Molecular characterization of *Eschericha coli* isolated from raw milk samples with emphasis on their antibiotic resistance and virulence markers

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Abstract:

In the present study (10) *E.coli* strains previously isolated from (450) raw milk samples of different sources were analyzed for the determination of their antibiogram and screened for the presence of alkaline phosphatase gene (*pho*A) and virulence markers for Shiga toxin producing *E.coli* (STEC); *stx*1, *stx*2, and the occurrence of antibiotic resistance genes; nonspecific TEM β-Lactamase (*blaTEM*),Tetracycline A (*tet*A), which were investigated by Polymerase chain reaction. Ten *E.coli* strains were tested for the antibiotic resistance by using the AST card of the VITEK2. The antibiotic sensitivity test showed a high resistance towards Ampicillin (100%), Tetracycline (80%), Piperacillin(50%) and Chloramphenicol, Cephaexin and Sulfamethaxole-Trimethoprim (40%) for each and 30% of the isolates showed intermediate resistance toward Amoxicillin and Clavulinic acid. The polymerase chain reaction (PCR)was used to detect the genes encoding for alkaline phosphatase *(pho*A) , Shiga toxin type 1(*stx*1),Shiga toxin type 2(*stx*2) , Nonspecific TEM β-Lactamase (*blaTEM*),Tetracycline A (*tet*A).The results showed that all the investigated *E.coli* strains(n=10) harbored the alkaline phosphatase gene(*pho*A),100%.The Shiga toxin type one encoding gene(*stx*2) was found in one *E.coli* strain (O158), while none of *E.coli* strains has the Shiga toxin type 1 gene (*stx*1).One hundred percentage of the screened *E.coli* strains carried the *blaTEM gene*, while 80% of the strains harbored the *tet*A gene.

Keywords: *E.coli*, antibiogram, *pho*A gene, virulence genes, resistance genes

Introduction:

Echerichia coli is one of the microorganisms which present normally in the intestines of animals and human but its isolation from food materials may have risk on the health of human being due to the probability of the existence of enteropathogenic and toxigenic strains which result in

concern about its relation to the presence of resistant bacteria in humans ***(Wegener et al., 1999)***.

The commensals *Echerichia coli* are often applied as indicator bacteria for estimation and detection of the antibacterial resistance levels which exists in the society ***(Kijima-Tanaka et al., 2003)***.

The distribution of antimicrobials resistant bacteria in the environment relies on the occurrence and transmission of resistance genes among different microorganisms, mutations, and the selection pressure to maintain such genes in a certain population ***(Levy, 2001).***

So; the aim of the work is to study the molecular characterization of *Echerichia coli* previously isolated from raw milk and to determine some of their virulence and resistance genes.

Material and methods:

Antibiotic sensitivity test: *E.coli* strains (n=10) which have been isolated from milk obtained from a previous study as ; O1(two isolates),O6(two isolates),O55(one isolates),O86(one isolates),O128(two isolates) and O158(two isolates) were subjected to antibiotic sensitivity test by using the AST card of the VITEK 2 apparatus, which screened for the *E.coli* susceptibility towards various types of antibacterial agents named as; Ampicillin, Amoxyicilin-Clavulenic,Cefpodoxime,Ceftiofur, Cefpirome, Cefalexin, gastrointestinal disturbances or may be life threatening syndromes caused by some toxigenic strains or pathotypes, named as Shiga toxin producing *Echerichia coli* (STEC), also called verotoxin producing *Echerichia coli* which is identified as strains of *Echerichia coli* that give one member or more of a class of virulent cytotoxins named as the Shiga toxin. ***(Soomro et al.2002)***

The two main virulence determinants of STEC are the cytotoxins, Shiga toxins type one and two (encoded by the abbreviations; *stx*1 and *stx*2 genes respectively) ***(Solomakos et al., 2009).***

Earlier studies recorded the occurrence of *Echerichia coli* possessing virulence markers in untreated milk and its products in Egypt ***(Amal 2014*** and ***Ombarak et al., 2016)***.

