Benha Veterinary Medical Journal 47 (2024) 124-128

Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Molecular identification of antibiotic resistance genes in *E. coli* isolated from dairy cattle

Alaeldin Hussein Mustapha[1] , Wafaa F. Omara[2], Hanaa F. Salama[3]

[1] *Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt.*
[2] *Microbiology Department, Shebin El-Kom Lab., Animal Health Research Institute (AHRI), Agriculture Research Centre (ARC), Egypt.*
[3]*Food Hygiene Department. Damanhur Lab., Animal Health Research Institute (AHRI), Agriculture Research Centre (ARC), Egypt.*

ARTICLE INFO

Keywords

E. coli

Cattle

VITEK

Resistance genes

Received 02/07/2024
Accepted 23/07/2024
Available On-Line
01/10/2024

ABSTRACT

Escherichia coli O157 is a leading cause of sub-clinical mastitis in dairy cows and is implicated in antibiotic resistance. So, this study aimed to isolate and characterize the phenotypic properties of *E. coli* O157 from apparently normal cow 's milk (150 milk samples) and then investigate their antibiotic resistance patterns and genes-related resistance using polymerase chain reaction (PCR). The biochemical evaluation showed that the isolates were positive for Indole, Methyl red, Nitrate reduction, Lactose, and Arabinose sugar fermentation tests, while Voges-Proskuaer, Citrate utilization, Urease, and H2S tests were negative and these results were confirmed by using VITEK® 2 compact. Of the milk samples that appeared normal, only 28 (19%) were positive for *E.coli*, whereas 122 (81%) of the samples were negative. Serological characterization revealed that out of 28 *E. coli* strains, 8 strains were containing O157. The antimicrobial susceptibility test indicated that the *E. coli* were highly resistant to gentamicin, ciprofloxacin and tetracycline (100%), ampicillin, cephalothin, kanamycin, nalidic acid and norfloxacin **(**87.5%), Trimethoprim/ sulfamethoxazole (75%) while erythromycin was (50%)**.** Furthermore, molecular characterization of the *bla$_{TEM}$* (Beta-lactams) gene at 516bp revealed that 7 of the isolates of *E. coli* O157 strains carried the Beta-lactams gene (516bp) while one isolate was negative. All isolates contained *tet*O (Tetracyclines) and *Aada1* (Aminoglycosides) genes that were identified at 559bp and 484bp, respectively. It can be concluded that the wide distribution of multidrug resistance O157. Furthermore, continued surveillance of *E. coli* isolates should be conducted on dairy cows to detect subclinical mastitis.

1. INTRODUCTION

Mastitis is the most critical causing bad quality milk since it is a complex disease that is divided into two distinct forms: subclinical mastitis, which is without symptoms, and clinical mastitis both of which have serious consequences for dairy herds (Oliver and Murinda, 2011). Thus, mastitis is possibly the most commercially significant disease of dairy cattle. Fecal pollution may cause *E. coli* colonization in mammary glands. This is notably evident during early breastfeeding due to a lack of neutrophil numbers and function (Güler and Gündüz, 2007).

The optimal growth of *E. coli* occurs at 37°C (98°F) but some laboratory strains can multiply at temperatures of up to 49°C. It takes as little as 20 min to reproduce in favorable conditions (Basavaraju and Gunashree, 2022) it also, commonly inhabits the environment, foods, and warm-blooded animals' lower gut (Campbell and Reec, 2002).

Consumption of unpasteurized milk and dairy products is very common and popular this underlines the potential role of milk as a vehicle for transferring the bacterium to the human food chain. In addition to cattle feces, other various sources of Shiga toxin-producing *E. coli* (STEC) contamination include the contaminated environment, water, equipment, infected workers or unhygienic handling, and marketing of milk constitute (Ahmadi et al., 2020).

Milk becomes contaminated with *E. coli* O157 when it comes into direct touch with excrement due to poor handling procedures (Bélanger et al., 2011). The high prevalence of *E. coli* O157 in milk could be due to poor milking hygiene and a lack of teat dipping after milking. Furthermore, its prevalence was high in dairy farms with no obvious farm therapy (Constable et al., 2016).

The VITEK 2 system (BioMérieux) is a new automated bacterial identification and susceptibility testing system that uses fluorescence-based technology. Previous studies showed that this system could give reliable identification and susceptibility results with pure bacterial cultures (Duraye et al., 2024)

Antibiotic-resistant *E. coli* strains from human clinical specimens and dairy cow mastitis are becoming increasingly common, causing global public health concerns (Copur-Cicek et al., 2014). There is compelling scientific evidence relating resistance development in *E. coli* strains generated from human clinical samples to those obtained from animals (Walther et al., 2018).

