats that inhibit phagocytosis and surface appendages that bind to the host cells **(**Peterson, 1996**).** Bacterial attachment is thought to enhance virulence by promoting colonization of the urinary tract and by attacking tissue. Uropathogenic express several classes of fimbriae-associated adhesins that mediate attachment through specific binding to

different glycoconjugate receptors (Leffler and Svanborg Ede´n, 1990). The production of Shiga toxins or Verotoxins is one of the defining characteristics of *E. coli* O157; these toxins are thought to be responsible for the principal manifestations of hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) (Paton, 1998). Certain studies have suggested a link between avin pathogenic *Escherichia coli* (APEC) and human diseases, thus, the enhanced control of avian colibacillosis proved to have benefits to both animal and human health (Ewers et al., 2004). The use of natural compounds has gained significant attention due to increasing concerns about the safety of synthetic chemicals and emerging antibiotic resistance in bacteria (Abee et al., 1995; Salamci et al., 2007). Garlic (*Allium sativum*) is one of those natural compounds that was seriously investigated over the years. It has been used for centuries to fight infections (Onyeagba et al., 2004). The early Egyptians used it to treat diarrhoea, the ancient Greeks used it to treat intestinal and extra intestinal diseases, while the ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever. In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhoea, otitis media and respiratory tract infections (Jaber and Al-Mossawi, 2007). Allicin (one of the active principal component of garlic) had been identified as antimicrobial ingredient (Ankri and Mirelman, 1999), which does not exist in garlic until it is crushed or cut; injury to the garlic bulb activates the enzyme allinase, which metabolizes alliin to allicin (Peyman et al., 2013). Allicin showed antibacterial activity primarily by partially inhibiting DNA and protein synthesis then by total inhibition of RNA synthesis as a primary target (Eja et al., 2007; Feldberg et al., 1988). Previous studies reported that exploring herbal plants efficacy on bacterial virulence genes' expression were sparse, for instance, Trans-cinnamaldehyde and clove oil that were evaluated as natural alternative to chemotherapeutics in down-regulation of Salmonella and Aeromonase virulence genes expression in poultry and fish (Abd El-Hamid et al., 2016; Kollanoor-Johny et al., 2012) respectively. IN contrast, there is no studies investigated the similar down-regulated expression effect for the garlic in virulence genes of such *E. coli*.

Therefore, this study was aimed to assess the *in vitro* antibacterial activity of garlic against *E. coli* as well as to shed spot light upon its efficacy in reducing *E. coli* infection in chicken (adhesion and toxins production) through reducing some of their virulence gene expression profiles using qRT-PCR.

2. MATERIALS AND METHODS

2.1. Samples

Two hundred and forty five tissue specimens obtained from intestine 119, liver 68 and heart 58 of freshly died and diseased birds that showed signs of diarrhoea and lesions of coli-septicemia (colicystitis and air saculitis) were bacteriologically examined for the presence of *E. coli*. The examined samples were collected randomly from different farms and localities as 140 in El-Sharkia and 105 El-Dakahlia Governorates, Egypt.

2.2. Garlic extracts

Fresh garlic bulbs were purchased from a retail store. After its cleaning and sterilization, aqueous and ethanolic extracts were prepared according to the methods of Martha et al. (1998) and Gaherwal et al. (2014) respectively.

2.3. Isolation and identification of E. coli isolates:

Classical identification of *E. coli* depends mainly on its growth on MacConkey`s agar plates, streaking on Eosin Methylene Blue (EMB)medium and biochemical examination of its typical colonies by IMVC and TSI (Quinn et al., 2002).

2.4. Serogrouping of E. coli isolates.

Serogrouping of pathogenic *E. coli* isolates was carried out by slide agglutination method using specific polyvalent and monovalent sera of *E. coli* (DENKA SEIKEN CO., LTD., 3-4-2 Nihonbashikaya-cho, Chuo-ku, Tokyo, Japan) and was performed in the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt. (Finegold et al., 1978).

2.5. Antimicrobial susceptibility testing.

The susceptibility of *E. coli* isolates were determined by the standard disk diffusion method (Ortez, 2005) against eight antimicrobial agents including colistin (10µg), doxycycline (30µg), amoxicillin+clavulanic acid (30µg), ampicillin (10µg), gentamicin (10µg), spiramycine (100µg), ciprofloxacin (5µg), and sulfamethoxazole+trimethoprim (25µg) (Oxoid, 1998). By using Agar well-diffusion assay, the antibacterial activity of garlic aqueous extract was tested in different concentrations (100%, 50% and 25%) against 25 different isolated *E. coli* (previously showed a MDR by the disk diffusion) according to Srinivasan et al. (2009).