E.coli infection like other infectious diseases caused by bacteria need antimicrobial therapy; either in veterinary field or in human medicine; but unfourtionally the efficacy of treatment by such antibiotics and antimicrobial agents have been diminished by the over use of certain drugs by many years or decades. The effects of antibacterial over usage are numerous. As it is one of the necessary applications in veterinary field; it is highly obvious that the antimicrobial agents are largely used either for prophylaxis or for treatment procedures, and their applications have an elevated

*pho*A gene, ***(Dipineto et al., 2006)***, for detection of *stx*1 and *stx*2 genes**,** ***(Colom et al., 2003*)** for detection of *blaTEM* gene and ***(Randall et al., 2004)*** for detection of *tet*A gene, as shown in table (1).

Temperature and time conditions of the primers during PCR are summarized in table (2) according to the specific authors and Emerald Amp GT PCR mastermix (Takara) kit.

Amplified gene products were verified by gel electrophoresis (1% agarose) for about 30 minutes and visualized under ultraviolet light, according to ***(Sambrook et al.1989)***, with modification.

The gel was photographed by a gel documentation system (Alpha Innotech) and the data was analyzed through computer software.

Marbofloxacin, Nitrofurantion, Tetracyciline, Trimethoprim-Sulfamethaxole and Chloramphenicol. *E.coli* isolates also tested for the presence of extended spectrum β-lactamase producers.

The minimum inhibitory concentration guideline was adjusted according to the Global ***CLSI-basis (2012)***.

Polymerase chain reaction (PCR):

E.coli strains were screened for the presence of alkaline phosphatase gene, virulence associated genes and antibiotic resistance genes by using the PCR technique. The PCR was standardized for the determination of *pho*A, *stx*1, *stx*2, *blaTEM*, *tetA* genes by following the methodology as described by ***Hu et al. (2011)***, for detection of

Table (1): *Oligonucleotide primers sequences Source: Midland Certified Reagent Company_ oilgos (USA).*

Gene	Primer Sequence 5'-3'	Amplified product	Reference
*tet*A(A)	(F)GGTTCACTCGAACGACGTCA	576 bp	**Randall *et al.* 2004**
	(R)CTGTCCGACAAGTTGCATGA		
blaTEM	(F)ATCAGCAATAAACCAGC	516 bp	**Colom *et al*., 2003**
	(R)CCCCGAAGAACGTTTTC		
stx1	(F)ACACTGGATGATCTCAGTGG	614 bp	**Dipineto *et al.*, 2006**
	(R)CTGAATCCCCCTCCATTATG		
stx2	(F)CCATGACAACGGACAGCAGTT	779 bp	
	(R)CCTGTCAACTGAGCAGCACTTTG		
*pho*A	(F)CGATTCTGGAAATGGCAAAAG	720 bp	**Hu *et al.*, 2011**
	(R)CGTGATCAGCGGTGACTATGAC		

Table (2): *Cycling conditions of the different primers during cPCR*

Gene	Initial denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
*tet*A(A)	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
blaTEM	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
*pho*A	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
*stx*1,2	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.

Detection of antibiotic resistance genes; *blaTEM* and *tetA*(A) in *E.coli* isolates: The distribution of antibiotic resistance genes in *E.coli* serogroups isolated from various milk samples revealed that; all *E.coli* strains examined for presence of *blaTEM* gene were positive(100%).; and those examined for *tetA* "except for two *E.coli* strains (O1,O158)" were negative for *tetA* (80%),(as shown in figures 2;3).

Detection of Shiga-toxins genes; *stx*1 and *stx*2 in *E.coli* isolates:

None of the investigated isolates reacted positively with the *stx*1 specific primers, while only one isolate (10%) had *stx*2 gene in its sequence (O158), (as shown in figure 4).

