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Prevalence of Toxins and Antimicrobial Resistance among *E. coli* Isolated from Meat

Shaymaa H. Abdel-Rhman[1], Sara M. Khalifa[2], Khaled H. Abd El Galil[1], Rasha M. Barwa[1*]

[1]Microbiology and Immunology Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt
[2]Ministry of Health, Mansoura, Egypt
Email: [*]rasha2000@gmail.com

Abstract

Aim: The present study aims to evaluate the occurrence and characterize *E. coli* in meat and meat products marketed in Egypt based on their antimicrobial-resistance pattern and production of enterotoxins. Methods: A total of 250 meat samples, categorized as 80 fresh beef, 85 ground beef and 85 beef burger purchased from supermarkets and butchers' shops were used for isolation of *E. coli*. All isolates were screened for antimicrobial susceptibility. Plasmid profile analyses were done. Polymerase chain reactions were performed for detection of enterotoxin-encoding genes (*astA, eaeA, stx*1 and *stx*2). Results: Twenty-five samples were isolated and identified as *E. coli.* 14 isolates were multidrug resistant. Plasmids isolation from all isolates revealed that 76% of these isolates harbored plasmids. *astA* gene was amplified in 7 isolates (28%). Eight (32%) isolates harbored *eaeA* gene. However, none of the isolates harbored *stx*1 *or stx*2 genes. Analysis of multiple drug resistant isolates revealed a significant relation between multiple drug resistance and both *astA* and *eaeA*. Conclusion: The study confirmed the prevalence of enterotoxin genes (*astA* and *eaeA*) in *E. coli* isolated from meat product and the association between the presence of these genes and multiple drug resistant phenomena.

1. Introduction

Meat is a nutritive, protein-rich food which is very perishable and has a short shelf-life unless preservation methods are applied [1]. Shelf life and keeping of the meat quality are affected by a number of interrelated factors including holding temperature, which can cause deleterious changes in the quality attributes of meat. Spoil-

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age due to microbial growth is the most important factor in relation to maintaining quality of meat [2].

In most developing countries, fresh meat constitutes a considerable proportion of meat intake [1]. To avoid spoilage, it is either eaten cooked or processed into other forms. The main causative factor of such spoilage is associated with unavailability of necessary storage facilities and suitable ambient temperature that is usually prevalent in developing countries that are in tropical regions [3].

The Gram-negative bacterium, *Escherichia coli* is a major member of the bacterial microbiota of the environment and in the feces of many species of mammals and birds. *E. coli* constitutes 14% of all cultivable bacteria of the colon, and up to 10^{10} CFU *E. coli* can be detected in 1 g of feces [4].

Commensal *E. coli* strains are very important since they maintain the physiological milieu of the gut and support digestion as well as protect against enteric pathogens. Other *E. coli* strains harbor and express virulence genes that cause serious outbreaks of diarrhea (diarrheagenic *E. coli*) [5]. These virulence genes are often located on transmissible genetic elements and therefore can be transmitted to receptive *E. coli* recipient strains [6]. Cattle are considered the main reservoir of diarrheagenic *E. coli* especially enterohemorrhagic *E. coli* and an important source for contamination of human food supply.

E. coli can be transferred into meat products through various routes. The animal feces can be transferred on the carcass and hides; the equipment can be contaminated; personnel might not follow proper hygienic practices; airborne contamination, and rodents, insects, and other animals are all possible sources [7].

In several epidemiological studies, five categories of *E. coli* have been well associated with diarrhea. These categories are enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) which are mostly regarded as shiga toxin-producing *E. coli* (STEC) [8].

The virulence mechanisms that characterize the different categories of *E. coli* are genetically encoded by chromosomal, plasmid, and bacteriophage DNAs and are represented by various virulent genes. These genes include *eae* (attaching and effacing lesions), *ipaH* (enteroinvasive mechanism), the gene encoding enteroaggregative adherence, *bfpA* (localized adherence), the genes encoding heat-labile toxin (*LT*) and heat-stable toxin (*ST*), and *stx*1 and *stx*2 (Shiga toxins) [9].