2.6. Determination of SICs of garlic.

The MIC as well as SIC of garlic extracts against *E. coli* isolates were determined using a modification of broth macrodilution method according to Kollanoor et al. (2010) **.** Duplicate 50-ml tubes containing 20 ml brain heart infusion broth (BHI) were separately inoculated with 4-5 × 10^6 CFU/ml of each *E. coli* strain. The bacteriological suspensions were distributed in eleven tubs (1ml each). Garlic extract was added with an increment of 0.1 mg/µl each from 0.1 to 1 mg/µl to the corresponding tubes and incubated at 37°C for 24 h. After the incubation, samples were drawn from each tube, diluted (1/10) in sterile PBS (pH 7.2) and plated on TSA plates. The highest concentration of garlic that did not inhibit the bacterial growth after 24 h of incubation was taken as the SIC.

2.7. Genotypic detection of the three common virulence genes in E. coli isolates

Specific primer sequences were used to amplify the three *E. coli* virulence genes such as increased serum survival (*iss*), verotoxine *(vt2*e) and intimin (*eae*A). Each strain of *E. coli* (either treated or untreated with garlic) was grown in BHI medium with or without SIC of garlic to mid-log phase at 37ºC (Table 1).

2.7.1. PCR amplification.

DNA was extracted by ABIO pure genomic DNA extraction kit (Metabion, Germany) according to the manufacturer instructions. PCR amplification was performed with a PTC-100 programmable thermal cycler in a final volume of 25 µl consisting of 12.5 µl of Dream*Taq* TM Green Master Mix (2X) (Fermentas, USA), 1µl of each primer,7 µl of template DNA and nuclease-free water up to 25 µl. Amplified PCR products were electrophoresed on 1.5% agarose gel in tris acetate EDTA and visualized by UV transilluminator. PCR amplification cycles for *E. coli* strains were illustrated in Table (1) as, initial denaturation step at 95˚C/5 min for all genes, and final denaturation step was at 72 ˚C /45 sec in both *eae*A and *vt2e* genes while at 72 ˚C /30 sec *in iss* gene.

Table (1): Oligonucleotide primer sequences and cycling protocols used for genotypic identification of *E. coli* virulence genes.

Target gene	Sequence 5'-3'	PCR amplification cycles	Product size (bp)	Reference
eaeA	F : GACCCGGCACAAGCATAAGC R: CCACCTGCAGCAACAAGAGG	94˚C45 sec/ 54˚C 45 sec./ 72˚C 45 sec. 35	384	Wen-Jie et al. (2008)
vt2e	F:CCAGAATGTCAGATAACTGGCAC R: GCTGAGCACTTTGTAACGGCTG	94˚C 45 sec/ 57˚C 45 sec/ 72˚C45 sec. 35	322	Orlandi et al. (2006)
Iss	F:ATGTTATTTCTGCCGCTG R:CTATTGTGAGCAATATC	94˚C 30 sec/.54˚C 30 sec./ 72˚C 30 sec. 35	266	Yaguchi et al. (2007)

2.7.2. Semi-quantitative reverse transcriptase real time PCR (RT-PCR)

Semi-quantitative RT-PCR was performed to study the effect of garlic SIC on the mRNA expression profile of three *E. coli* virulence genes after RNA isolation and complementary DNA (cDNA) synthesis. Total RNA extraction was performed according to manufacture instructions of QIAamp RNeasy Minikit (Qiagen, Valencia, cA). The RNA quantities were evaluated by measuring the optical density at wave lengthes of 230, 260 and 280nm, using Nanodrop technique (Technical Bulletin Nanodrop 2000/2000c-Thermo Fisher Spectrophotometer) (Rock Land DE, USA) (V1.0 User Manual). This enables the measurement of very highly concentrated samples without the need for dilutions.

Cycling conditions for SYBR green real time PCR of *eae*A, *vt2*e and *iss* genes were carried out as follows: reverse transcription was performed firstly for each of these genes at 50 ºC for 30 min (1 cycle) then, primary denaturation at 94 ºC /1min (1cycle) . While the amplification process was at 3 steps in 40 cycles: secondary denaturation at (94 ºC / 45 sec, 94 ºC / 30 sec and 94 ºC / 45 sec) annealing at (54 ºC /45 sec, 57ºC / 30 sec and 54 ºC /30 sec) and extension at (72 ºC /45 sec, 72 ºC/ 30 sec and 72 ºC/ 30 sec) for *eae*A, *vt2e* and *iss* respectively. Dissociation curve were generated by a cycle of (94 ºC \1min, 52 ºC /1 min and 95 ºC /30sec) for *vt2*e and (95 ºC / 1 min, 50 ºC / 1 min and 95 ºC /30 sec) for *iss* genes.