Results:

The results indicated that all *E.coli* isolates were negative to extended-spectrum β-lactamase (ESBL), the isolates recorded variable resistance to various types of antibiotics as presented in table (3).

On the other hand, all the *E.coli* isolates were sensitive to Cefpodoxime, Ceftiofur, Cefpirome, Imipenem, Amikacin, Gentamycin, Tobramycin, Enrofloxacin, Marbofloxacin and Nitrofurantoin, by a percentage of 100%.

Polymerase chain reaction (PCR) for *E.coli* isolates

PCR used for identification of *E.coli* by detection of *pho*A gene. The results revealed that all of the samples were *E.coli* positive for alkaline phosphatase gene (100%), (as shown in figure 1).

Table (3) *Antimicrobial resistance percentages of Escherichia coli isolates (n=10)*

Antimicrobial agent	Number of isolates	Resistance (%)
ESBL	10	100%
Ampicillin	10	100%
Tetracycline	8	80%
Piperacillin	5	50%
Cefalexin	4	40%
Chloramphenicol	4	40%
Sulfa/Trimethoprim	4	40%

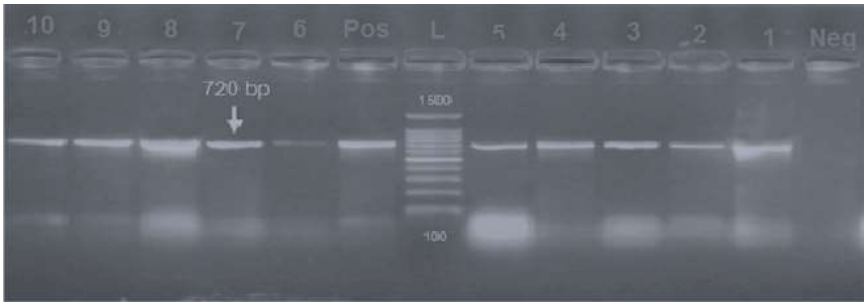

Figure (1): *Agarose gel electrophoresis of amplified phoA gene PCR product at (720bp), one-step ladder; 100 bp, Lane 1-10 represent the positive strains for phoA gene.*

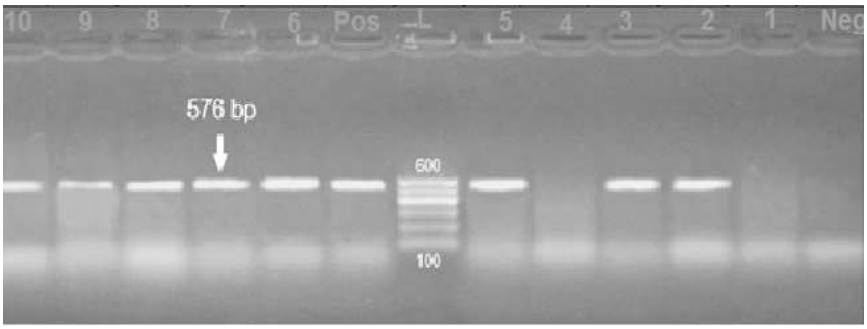

Figure (2) *Agarose gel electrophoresis of amplified tetA (A)gene PCR product at (576bp),1 and 4 lanes represent the negative lanes for tetA (A)gene, Lanes 2,3,6,7,8,9 and 10 are positive strains for tetA (A)gene.*