The misuse of antibiotics has led to the emergence of antimicrobial resistance in multiple bacterial isolates [10]. Foods contaminated with antibiotic resistant bacteria could be a serious threat to public health because there is a great possibility that genes encoding antibiotic resistance determinants that are carried on mobile genetic elements may be transferred to other bacteria of human clinical significance [11]. Development of such resistant bacteria may establish a problem in treatment of acute infections in man and animals. The antimicrobial agents have an important value for devising therapeutic measures against bacterial infections. However, the frequency of R-factor and its transmissibility in *E. coli* is a serious problem in treating such infections. Thus, with great expansion of sheep meat industry, *E. coli* is considered a problem of economic concern to all stages of meat industry—from production to marketing to consumer health significance, to the clinicians—due to emergence of multiple drug resistant bacteria, and to veterinarians due to reservoirs of infection.

The present study aims to evaluate the occurrence and characterize *E. coli* in meat and meat products marketed in Egypt. This characterization was based on the ability of the isolates to produce enterotoxins, on the antibiotic susceptibilities in order to determine the presence of multiple drug resistant isolates. Furthermore, the relationship between the different origins of the isolates and the ability of these isolates to produce enterotoxins and their resistant pattern was also evaluated.

2. Materials and Methods

2.1. Meat Sample Collection

A total of 250 meat samples, categorized as 80 fresh beef samples besides 85 ground beef and 85 beef burger purchased from supermarkets and butchers' shops distributed in Egypt. Each individual meat sample was aseptically packaged into a polyethylene bag then marked and transferred in an icebox to the laboratory of Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University, wherein the bacterial isolation were done. Samples were stored at 4°C till further analysis.

2.2. Isolation of *E. coli*

Twenty five grams from each meat sample were separately blended for 1 min in a sterile food blender, with 225

ml of sterile tryptone soya broth. The resultant suspension was then incubated at 37˚C for 18 - 20 h. For isolation of *E. coli*, MacConkey agar (MCA) and Eosin-methylene blue (EMB) agar were used as differential and selective medium respectively, all Oxoid products. The MCA and EMB agar were incubated at 37˚C for 24 hr. Then each morphological consistent colony was purified and subjected to biochemical characterization according to Barrow and Felthman (1993) [12]. All the purified isolates were maintained in glycerol 30% at −80˚C.

2.3. Antimicrobial Susceptibility Testing

Susceptibility to antimicrobials was determined by the disc-diffusion method using Mueller-Hinton agar (Becton, Dickinson and company, USA) according to the Clinical and Laboratory Standards Institute (CLSI) 2011 [13]. Twelve antimicrobial discs were used including: ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefipeme (30 µg), imipenem (10 µg), erythromycin (15 µg), gentamicin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), norfloxacin (10 µg) and chloramphenicol (30 µg). All discs were supplied from Oxoid products, UK.

2.4. Molecular Studies

2.4.1. Preparation of Plasmid DNA Content of the Isolated Strains Using DNA-Spin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Inc.)

The plasmid contents of all *E. coli* isolates were prepared using DNA-spin™ plasmid DNA Purification kit cat. No. 17091 ISO9001 (iNtRON Biotechnology, Inc.), according to manufacturer instructions. Plasmid DNA was visualized by electrophoresis on horizontal gels containing 0.8% agarose stained with ethidium bromide and illuminated under UV translluminator and photographed.

2.4.2. Isolation of Chromosomal DNA from Bacterial Plate Cultures Using I-Genomic BYF DNA Extraction Mini Kit (iNtRON Biotecnology, Inc.)

The genomic DNA of all *E. coli* isolates was prepared using i-genomic BYF DNA Extraction Mini kit (Cat. No. 17361, iNtRON Biotecnology, Inc.), according to manufacturer instructions. Genomic DNA was visualized by electrophoresis on horizontal gels containing 0.8% agarose stained with ethidium bromide and illuminated under UV translluminator and photographed.