3. RESULTS

3.1. Recovery rate of E. coli isolates.

One hundred and thirteen (46.12%) of 245 isolates were identified as *E. coli* that were highly recorded in intestinal samples with a percentage of 64.60% (73/113) followed by liver 24.77% (28/113) then heart 10.6% (12/113), the recovery rate was 61.34%, 41.17% and 20.68% respectively from organs (/total organ No.) of freshly died and diseased chickens with the total rate of 67(47.85 %) in El-Sharkia and 46 (43.80 %) in El-Dakahlia Governorates.

3.2. Serogrouping of E. coli isolates.

Nine different serotypes were identified among the selected 14 *E. coli* isolates, and the most predominant one was O146 with a percentage of 21.42% followed by O157, O158 and O1 with the same percentage of 14.28% respectively (Table 2).

3.3. Garlic susceptibility pattern

It was noted that the *E. coli* strains showed high sensitivity against the aqueous garlic extract of concentration 100% represented by the widest inhibition zone (16-28 mm) followed by 50% (18-19 mm) and 25% (16-17 mm).

3.4. Garlic SIC against E. coli.

The tested *E. coli* cultures were inoculated with increment concentrations of crude garlic extract of 0.1 mg/µl each from 0.1-1 mg/µl. The results revealed that the SIC of garlic against *E. coli* isolate was 1% (0.01mg/µl), where no growth was shown in the tube after that. The initial average of *E. coli* population in the control (0mg/µl) and garlic treated samples (0.1mg/µl) was approximately the same about 5 Log10 CFU/ml. After 24hr. incubation at 37ºC approximately 8.0 Log10 CFU/ml of bacteria was recovered from control and treated samples. Thereby, confirming that a fore mentioned concentration of garlic was not inhibitory for *E. coli*.

3.5. Antimicrobial resistance phenotype of E. coli isolates.

The resistance profile of 50 *E. coli* isolates (El-Sharkia 37 and El-Dakahlia 13) revealed that, the highest resistance was for ampicillin (84%) followed by amoxicillin/ clavulanic acid (72%), sulfamethoxazole/trimethoprim and colistin that was 60% for each, then spiramycin (56%), gentamicin (32%), doxycycline (24%), while the lowest one was reported for ciprofloxacin as 16%. Multiple drug resistant strains were detected among most of the *E. coli* isolates and represented 68% (34/50).

3.6. Genotypic identification of the E. coli isolates

3.6.1. E. coli virulence genes before and after garlic treatment by PCR amplification

The obtained results revealed that *iss*, *vt*2e and *eae*A genes were detected in 10, 6, 5 of the 14 examined isolates (Table 2) at 384, 322 and 266 bp before garlic treatment and in 10, 4, 0 of the same isolates after garlic treatment respectively (Table 3 and Fig 1,2 & 3).

3.6.2. Effect of the SIC of garlic on expression of E. coli virulence genes by qRT-PCR in different E. coli isolates.

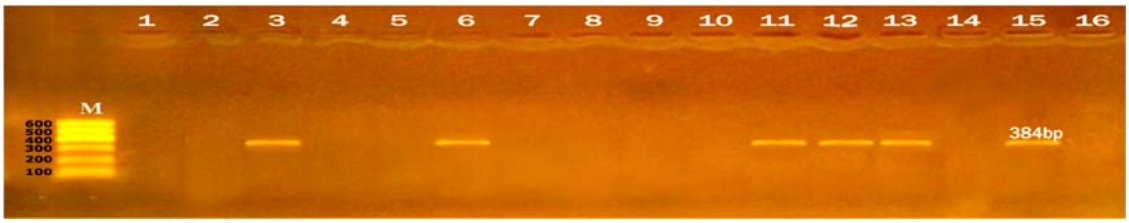
Table (2): Serogroupes and virulence genes of 14 *E. coli* strains of chicken origin.