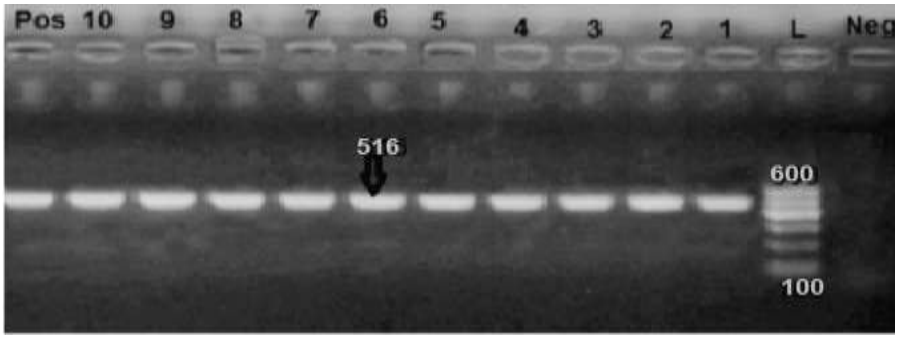

Figure (3) *Agarose gel electrophoresis of amplified blaTEM gene PCR product at (516bp), all Lanes (1to 10) are positive for blaTEM gene***.**

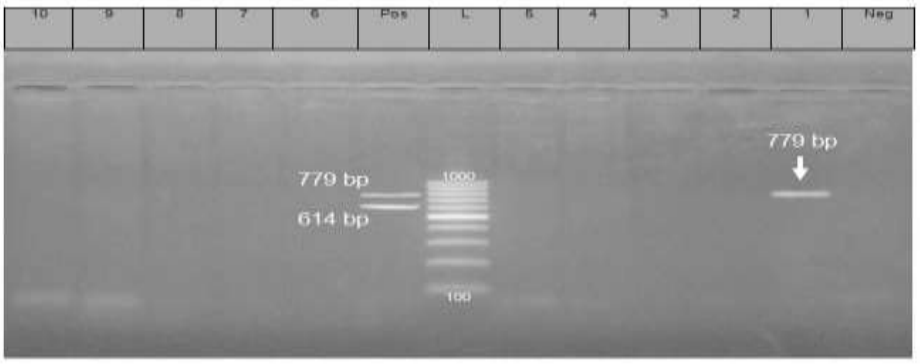

Figure (4) *Agarose gel electrophoresis of amplified stx1 gene and stx2 gene , PCR product at (779 bp) pointed the positive isolate of Stx2 at Lane 1,Lanes: 2-10 negative for stx2 gene, all Lanes are negative for stx1 gene(Lanes: 1-10).*

Antimicrobial susceptibility test was carried out for *E.coli* isolates from contaminated raw milk using the Vitek2 AST cards designed for Gram negative bacteria according to the manufacturer′s recommendations (The VITEK2 AES system).

A high antibiotic resistance was demonstrated for *E. coli* isolates to Ampicillin (100%), Tetracycilin (80%), Piperacillin(50%), Chloramphenicol (40%) and

Discussion:

Diseases caused by *E. coli* often require antimicrobial therapy; however, antibiotic-resistant strains of this bacterium cause longer and more severe illnesses than their antibiotic-susceptible counterparts ***(Dehkordi et al., 2014)****.*

Studies showed that the antibiotic resistance bacteria which present in the milk of infected animals can be transmitted to human by the ingestion of raw milk or milk products ***(Virpari et al., 2013).***

and molecular cloning ***(Suresh and Das, 2014).*** Alkaline phosphatase of *Escherichia coli* is considered the most studied prokaryotic alkaline phosphatase ***(Wanner, 1987).***

In the present study, all the *E.coli* isolates showed positivity for the alkaline phosphatase gene (*pho*A), with an incidence of 100%.

These results were totally come in agreement with the finding of ***Abdulgayeid et al. (2015)***, where PCR was conducted for identification of *E.coli* strains by detection of *pho*A gene and the results revealed that 100% of the samples were *E.coli* positive.

Nowadays, Shiga toxigenic *Escherichia coli* (STEC) strains are the most important emerging groups of foodborne pathogens and is often related to the consumption of contaminated food, uncooked beef, water and raw milk ***(Griffin and Tauxe ,1991 and Beutin et al., 2002).***

Earlier studies documented the presence of *E.coli* possessing virulence markers from raw milk and its products in Egypt ***(Amal, 2014 and Ombarak et al., 2016)*** and also, all over the world ***(Rey et al., 2006; Farhan et al., 2014*** **and** ***Nobili et al., 2016),*** this gives an indication for the wide distribution of *E.coli* with its virulence factors in milk and the significance of monitoring the STEC, which is potentially pathogenic for humans.