2.4.3. PCR Experiments

PCR experiments were done according to El Naggar *et al.*, 2011 [14]. The genomic DNA from every isolates of *E. coli* was used as template for PCR experiments. The sequences of the oligonucleotides primers used in this study are shown in **Table 1**. The PCR reaction was: 12.5 µl Dream Taq™ Green PCR Master Mix (2X) (Dream Taq™ DNA polymerase, optimized Dream Taq™ Green buffer, MgCl$_2$, dNTPS), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 1 µl of the DNA template and 9.5 µl of nuclease free water were added for a total 25 µl per reaction. The PCR conditions were started with initial denaturation step at 95˚C for 5 min, followed

Table 1. Oligonucleotide primers used to amplify the tested genes.

Gene name		Sequence	Annealing temperature	References
*stx*1	Fw	5'… ATAAATCGCCATTCGTTGACTAC	58˚C	This study
	Rv	5'…AGAACGCCCACTGAGATCATC		
*stx*2	Fw	5'…GGCACTGTCTGAAACTGCTCC	58˚C	This study
	Rv	5'…TCGCCAGTTATCTGACATTCTG		
eaeA	Fw	5'… GACCCGGCACAAGCATAAGC	60˚C	This study
	Rv	5'…CCACCTGCAGCAACAAGAGG		
astA	Fw	5'... GCCATCAACACAGTATATCC	58˚C	This study
	Rv	5'... GAGTGACGGCTTTGTAGTCC		

Fw: forward; Rv: reverse.

by 40 cycles of denaturation at 95°C for 30 second (s), annealing temperatures for each primer were listed in **Table 1** for 40 s, and extention at 72°C for 1 min and final extention at 72°C for 5 min. The generated amplicons were visualized by electrophoresis on 1.2% agarose gel stained with ethidium bromide and illuminated under UV translluminator and photographed.

2.5. Statistical Analysis

Statistical analysis was performed using the χ^2 test. Data was analyzed using the GraphPad Instate software package (version 3.05) according to the TukeyeKramer multiple-comparison test at a $p < 0.05$ for significance.

3. Results

3.1. Incidence of *E. coli* in Meat Types

A total of 25 *E. coli* were isolated and purified from 250 meat samples. The prevalence of *E. coli* isolates in tested samples of fresh beef, ground beef, and beef burger was 4, 7 and 14 respectively.

3.2. Antimicrobial Susceptibility of the Tested Organisms

In term of overall response of *E. coli* isolates to tested antimicrobials, ampicillin, amoxicillin/clavulanic acid, ceftriaxone and ceftazidime showed the least overall potency (4% - 24%). For gentamicin, ciprofloxacin, norfloxacin, chloramphenicol and erythromycin; the overall potency against *E. coli* isolates was (48% - 64%). While for imipenem, cefepime and tobramycin; the overall potency was around (80% - 100%) as indicated in **Table 2**. Multiple drug resistance (MDR) was demonstrated in 14 isolates (56%), showing simultaneous resistant to more than two classes of antibiotics).

The relation between the percentage of resistant isolates and the number of the antibiotics were shown in **Figure 1**. Out of 25 *E. coli* isolates, 15 isolate (60%) were resistant to 5 or more antibiotic but no isolate were resistant to all the 12 antimicrobials. In addition, no isolate exhibited complete sensitivity to all antimicrobials.