Isolates Code No.	Serogroup	(%)	virulence genes		
			iss	*vt*2e	*eae*A
51H			-	+	+
57 H	O146	21.42	-	+	-
80 L			+	-	-
21 I	O158	14.28	+	-	-
62 I			-	-	+
8 I	O157	14.28	-	+	+
50 I			+	-	-
77 H	O1	14.28	+	-	-
83 L			+	+	-
3 I	O152	7.14	+	+	+
26 L	O15	7.14	+	-	+
37 H	O115	7.14	+	-	-
52 L	O18	7.14	+	+	-
97 L	O126	7.14	+	-	-

Table (3): Isolation percentage of *E. coli* virulence genes of chicken organs using conventional PCR

Virulence gene (s)	No. (%) of positive isolates and their virulence genes before garlic treatment				after garlic treatment			
	Positive Isolates No.(%)	gene No. (%) / intestine	liver	heart	Positive Isolates No.(%)	gene No. (%) / intestine	liver	heart
Iss	10/14 (71.42)	5\5 (100)	3\5 (60)	2\4 (50)	10/14 (71.42)	5\5 (100)	3\5 (60)	2 \4 (50)
*vt*2e	6/14 (42.85)	4\5 (80)	2\5 (40)	0 (0.0)	4/14 (28.57)	3\5 (60)	1\5 (20)	0 (0.0)
*eae*A	5/14 (35.71)	2\5 (40)	1\5 (20)	2\4 (50)	0/14 (0.00)	0 (0.0)	0 (0.0)	0 (0.0)
Sum	21	11	6	4	14	8	4	2

A)



B)

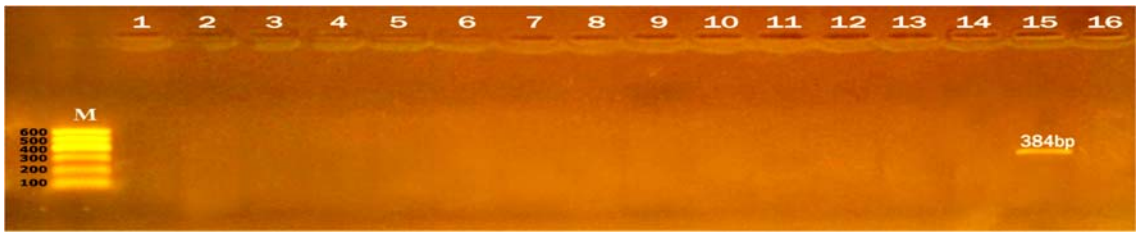
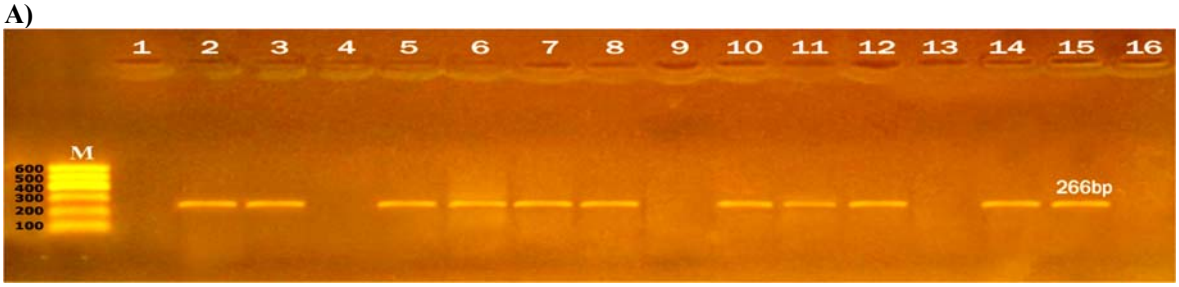


Fig (1) Agarose gel electrophoresis pattern showing typical amplification products of *eae*A virulence gene in uniplex PCR for the isolated fourteen *E. coli* strains. M: 100 bp DNA ladder "Marker". A) Before treatment, lanes 3 ,6, 11,12,13 showed amplification at 384 bp for 5 eaeA gene, B) After treatment, all lanes had no *eae*A gene. Lanes 15: control positive and lane16: control negative,

A)



B)