The emerging multidrug-resistant *E. coli* is considered a public health threat. The antimicrobial resistance in *E. coli* is mainly attributed to the Extended-Spectrum Beta-Lactamases (ESBLs); which could destroy various β-lactam antimicrobial agents such as penicillins, various generations of cephalosporins, and carbapenems (Magiorakos et al. 2012). ESBLs are encoded by specific ESBL genes such as; *bla*$_{TEM}$ (encoded for penicillins-resistance), *bla*$_{KPC}$ (encoded for carbapenems-resistance), and *bla*$_{CTX}$ (encoded for cephalosporins-resistance) (Enany et al., 2019; Eid et al., 2019; Algammal et al., 2020)

Therefore, the current study aimed to isolate *E. coli* O157 from seemingly normal milk samples and characterize its

* Correspondence to: wafaaomara83@gmail.com

antimicrobial resistance in subclinical mastitic cow's milk samples.

2. MATERIAL AND METHODS

2.1. Sample Collection

From August 2021 until the end of 2022, 150 sub-clinically mastitic milk samples have been collected in equal parts over the year in Menoufia governorate, Egypt. The samples were diagnosed using the CMT (California Mastitis Test). After sanitizing the udder, discard the first milk before milk samples collection, and then about five ml of milk was collected in sterilized glass vials (Quinn et al., 1999). The samples were kept refrigerated until they were delivered to the microbiology laboratory at the Shebin El-kom branch of the Animal Health Research Institute, Menoufia governorate, Egypt, within two hours.

2.2. Screening for Enteropathogenic E. coli

2.2.1. Enrichment

MacConkey broth tubes (HIMEDIA, M1125 supplemented with reversed Durham's tubes. Inoculated tubes were incubated at 37°C for 24 hours. One milliliter of a positive MacConkey tube was transferred into another MacConkey broth tube and incubated at 44°C for 24 hrs. Loopfuls of positive MacConkey broth tubes were streaked on Eosin Methylene Blue agar medium (EMB) (HIMEDIA, M317) and incubated at 37°C for 24 hours. The putative colonies were metallic green in color. The suspected colonies were purified and placed in slope nutrient agar tubes (Oxoid, 75312) for further study (ISO 16649-2, 2001).

2.2.2. Identification of Enteropathogenic E. coli

Tested morphologically as Gram-negative bacilli then the purified isolates were tested biochemically by sugar fermentation tests, indol, methyl red, Voges-Proskaur, citrate utilization, H2S production, urease, catalase, oxidase, and nitrate reduction tests according to Quinn et al. (2002).

2.2.2.5. Bacterial isolates characterization was performed with the VITEK®2 compact

According to the company's instructions (Biomeriux, 2006). The turbidity was adjusted to the equivalent of 0.5-0.6 McFarland turbidity. Analysis was done using Gram-negative bacterial identification cards.

2.2.3. Serological identification of E. coli

The isolates were serologically identified for O antigen according to Kok et al. (1996) by using rapid diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types at the animal health research institute, Cairo**.**

2.2.4. Antimicrobial sensitivity testing according to Patel et al. (2015)

The Kirby-Bauer diffusion technique using disks for Ampicillin, Cephalothin, Gentamicin, Kanamycin, Tetracycline, Erythromycin, Nalidic acid, Ciprofloxacin, Norfloxacin, Trimethoprim/sulfamethoxazole on Mueller-Hinton agar (Sigma-Aldrich, USA) was used to assess antimicrobial sensitivity.

2.2.5. DNA Extraction and Molecular Identification of E. coli.

Using the QIAamp DNA Mini Kit (catalog number 51304). The QIAamp DNA Mini Kit purifies nucleic acids from a variety of sources using a silica membrane. Because the spin-column process eliminates the need for mechanical homogenization, the total hands-on preparation time is only 20 minutes. The studied virulence genes (*bla*TEM, *Tet*O, and *aada*1) in Table (1) were detected by PCR (Biometra, Germany), with amplified products at 516, 559, and 484 bp, respectively, as previously described (Sambrook et al., 1989). Fermentas (USA) furnished a 100-bp DNA ladder (Cat. No. SM0243).