3.3. Plasmid Profile Analysis

The plasmid DNA profile **Figure 2** illustrated that fifteen isolates had similar plasmid band patterns, possessing a single plasmid with a size (23 kbp). Additionally, three isolates harbored two plasmids for each with sizes (23 kbp and 9.4 kbp). Another three isolates harbored 3 plasmid bands with sizes (23 kbp, 9.4 kbp and 6.5 kbp) for isolate No. 2; sizes (23 kbp, 9.4 kbp and nearly 5.4 kb) for isolate No.6 and sizes (23 kbp, 9.4 kbp and 2 kbp) for isolate No. 8. On the other hand, four isolates (No. 4, 12, 14 and 25) were devoid of plasmids.
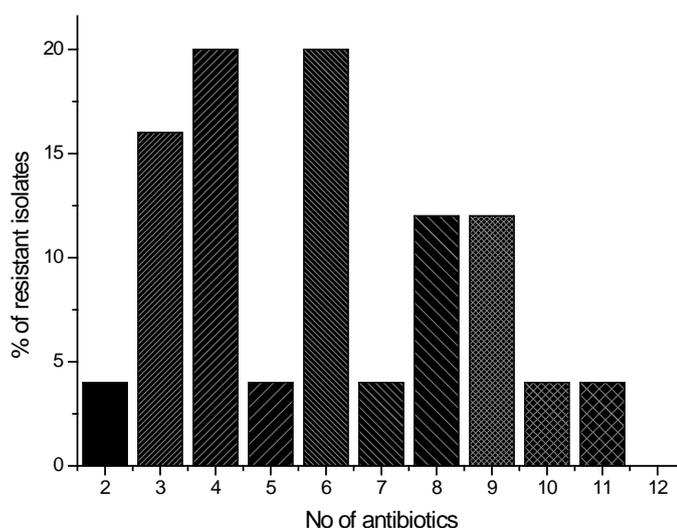
Figure 1. The relation between the percentage of resistant isolates and the number of the antibiotics.
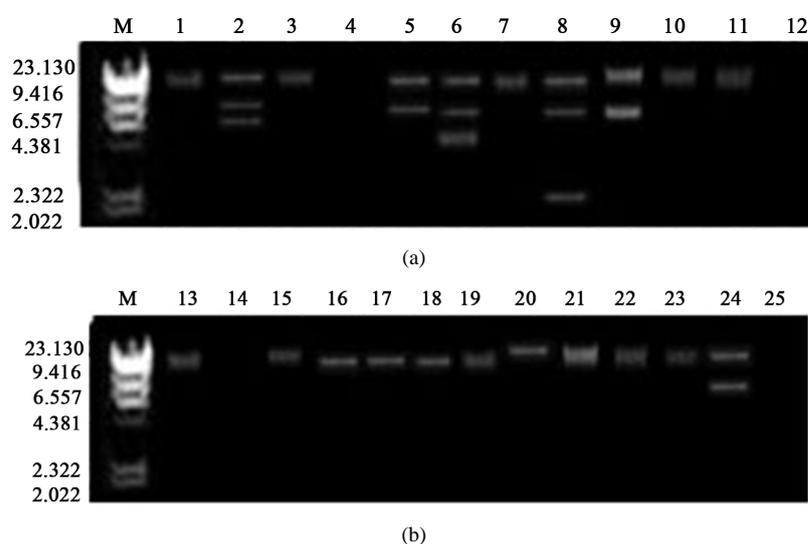
Figure 2. Plasmid DNA profiles of *E. coli* isolates. (a) Lanes 1-12 represent isolates No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 respectively. Lane M: λ Hind III marker; (b) Lanes 13-25 represent isolates No. 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 respectively. Lane M: λ Hind III marker.

Table 2. Antimicrobial susceptibility of *E. coli* isolates.

Antimicrobial agents	No. of *E. coli* isolates (n = 25)		
	R	**I**	**S**
Ampicillin	24	-	1
Amoxicillin/clavulanic	23	-	2
Ceftriaxone	22	-	3
Ceftazidime	19	4	2
Cefepime	1	1	23
Imipenem	-	-	25
Erythromycin	10	1	14
Gentamicin	13	-	12
Tobramycin	5	3	17
Ciprofloxacin	12	5	8
Norfloxacin	10	5	10
Chloramphenicol	9	7	9

S, Sensitive; I, Intermediate; R, Resistant.