Therefore, the occurrence of *stx*1 (encoding for Shiga toxin type 1) and *stx*2 (encoding for Shiga toxin Trimethoprim\ Sulfamethoxazole (40%).

In the current study, all of the *E.coli* isolates were resistant to at least one of antimicrobial agent investigated, showed a different patterns of antibiotic resistance.

In this study, None of *E.coli* strains yielded the extended spectrum beta –lactamase (ESBL), which go in accordance with the results of (***Martin et al., 2002*** *and* ***Geser et al., 2012)*** .

The frequent type of resistance was found against Ampicillin and Tetracycline which go matching with the results of ***Baptista and Marin (2006)***.

Many researchers have recorded a high *E.coli* resistance against Tetracycline, (***Dehkordi et al., 2014****;* ***Abike et al., 2015*** and ***Mosolo 2016)***.This is may be due to the abuse usage of Tetracycline drug in the veterinary field.

Also an elevated Ampicillin resistance for *E.coli* strains has been estimated in many researches which conducted on dairy samples ***(Gundogan*** and ***Avic 2014***, ***Sarker 2014*** and ***Sharma et al., 2014***.*)*

Other researchers; ***(Haque et al., 2014*** *and* ***Sanusi et al., 2015)*** recorded that all *E.coli* isolates were completely resistant against Ampicillin, in other words; All *E.coli* isolates showed 100% resistance against Ampicillin, which is the same result recorded in the present study.

Alkaline phosphatases play a vital role in DNA sequencing analysis

antibiotic resistance genes often exist in *E.coli* strains and may play a significant role in getting of various types of antimicrobials resistance in food.

As a consequence of high antibiotic resistance observed in this study for *E.coli* isolates towards Ampicillin (100%) and Piperacillin(50%),and the minimum inhibitory concentration(MIC) of Amoxycillin and Clavulenic acid which indicated intermediate resistance for 30% of the isolates ; which belong to the betalactam group of antibiotics; The sequence of *blaTEM* gene was targeted in The *E.coli* isolates (n=10) by using PCR .

In the current study; all the investigated *E.coli* isolates for the occurrence of *blaTEM* encoding gene were positive by a percentage of 100%.

The same results were demonstrated by ***Timofte et al. (2014)***; who identified that all *E.coli* isolates from raw milk samples were found to be positive for the *blaTEM* gene, which was further identified to be *blaTEM*1.

Also; a high incidence of harboring the *blaTEM* gene in *E.coli* isolates from raw milk was reported by; ***Ahmed and Shimamoto (2011)*** and ***Koraney (2016)*** with a percentage of 60% and 76.4% respectively

Because of the high resistance estimated for the Tetracycline drug for the *E.coli* isolates in the current study represented by an eighty percentage, *tetA* encoding gene was type 2) were investigated using the PCR assays in *E.coli* strains isolated from milk samples, (n=10).

In the current study; there was only one strain which harbor the Shiga toxin type two genes in its sequence (O158) ;(as shown in figure 4).

A similar result was detected by ***Picozzi et al. (2005)*** who recorded the presence of Shiga toxin type two genes in 2 strains (20%).

The same as reported by ***Farhan et al. (2014),*** who demonstrated only two strains encoded for Shiga toxin type two genes by using multiplex PCR.

On the contrary to ***Amal (2014)*** study which screened for the presence of virulence associated genes(*stx*1and *stx*2) in *Escherichia coli* isolates, and demonstrated that only one isolate from farm 1 (bulk tank milk) encoded for the *stx*1.