3. RESULTS

Data in Fig. (1) showed that most of the examined samples were negative for *E. coli* 122 (81%) while only 28 (19%) of the apparently normal milk samples were contaminated with *E. coli*.

The serological identification showed that out of 28 *E. coli* strains 5 strains contained O127 (*E. coli* O127) (Fig. 2).

As presented in Table (2) the biochemical evaluation for the isolated *E. coli* showed that Indole, Methyl red, and Nitrate reduction tests were positive also, the isolates were positive for Lactose and Arabinose sugar fermentation while, Voges Proskuaer, Citrate utilization, Urease, and H2S tests were negative.

These results were confirmed using VITEK® 2 compact as the results revealed that the isolate gave 96 % (excellent identification) similar to the characteristic features of *E. coli*, likeness to those detected by the standard cards of gram-negative everywhere, as shown in (Table 3).

The antibiotic susceptibility test in Table (4) indicated that high resistance rates of *E. coli* 157 strains that isolated in this study were determined against gentamycin, ciprofloxacin and tetracycline (100%), ampicillin, cephalothin, kanamycin, nalidic acid and norfloxacin **(**87.5%), Trimethoprim/sulfamethoxazole (75%) while Erythromycin was (50%)**.**

Furthermore, in Figure (3) the molecular characterization of the bla_{TEM} (Beta-lactams) gene (516bp) revealed that 7 of the isolates of *E. coli* O157 strains carried Beta-lactams gene (516bp) while one isolate was negative.

Moreover, all isolates of *E. coli* O157 isolated from milk samples were contained TetO (Tetracyclines) and Aada1 (Aminoglycosides) genes that identified at 559bp and 484bp, respectively (Fig. 4 & 5)

Table (1) Oligonucleotide primers sequences and cycling conditions of the primers during cPCR.

Target gene	Primer sequence (5'-3')	Length of amplified product	Final extension	No. of cycles	Extension	Annealing	Secondary denaturation	Primary denaturation	Reference
bla_{TEM}	ATCAGCAATAAACCAGC	bp16 5	72˚C	35	72˚C	55˚C	94˚C	94˚C	Colom *et*
	CCCCGAAGAACGTTTTC		10 min.		45 sec.	45 sec.	30 sec.	5 min.	*al*., 2003
TetO	GGCGTTTTGTTTATGTGCG	559 bp	72˚C	35	72˚C	50˚C	94˚C	94˚C	Gibreel *et*
	ATGGACAACCCGACAGAAGC		10 min.		45 sec.	40 sec.	30 sec.	5 min.	*al*., 2004
Aada1	TATCAGAGGTAGTTGGCGTCAT	484 bp	72˚C	35	72˚C	54˚C	94˚C	94˚C	Randall *et*
	GTTCCATAGCGTTAAGGTTTCATT		min.		45 sec.	40 sec.	30 sec.	5 min.	*al.* 2004

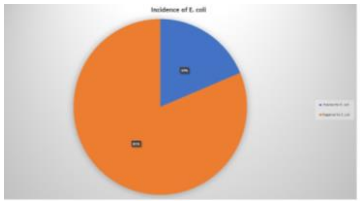

Fig. (1) the incidence of *E.coli* isolated from subclinical mastitic milk samples

Table (2) Biochemical reactions for identification of *E. coli* isolated from subclinical mastitic milk

Biochemical test	*E. col.*
Motility	V
Indole	+
Aletbyle red	+
Voges Proshaer	-
Citrate utilization	-
Urease	-
11i25	-
Nitrate reduction	+
Gelatin liquefaction	
ODC	V
LDC	V
Arginiue dibydcolase	V
ONPG	+
Sugar fermentation	
Lactose	+
Sucrose	V
Duleitol	V
Salicin	V
Arabinose	+
hositol	-
Xylose	V

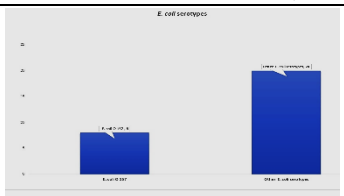

Fig. (2) the incidence of *E. coli* O157 serotype that isolated from subclinical mastitic milk samples

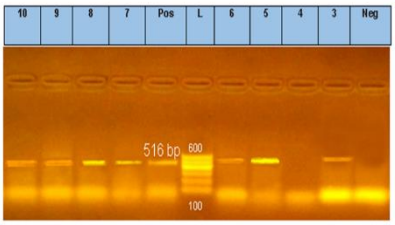

Fig. 3 Agarose gel electrophoresis of *bla$_{TEM}$* (Beta-lactams) (516bp) gene identified by PCR from *E.coli O157* strains isolated from subclinical mastitis milk. Lane L: 100 bp ladder as molecular size DNA marker. Lane Pos: Control positive Beta-lactams (516bp) gene. Lane Neg: Control negative. Lane 3, 5, 6, 7, 8, 9 and 10 are positive for Beta-lactams (516bp) gene. Lane 4 is negative for the positive Beta-lactams (516bp) gene.