3.4. Polymerase Chain Reaction for Amplification of Enterotoxin-Encoding Genes on Genomic DNA of *E. coli*

PCR of heat-stable enterotoxin gene (*astA*) reveled that it was harbored by chromosomal DNA of seven isolates (28%) with amplicon size of 390 bp **Figure 3**. With regard to *stx*1 *and stx*2 genes, none of the *E. coli* isolates possessed these genes. However, *eaeA* gene was presented in 8 isolates (25%) with size 550 bp **Figure 4**. The distribution of *eaeA* and *astA* gene among *E. coli* isolates with regard to their isolation source was shown in **Table 3**. Estimation of MDR phenomena among *E. coli* isolates revealed that 100% of *astA* harbored *E. coli* isolates and 100% of *eaeA* harbored *E. coli* isolates were MDR **Figure 5**. There was significant relation between the presence of both *astA* and *eaeA* and multiple drug resistance phenomena ($p < 0.0001$).
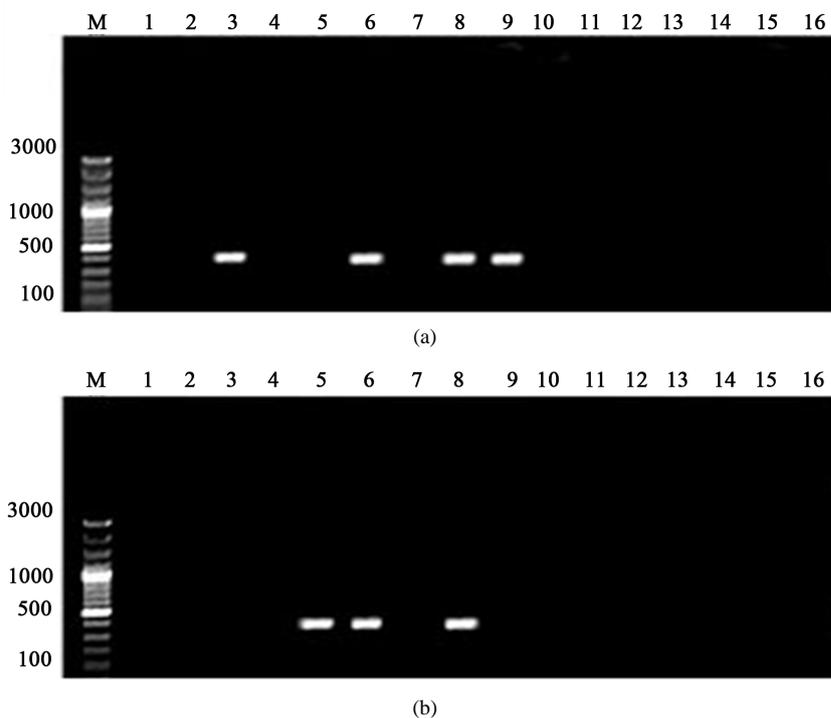
Figure 3. Agarose gel electrophoresis of *astA* gene amplicone from genomic DNA of *E. coli*. Lane M was 100 bp plus DNA ladder. (a) Lanes from 1 to 16 were amplicones from isolates No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 respectively; (b) Lanes from 1 to 9 were amplicones from isolates No. 17, 18, 19, 20, 21, 22, 23, 24 and 25 respectively. Lane 10 was negative control.
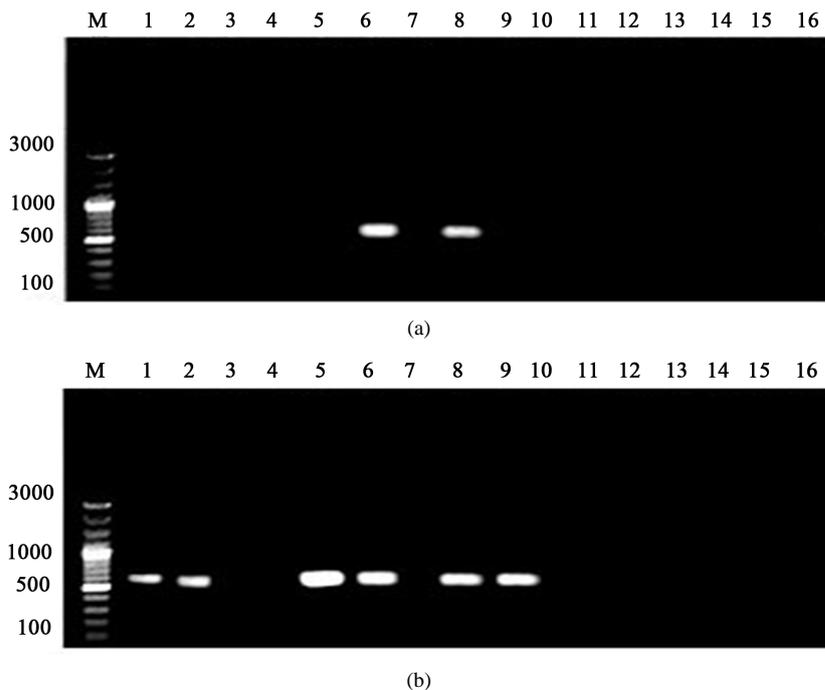
Figure 4. Agarose gel electrophoresis of *eaeA* gene amplicone from genomic DNA of *E. coli*. Lane M was 100 bp plus DNA ladder. (a) Lanes from 1 to 16 were amplicones from isolates No. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 respectively; (b) Lanes from 1 to 9 were amplicones isolates No. 17, 18, 19, 20, 21, 22, 23, 24 and 25 respectively. Lane 10 was negative control.
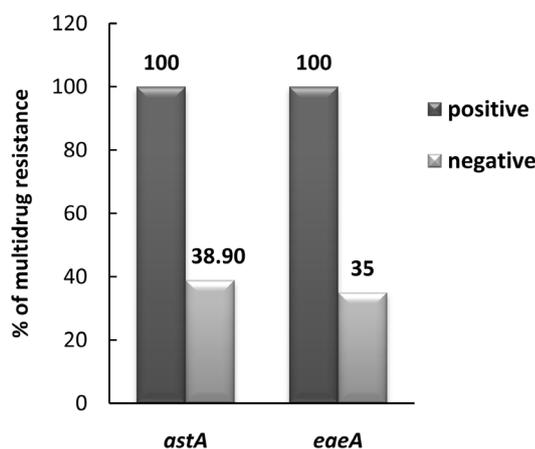
Figure 5. Correlation between the presence of *astA* and *eaeA* gene and multiple drug resistance.