On the other hand; None of the investigated *Escherichia coli* isolates in the present study revealed positivity in PCR for the Shiga toxin type one gene (*stx*1).These results agreed with the findings obtained by ***Nehar et al. (2015)*** which recorded the presence of *stx*2 gene sequence in three isolates from raw milk and milk products samples and the absence of any isolate which carry the *stx*1 gene sequence.

The genetic characterization of *E.coli* strains is carried out for the aid of better understanding of the antibiotic resistance profile of *E.coli* strains recovered from milk samples; by the determination of

the burden of antibiotic resistance genes which can transfer resistance to other pathogenic bacteria, beside failure of treatment strategies for human being who are consuming raw unpasteurized milk directly or indirectly by taking its products.

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chosen to be screened in the *E.coli* strains(n=10).

It was found that out of the ten *E.coli* strains screened for the possession of *tetA* gene; 8 *E.coli* strains carried the sequence of *tetA* encoding gene; with a percentage of 80%, with the exception of 2 strains; O1, O158 *E.coli* strains.

These results are matched with the finding of ***Lollai et al. (2005)***; where three strains out of ten *E.coli* strains were resistant to Tetracycline and were consequently screened for the presence of Tetracycline resistance genes; it was demonstrated that two strains out of three were harboring the *tetA* gene and that gene was the predominant among Tetracycline resistant genes investigated.

A higher percentage was recorded in ***Abdulgayeid et al. (2015)*** study; where the PCR teqnique was carried out for detection of antibiotic resistance genes; *tetA* and *sul1* in *E.coli* isolates; as the *tetA* gene was detected by 100%.

On the other hand, a varied incidence for the distribution of the *tetA* gene determinant among *E.coli* isolates were reported by; ***Dehkordi et al. (2014)***, ***Sanusi et al. (2015), Koraney (2016)*** *and* ***Ranjba et al. (2016)*** by percentages of 76%,40%,35.3% and 30% respectively.

Conclusion:

From previously mentioned results, it is concluded that the increasing alarm of Antibiotic resistance in *E.coli* isolates from raw milk and

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الملخص العربي

التوصيف الجزيئي للاشيريشيا كولاي المعزولة من اللبن الخام مع التركيز على عوامل الضراوة و العوامل المقاومة للمضادات الحيوية

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في الدراسة الحالية تم تحديد الحساسية لعدد من المضادات الحيوية مع تحديد وجود عدد من الجينات المحددة لعوامل الضراوة و الجينات المقاومة للمضادات الحيوية و ذلك لعدد 10 من معزولات الاشيريشيا كولاي و التي قد تم عزلها في دراسة سابقة من عينات ألبان خام.تم اجراء اختبار الحساسية ضد المضادات الحيوية باستخدام كارت الحساسية لجهاز الفيتك 2 ؛ و قد أسفرت النتائج عن وجود مقاومة عالية ضد مضاد الأمبسيلين (100%) ، التيتراسيكلين (80%) ، بيبراسيلين (50%) ، و مقاومة 40% لمضادات الكلورمفينيكول ، سيفاليكسين و السلفاميثاكسول ترايمثوبريم كما أظهر 30% من المعزولات مقاومة متوسطة لمضاد الاموكسيسيلين و الكلافيولينيك أسيد. و لقد تم استعمال تفاعل البلمرة المتسلسل لتعيين وجود الجينات االمحددة للالكالين فوسفاتاز و الشيجا توكسين من النوع 1 و الشيجا توكسين من النوع الثاني ، و البلاتيم جين و التيتراسيكلين من النوع أ ؛ حيث بينت النتائج أن جميع المعزولات (10) التي تم البحث عنها تحمل جين ألالكالين فوسفاتاز و البلاتيم جين بنسبة 100%، في حين وجد أن 80% من المعزولات تحمل جين التيتراسيكلين من النوع أ، كما تم العثور على جين شيجا توكسين من النوع الثاني في واحدة من المعزولات (10%) ، كما أنه لم يتم العثور على جين شيجا توكسين من النوع 1 في أي من المعزولات.