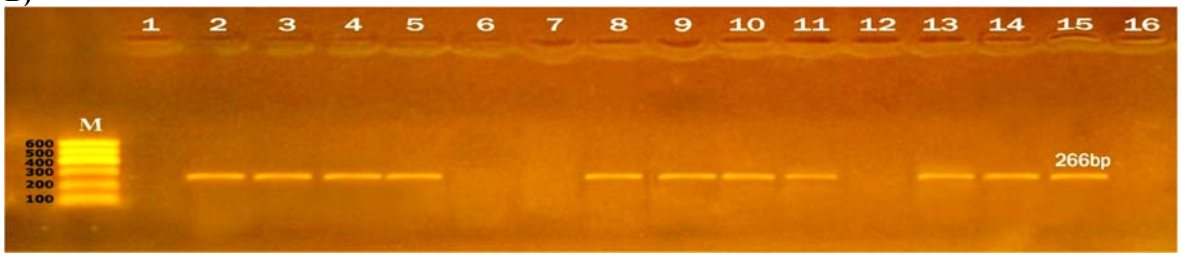


Fig (2) Agarose gel electrophoresis pattern showing typical amplification products of *iss* virulence gene in uniplex PCR for the isolated fourteen *E. coli* strains. M: 100 bp DNA ladder "Marker". A) Before treatment, lanes 2,3,5,6,7,8,10,11,12 and 14 showed amplification at 266 bp for *iss* gene, B) After treatment, lanes 2,3,4,5,8,9,10,11,13 and 14 of ten *E. coli* isolates had 266 band of *iss* virulence genes. Lanes 15: control positive and lane16: control negative,

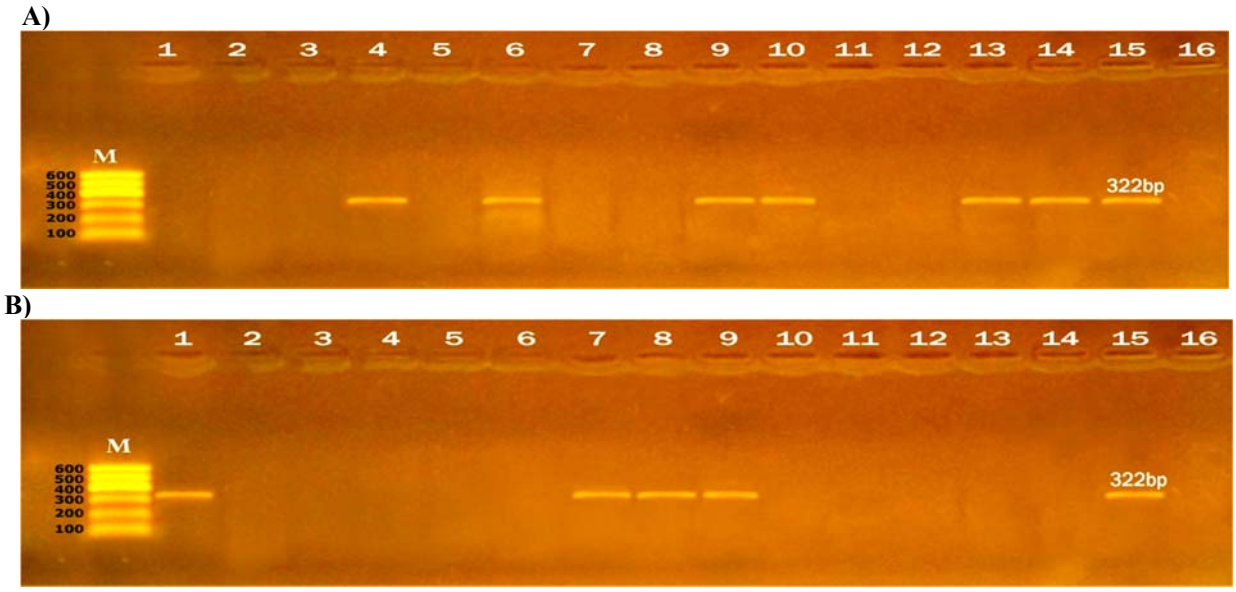


Fig (3) Agarose gel electrophoresis pattern showing typical amplification products of *vt2e* virulence gene in uniplex PCR for the isolated fourteen *E. coli* strains. M: 100 bp DNA ladder "Marker" A) Before treatment, lanes 4,6,9,10,13and 14 showed amplification at 322 bp for 6 *vt*2e gene, B) After treatment, lanes 1,7,8 and 9 of four *E. coli* isolates had 322 bp bands of *vt*2e virulence genes. Lanes 15: control positive and lane16: control negative,

Table (4): Data sheet of q RT-rtPCR showing *E. coli vt*2e (in two isolates) and *iss* genes, before and after garlic treatment.