Table 3. Prevalence of enterotoxins *astA* and *eaeA* in *E. coli* isolates from different meat samples.

Gene name	Isolation source			Total (n = 25)
	Fresh beef from butcher's shop (n = 3)	Ground beef (n = 7)	Beef burger (n = 15)	
eaeA	1 (33.3%)	1(14.28%)	1 (6.66%)	3 (12%)
astA	2 (66.6%)	- (0%)	- (0%)	2 (8%)
eaeA + *astA*	- (0%)	2 (28.5%)	3 (20%)	5 (20%)

4. Discussion

The prevalence of *E. coli* isolates in tested samples of fresh beef, ground beef, and beef burger was 16%, 28% and 56% respectively. It is noteworthy that, amongst the meat products tested, fresh beef was considered the lowest contaminated product with *E. coli*, followed by ground beef whilst beef burger was the highly contaminated product which indicated that more than half of the tested samples positive for *E. coli* were isolated from beef burger. The relatively higher incidence of *E. coli* strains in tested beef burger and ground beef samples may be attributed to the fact that these two products are manufactured from imported frozen meat which undergo subsequent processing as grinding and addition of non-meat ingredients, along with different hygienic measures and sanitation levels during processing, packaging, handling, distribution and storage [15].

Food is a leading factor for the transfer of antibiotic resistances. This transfer can occur by means of antibiotic residues in food, through the transfer of resistant food-borne pathogens or due to the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms [16]. In addition to the misuse of antibiotics, the rate of resistance to these antimicrobial agents in developing countries is high which attributed to the use of these drugs in gastroenteritis and self medication which are often the causes of resistance. While the causes of resistance in developed countries may be due to, the use of these antibiotics in animal feeds and by traveling to developing countries [17].