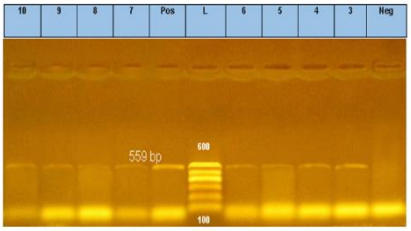

Fig. 4 Agarose gel electrophoresis of TetO (Tetracyclines) (559bp) gene identified by PCR from *E. coli O157* strains isolated from subclinical mastitis milk. Lane L: 100 bp ladder as molecular size DNA marker. Lane Pos: Control positive TetO (Tetracyclines) (559bp) gene. Lane Neg: Control negative. Lane 3, 4, 5, 6, 7, 8, 9 and 10 are positive for TetO (Tetracyclines) (559bp) gene.

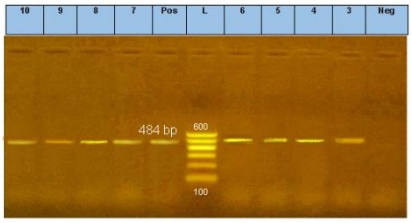

Fig. 5 Agarose gel electrophoresis of Aada1 (Aminoglycosides) (484bp) gene identified by PCR from *E. coli O157* strains isolated from subclinical mastitis milk. Lane L: 100 bp ladder as molecular size DNA marker. Lane Pos: Control positive Aada1 (Aminoglycosides) (484bp) gene. Lane Neg: Control negative. Lane 3, 4, 5, 6, 7, 8, 9 and 10 are positive for Aada1 (Aminoglycosides) (484bp) gen

Table (3) the biochemical details of *E. coli* isolated from subclinical mastitic milk using VITEK® 2 compact

2	APPA		3	ADO		4	PyrA	
5	IARL		7	dCEL		9	BGAL	+
10	H25		11	BNAG		12	AGLTp	
13	dGLU	+	14	GOT		15	OFF	+
17	BGLU		18	dMAL	+	19	dMAN	+
20	dMNE	+	21	BXYL		22	BA1ap	
23	proA	+	26	LIP	-	27	PLE	-
29	TyrA	+	31	URE	-	32	(ISO R	+
33	SAC	+	34	dTAG	+	35	dTRE	+
36	CIT		37	MNT		39	5KG	
40	I LATK		41	AGLU		42	S U CT	+
43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GIyA	-	47	ODC	+	48	LDC	+
53	IHISa		56	CMT	+	57	BLUR	+
58	0129R	+	59	GGAA		61	IMLTa	
62	6 LLM	-	64	I LATa	-			

Table (4) the antibiotic sensitivity testing (number and percentage) of different antibiotic agents against *E. coli* O157 isolated from subclinical mastitic milk

Antibiotic Agents	Sensitive	Intermediate	Resistant
Ampicillin (AM)	0 (0%)	1 (12%)	7 (87.5%)
Cephalothin (KF)	1 (12.5%)	0 (0%)	7 (87.5%)
Gentamicin (GN)	0 (0%)	0 (0%)	8 (100%)
Kanamycin (K)	0 (0%)	1 (12.5%)	7 (87.5%)
Tetracycline (TE)	0 (0%)	0 (20%)	8 (100%)
Erythromycin (E)	3 (37.5%)	1 (12.5%)	4 (50%)
Nalidic acid (NA)	0 (0%)	1 (12.5%)	7 (87.5%)
Ciprofloxacin (CIP)	0 (0%)	0 (0%)	8 (100%)
Norfloxacin (NOR)	0 (0%)	1 (12.5%)	7 (87.5%)
Trimethoprim/ sulfamethoxazole (SXT)	1 (12.5%)	1 (12.5%)	6 (75%)

4. DISCUSSION

E. coli is the most common cause of subclinical mastitis and is tolerant to a variety of medicines. Nonetheless, the presence of virulent *E. coli* in the environment is frequently ignored (Farhad et al., 2021). So, isolating *E. coli* and identifying the genes responsible for antibiotic resistance is crucial. Phenotypic characterization of *E. coli* revealed that the colony features found in this study on EMB agar were similar to those previously reported as metallic sheen with a dark center (Khmnh et al., 2005; Kabir et al., 2017a). Gram staining showed that the isolated bacteria were pink, coccobacilli, and Gram-negative. Several authors supported these findings (Mamun et al., 2016; Hassan et al., 2017).