Well Name	C_t	Tm Product
*vt*2e (isolate 8I) (untreated with garlic)	18.60	80.55
*vt*2e (isolate 8I) (treated with garlic)	19.42	80.55
*vt*2e (isolate 3I) (untreated with garlic)	21.66	80.55
*vt*2e (isolate 3I) (treated with garlic)	21.63	80.55
Iss (untreated with garlic)	14.36	79.72
Iss (treated with garlic)	14.42	79.72

$C_{(t)}$: Threshold cycle. Tm Product: the peak value of curve referring to second annealing temperature.

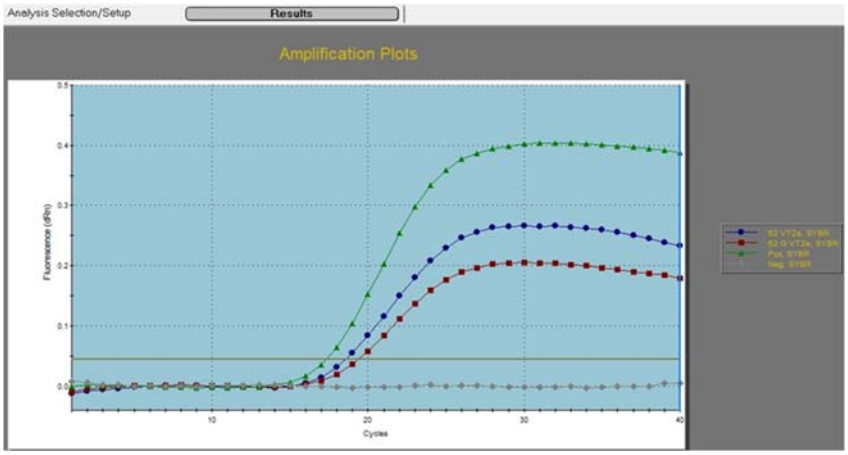


Fig (4A):

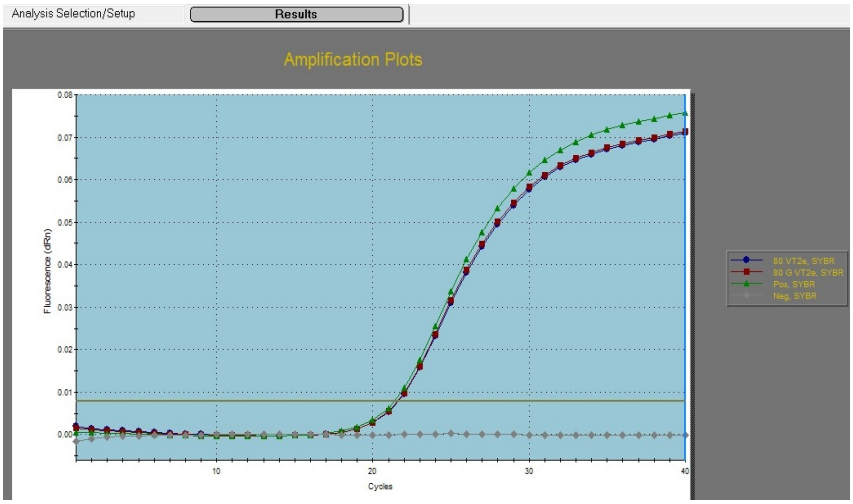


Fig (4B):

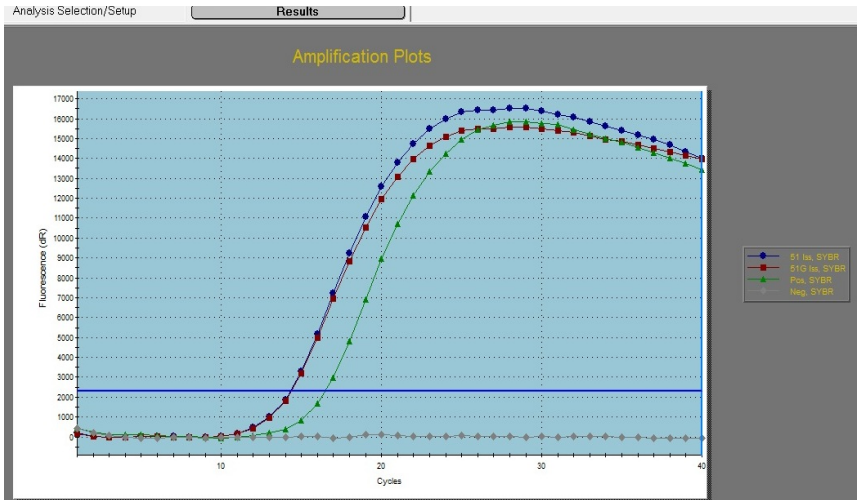


Fig (4C) A amplification curves of qRT-PCR for semi-quantitative detection of *E.coli vt*2e and *iss* genes expression before and after garlic treatment. A) *vt*2e (isolate 8I). B) *vt*2e (isolate 3I). C) *iss*.