Resistance pattern of the tested isolates were determined according to CLSI, 2011 [13] using disc diffusion method. The results showed significant multiple resistances. As shown in **Table 2** most of *E. coli* isolates showed high resistance to ampicillin and amoxicillin-clavulanic acid (96%, 92%) respectively. The results showed that the resistance of *E. coli* isolates to ciprofloxacin, norfloxacin and chloramphenicol were (48%), (40%) and (36%) respectively. Ten isolates (40%) showed resistance to erythromycin. For aminoglycosides antibiotics, it was found that thirteen isolates (52%) were resistant to gentamicin, while for tobramycin only five isolates (20%) were resistant. On the other hand (100%) of isolates were sensitive to imipenem.

Zhao *et al.* (2009) [18] reported that resistance rate of *E. coli* isolates to ampicillin was greater than 90%. Motina and Yadava (2005) [19] observed that 41.37% *E. coli* isolates were resistant to norfloxacin. Our results were in accordance with a study conducted in Egypt by El Naggar *et al.* [14] which showed that 96.7% of *E. coli* isolates were resistant to cefepime, 40% to chloramphenicol, 53.3% to gentamicin, 60% to ciprofloxacin; while

all isolates were sensitive to imipenem. While a study in Spain conducted by Álvarez-Fernández *et al.*, 2013 [20] showing the rates of resistance of *E. coli* isolates was (75.0%) to ampicillin, (73.3%) ciprofloxacin and (45%) to amoxicillin-clavulanic acid.

According to **Figure 1**, 36% of the isolates were resistant to seven or more antimicrobial. A lower percent (26.6 %) was found in the study of Yadav *et al.*, 2007 [21].

On the overall isolates; 56 % of them showed multidrug resistance MDR. The MDR isolates had a general drug resistance pattern of AMP-AMC-CRO-CAZ. A lower percent of multiple drug resistance was found in the study of Suojala *et al.*, 2011 [22] where only 20.1% of the *E. coli* isolates were reported as MDR. Van *et al.*, 2008 [23] has revealed that *E. coli* in raw foods is a significant reservoir of resistance and virulence genes. Multi-resistance observed in beef was (15%).

Multidrug-resistant *E. coli* is an emerging clinical challenge in domestic species. Treatment options in many cases are limited [24]. Our findings demonstrate significant MDR *E. coli* isolated from meat (56%).The anti-biogram phenotype of *E. coli* isolates shows that treatment options are limited. It seems that imipenem and cefepime can serve as a medication of choice for treatment infections caused by multidrug resistant *E. coli*. However, it should be noted that imipenem has some drawbacks with fluid restricted patients and unlimited use of imipenem and cefepime can gradually lead to rising antibiotic resistance.

MDR in microorganisms was known to develop as a result of antibiotics misuse or acquisition of transferrable plasmids [25]. In this study, plasmids isolation from all isolates revealed that 84% of *E. coli* isolates harbored plasmids (**Figure 2**). Of them 60% exhibited similar plasmid band size 23 kbp, 12% have 2 plasmids with sizes (23 kbp and 9.4 kbp) and 12% have 3 plasmids with sizes (23 kbp, 9.4 kbp) and the third plasmid with size of (6.5, nearly 5.4 or 2 kbp). While 16% have no plasmids. Mobile plasmids are usually less than 10 kb in size while conjugative ones are sized between 20 and 30 kb [26]. Conversely the heavy weight plasmids (23 kbp) detected in our MDR strains of *E. coli* may be conjugative therefore their resistance determinants may have been acquired by horizontal transfer from species, while the other light plasmids (2, 5.4, 6.55 and 9.4) are mobile plasmids.

Plasmid fingerprinting was the first molecular method has been used for demonstrating the similarity of clinical isolates of bacteria in epidemiological studies. Isolates from the same strain contain the same number of plasmids with the same molecular weights and generally the same phenotype. Origin of isolates was indicated more accurately by plasmid fingerprinting [27]. In fact, plasmid profiles of the isolates are generally a useful tool for obtaining knowledge about enterotoxin production, resistance to antimicrobials and transfer of a plasmid among closely related isolates from different sources. Plasmid profile is one of several useful methods for determining the relatedness and unrelatedness of bacterial strains that contain plasmid DNA.