All *E. coli* isolates fermented dextrose, sucrose, fructose, maltose, and mannitol after 24 hours of incubation, producing acid and gas, as described in other publications (Hassan et al., 2017; Kabir et al., 2017b). The isolates responded positively to the methyl red and Indole tests, but negatively to the Voges–Proskauer test. The incidence of isolated *E. coli* from subclinical mastitic milk samples was (19%), which is virtually identical to the results recorded by Farhad et al., (2021), as the incidence of *E. coli* in cow's milk with SCM was 16% and 16.25% by Abdel-Rady and Sayed (2008). Many investigations found a lower prevalence of *E. coli*, with Saidi et al. (2013) claiming an incidence of 7.5% and Mpatswenumugabo et al. (2017) reporting a prevalence of 1.5 percent. This might be due to changes in the environment and management approaches. However, Garbaj et al. (2016) found an incidence of 27.3% which is greater than in the present study.

Serological analysis revealed the presence of eight strains (28.5%) of O157 isolated from mild mastitic milk. Abdel-Fattah et al. (2023) reported an 18% incidence of *E. coli* from mastitic milk samples, with the detected *E. coli* serotypes being O26, O119, and O157. Furthermore, Farhad et al. (2021) recovered five of the total samples of O157

strains from mild mastitic milk. The incidence of *E. coli* O157 serotype in raw milk in Nigeria's Ogun State was 2% (Ivbade et al., 2014). The origin of STECO157:H7 isolates was the infected udder (Ahmadi et al., 2020), as well as contaminated feces and the environment; thus, in addition to mastitis control and treatment and promoting hygienic conditions in farms is important to reducing the rate of raw milk contamination with Shiga toxin-producing *E. coli* (STEC) strains (Mashak, 2018).

All eight *E. coli* O157 isolates were resistant to gentamicin, ciprofloxacin, and tetracycline (100%). Haftu et al. (2012), Thaker et al. (2012), and Hinthong et al. (2017) reported very identical results, with the authors reporting 100% ampicillin resistance. Rangel and Marin (2009) demonstrated that tetracycline was the most effective antibiotic. However, Tetracycline was discovered to be completely resistant in this investigation. Farhad et al. (2021) found that *E. coli* O157 was most susceptible to gentamicin (75%), followed by levofloxacin (62.5%), and tetracycline (50%).

The presence of the O157 strain and a high rate of treatment resistance among the isolates is relevant because it suggests a possible risk of human infection after consuming STEC-contaminated milk (Ahmadi et al., 2020). Variations in sensitivity patterns can be explained by test sensitivity, laboratory culture processes, bacterial identification, sampling methodologies, sample sources, and prior antibiotic exposure (Ismail and Abutarbush, 2020).

Multidrug-resistant *E. coli* has been isolated from both animal and human clinical samples at an increasing rate (Srinivasan et al., 2007). The antibiotic resistance profile of *E. coli* presented in this study indicates that all isolates carried most of the genes responsible for resistance against major antimicrobial groups. Similar results revealed by Ismail and Abutarbush (2020) as they isolated tetA, tetB, tetC, tetD, tetE, and tetG genes responsible for tetracycline resistance, aadA gene responsible for Aminoglycosides resistance and ampC, bla1 and bla2 genes responsible for Beta-lactams resistance. Moreover, *E. coli* strains from mastitis in cows were resistant to more than one antibiotic and carried multiple resistance genes, including tetA, tetB, ampC tetD, tetE, and tetG (Ashraf et al., 2018).

5. CONCLUSIONS

Raw milk containing *E. coli* O157 does not only reflect the status of the dairy herd additionally, it poses a serious threat to human health if it is consumed raw or used to make any type of value-added food product. The existence of high rates of multidrug resistance among O157strains represents a negative prognosis for the treatment of life-threatening infections caused by STEC in humans. Moreover, the propagation of resistance genes from *E. coli* strains to other pathogenic or commensal strains is the other aspect of the hazard in the public and dairy sector

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