Positive control Negative control

Sample untreated with garlic Sample treated with garlic

There was a clear difference in the amount of cDNA detected after gene expression assessment of *E. coli* strains before and after treatment with garlic extract. The absorbance of DNA in the standard samples was measured using ultra-violet spectrophotometer at 260 nm and the *vt*2e (isolate 8I) product was relatively quantitated as increased in samples untreated with garlic than those treated, at C_t value of 18.60 and 19.42, respectively.

The *vt*2e (isolate 3I) and *iss* products were relatively quantitated and revealed nearly the same result for the samples untreated and treated with garlic. The results of real time PCR of *vt2e* (8I), *vt2e* (3I) and *iss* are shown in Table (4) Fig 4 A, B and C respectively). From Table (4), the relative quantity estimation of *E. coli vt*2e in isolate (8I) proved that its concentration was reduced after garlic treatment (C_t value was increased) (Fig. 4A), which reflect the decreasing effect of garlic on mRNA expression of this gene. The above-mentioned data, proved that the garlic downregulated *E. coli* virulence genes expression.

4. DISCUSSION

Avian pathogenic *E. coli* (APEC) is responsible for extra intestinal diseases, called colibacillosis in avian species, which is considered as one of the principal causes of morbidity and mortality in poultry with high economic losses **(**Janßen et al., 2001**;** Omer et al., 2010**).** Regarding to the incidence of the infection with *E. coli* in specimens

of intestine, liver and heart of freshly died and diseased poultry that was 64.6%, 24. 7% and 10.6% respectively. These results were nearly similar to those detected by Sharada et al. (2010) and Wafaa (2012) who isolated *E. coli* from diseased chicken with total recovery rate 44.6% and 46,6% respectively. Higher incidence rates were previously recorded by Nashwa et al. (2010) and Shymaa (2013) who recorded *E. coli* with a percentage of 75% for each. On the other hand a lower incidence **(**15.8%) was detected by Momtaz et al. (2012)**.** Regarding to the incidence of the infection with *E. coli* in specimens of intestine, liver and heart of poultry that was 64.6%, 24. 7% and 10.6% respectively. In Egypt, a higher incidence of *E. coli* than that mentioned in the current research were documented previously in several studies in both liver and heart, (36% and 14.3%) **(**Heba et al., 2012**)**, (28.1% and 15%) **(**Ashraf et al., 2014**)** respectively and (20%) in heart only **(**Hassanin et al., 2014**).**

The 14 *E. coli* isolates recovered from broilers and layers suffering from diarrhoea were serotyped. The results showed that O146 was the most predominant serotype with a percentage of 21.4%, followed by O158, O157 and O1 (14.2%), the remained isolates were serotyped as O152, O115, O18, O15 and O126, one isolate for each serotype (7.1%). These results are in line with those recorded by Azucena et al. (2013) who stated that the most common *E. coli* serotype in avian colibacillosis was O146 serotype and Rezk et al. (2010) who detected *E. coli* O146 and O125 serogroups in diseased chickens collected from different localities in Ismailia Governorate. On the contrary to the currant results in Sharkia Province, the most obtained serotype was O78 from avian colibacillosis **(**Ammar et al., 2011**)** and O125 **(**Al-Ajimi, 2011**).** Moreover isolation of serogroup O157 is alarming that it was associated with outbreaks of sporadic cases of haemorrhagiccolites and haemolytic uremic syndrome in human **(**Gould et al., 2009**)** this serogroupe had been previously isolated from diseased chickens by Rezk et al. (2010) suggesting that poultry may act as a reservoir for *E. coli* and possess a zoonotic risk for human **(**Hussein et al., 2013**).** In fact, in avian species, O1 and O15 are reported to be common sources of colibacillosis in Italy and could be classified as avian pathogenic strains **(**Antonio et al., 2007**).** In our antimicrobial susceptibility test that was performed on 50 *E. coli* isolates, the results indicated that the isolates were resistant to ampicillin, amoxicillin/ clavulanic acid, colistin, sulphamethoxazole/ trimethoprime and spiramycin (84%, 72%, 60%, 60% and 56%, respectively) while they were susceptible to doxycycline, ciprofloxacin and gentamicin (60%, 50% and 44%, respectively). Similar results were detected by Salehi and Bonab (2006) who illustrated that *E. coli* isolates were sensitive to Ciprofloxacin followed by gentamicin. Olatoye et al. (2012) Ezzeldeen et al. (2013) and Fyre and Jackson (2013), showed that the tested *E. coli* samples were resistant to amoxicillin/clavulanic acid and ampicillin, while were sensitive to ciprofloxacin