PCR analysis for enterotoxin genes (*stx*1, *stx*2, *astA* and *eaeA*) revealed that enteroaggregative heat-stable enterotoxin gene (*astA*) is harbored by 28% *E. coli* isolates with band size 390 bp (**Figure 3**). Of these isolates; 42.5% were isolated from beef burger (No.8, 9 and 24), 28.5% isolated from ground beef (No. 6 and 22) and 28.5% isolated from fresh beef (No. 3, 21). Similar results achieved by Sousa and Dubreuil, 2001 [28] where the 358 bacterial strains studied using PCR showed that the *astA* gene was present 71 *E. coli* isolates (32.6%). Zahfer, 2013 [29] found that (*astA*) gene was predominant over the other detected enterotoxin genes; A higher percent was noticed in the study of Vu Khac *et al.*, 2006 [30] where 64% of the isolates harbor *astA* gene.

With regard to *stx*1 and *stx*2 genes, none of the *E. coli* isolates possessed these genes. Our results are in accordance with Shabana *et al.* (2013) [31] where all *E. coli* isolates were negative for the presence of shiga-toxin2 (*stx*2). Also these results are in accordance with Van *et al.* (2008) [23] where in this case, the *stx1* and *stx2* virulence genes associated with the STEC pathotype were not detected.

Regarding *eaeA* gene, it is harbored by 8 strains of *E. coli* isolates (32%) with band size 550 bp (**Figure 4**). Among them, 4 isolates (50%) were beef burger isolates (No. 2, 8, 9, and 24), three isolates (37.5%) were ground beef isolates (No. 5, 6 and 22), only one fresh beef isolate (12.5%). A lower percent were introduced by Mohmmed *et al.* (2014) [32] where PCR was performed for categorizing diarrheagenic *E. coli* other than STEC. It was found that 12% were designated as EPEC based on amplification of *eae*A gene. Gallien *et al.* (1999) [33] reported different results were found for strains isolated from minced meat in Germany. None of the 36 further characterized strains harbored the *eaeA* gene; however, 25% *astA* positive.

The correlation between the presence of enterotoxin genes and MDR phenomena among *E. coli* isolates was studied (**Figure 5**). It was found that 100% of *astA* harbored *E. coli* isolates were MDR while only 38.9% of *astA* negative isolates were multidrug resistant. With regard to *eaeA* gene 100% of *eaeA* harbored *E. coli* isolates

were multidrug resistant while only 35% of *eaeA* negative isolates were multidrug resistant. Statistical analysis revealed that there was significant relation between the presence of both *astA and eaeA* and multiple drug resistance phenomena ($p < 0.0001$). Multidrug-resistant *E. coli* is an emerging clinical challenge in domestic species. Our results demonstrate the high prevalence of enteroaggregative heat-stable enterotoxin (*astA*) and intimin (*eaeA*) gene in *E. coli* isolated from meat product which may be involved in food poisoning. In the study of Mohammad, 2012 [9] the presence of *astA* by was investigated PCR, (46.7%) of the EAEC isolates harbored the *astA* gene and multidrug resistance was observed in high levels.

5. Conclusions

In conclusion our investigation highlights that the presence of enterotoxigenic and multiple antimicrobial resistant strains of *E. coli* in Egyptian meat products may constitute high potential risk for consumers in the absence of strict hygienic and preventative measures to avoid the presence of resistant bacterial isolates and enterotoxin production in foods. The presence of multidrug resistant isolates causes alarm and underlines the necessity as to a careful choice and a cautious use of antibiotics in *E. coli* treatment.

The use of innovative techniques for the identification of the genes coding for the production of enterotoxin would enable the identification of strains that carry genes that might in suitable conditions produce as yet unknown toxins potentially capable of producing sickness in humans.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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