The results of the present study are similar to those of garlic aqueous extract examined by Indu et al. (2006) Srinivasan et al. (2009) and Fahad et al. (2013) who mentioned that garlic aqueous extract was active against *E. coli.* with inhibition zone of 30 mm, 33 mm, and 26mm for the three researchers respectively. The inhibitory effect of garlic was found to be due to allicin **(**Ankri and Mirelman, 1999**)**. In contrary**,** Packia Lekshmi et al. (2015) mentioned that garlic aqueous extract had a little antibacterial activity against *E. coli*. The minimum inhibitory concentration of garlic aqueous extract was found to be (20mg/ ml) (0.02mg/µl). The current detected results were similar to that detected on ethanolic extract of garlic by Musa et al. (2013) who found that the MIC was 20 mg/ml. Lower incidences of garlic MIC against *E. coli* than that mentioned in the present study were documented previously in several studies for aqueous extract as 3.2 mg\ml **(**Iwalokun et al., 2004**).** Higher results were detected on other plants as that detected by Kollanoor-Johny et al. (2012) who found that the minimum inhibitory concentration of *trans*-cinnamaldehyde (TC) extract against Salmonella was (200mg\ ml).

From the results, it was noted that the PCR products of the obtained virulence genes of *E. coli* isolates from chicken gave characteristic bands at 266, 322 and 384bp for *iss*, *vt2* and *eaeA* virulence genes, respectively, and that *iss* gene was the most prevalent virulence gene (71.42%), so it represents an additional virulence marker playing a role in the pathogenesis of poultry colibacillosis. These results are in accordance to Orlandi et al. (2006) Yaguchi et al. (2007) and Wen-Jie et al. (2008)**.** Higher incidence of *iss* virulence gene, obtained by Ewers et al. (2004), and Moemen et al. (2014) as (82.7%) , (73.8%) and (72.2%) respectively and add that the *iss* gene (the anti-complement) one of the most important genes that help in pathogenisity and virulence of APEC and is the most dominant gene isolated from chicken suffered from diarrhoea **(**Soon-Gu et al., 2008**).**

After treatment of *E. coli* isolates with garlic extract the virulence genes expression gave different results by RT-rtPCR, showed that 10 isolates (71.42%) expressed *iss*, the same as before treatment, while 4 (28.6) of 6 expressed *vt2*, finally *eae*A cannot be expressed in all examined isolates which affirm that garlic extract can completely

inhibit or downregulate the expression of virulence genes on *E. coli* mRNA. The forementioned results go hand in hand with that of different plant extracts on other genus by Kollanoor-Johny et al. (2012) who found that *trans*-cinnamaldehyde (TC) and eugenol (EG) reduced the motility and invasive abilities of *S.* Enteritidis and downregulated expression of the motility genes (*flhC* and *motA)* and invasion genes *(hilA*, *hilD*, and *invF).* And on the same genus of *Escherchia* Dorota et al. (2012) who detected that the *Urtica dioica* extracts significantly reduced the motility of the *E. coli* rods and biofilm activity and other extracts of *Vaccinium vitis-idaea* decreased the bacterial survival and virulence factors involved in tissue colonization and biofilm formation of the uropathogenic *Escherichia coli*.

5. CONCLUSION

After treatment of the *E. coli* isolates with garlic aqueous extract SIC, the reverse transcriptase cPCR (RT-PCR) technique showed that the expression profile of *vt2*e virulence gene was decreased while the *eae*A gene was disappeared completely in the representative isolates. That may reflect the garlic efficacy on transcription of the *E. coli* virulence genes. And relative quantitative (semi-quantitative) reverse transcriptase real time PCR (qRT-PCR) analysis confirmed the excellent role of garlic (previously detected by RT-cPCR) focused on the inhibition and down-regulation of *E. coli* virulence genes expression mainly *eae*A and *vt*2e gene. Therefore, the garlic supplementation through feed can reduce infection by *E. coli* via decrescent their toxin production and may be the adhesion ability to the intestinal mucosa of the host either animal or human.

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