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Detection and Molecular Characterization of Some Virulence Genes of Escherichia Coli Isolated from Milk in Dairy Cow Farms

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ABSTRACT

Coliform pathogens, primarily E. coli, were discovered throughout the farm, causing environmental mastitis and can be shed from the udder into the milk; they are concerned about severe gastrointestinal disruption and potential enteropathogenic and/or toxic strains, posing a risk to public health. The objectives of this study were to identify the incidence and harmful serotypes of pathogenic *Escherichia coli* and some of their virulent genes, which were isolated from the collected milk of some dairy farms in the Delta region and Cairo-Alexandria desert road farms, in Egypt during one year using the polymerase chain reaction (PCR) technique after bacteriological and serological identification as well as determine the antimicrobial susceptibility of the isolated strains. 150 milk samples in total were gathered (100 milk samples from bulk milk tanks and the other 50 samples from clinically mastitic dairy cows). According to our finding, the mean values of somatic cell count (SCC), standard plate count (SPC), and coliform count (CC) in the hundred bulk tank milk samples were 3.67 1.08×10^4/ml, $7.08\times10^4\pm$ 6.25×10^4 cfu/ml and $3.04\times10^2\pm1.43\times10^2$/ml, respectively. The bacteriological investigation exhibited that, the *Escherichia coli* incidences from bulk tank milk (BTM) and mastitic milk samples (MMS) were 12% and 18%, respectively. The detected *E.coli* serotypes including, O26, O44, O55:K99, O111, O119 and O157:H7 from MMS, while O1, O55, O78, O86, O124 and O158:H10 from BTM. Molecular virulence characterization of *E.coli* strains showed that, Shiga toxins 2 (stx2) gene is present in O157:H7, while the stx1 gene present in O26. The Intimin gene (eaeA) is involved in four strains, O44, O111, O119, and O157:H7. Positive amplification of a biofilm gene (adrA) appeared in all *E.coli* strains. The outcome of the antimicrobial susceptibility revealed high resistance to amoxicillin (85.71%), streptomycin (80.95%), ampicillin (71.43%), and flucloxacillin (61.90%). Meanwhile, the highest susceptibility was to ciprofloxacin (95.24%) followed by enrofloxacin (90.48%), neomycin (80.95%), and gentamycin (76.19%). Effective hygienic measurements are required to avoid toxigenic and pathogenic E.coli and more future studies should be performed to increase awareness in dairy farms.

Keywords: Antimicrobial susceptibility, *E.coli,* , Mastitis, PCR, STEC, Virulence genes.

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INTRODUCTION

Bovine mastitis is the most serious condition that poses the greatest threat to public health since it has the most financial impact on dairy husbandry which affects dairy farms around the world, negatively affects milk quality, lowers milk output, and is a source of contamination for raw milk and other dairy products **(Harjanti and Sambodho, 2020).** There are many various factors starting with animal health, handling and storage of milk that might affect the microbiological quality of raw milk, pasteurized milk

and other milk products (**Marri, *et al.,* 2020).** Despite the fact that E. coli is present everywhere as a common organism that causes mastitis and is linked to potential enteric risk **(Urseler, *et al.,* 2022).** In order to improve quality, sanitary practices would be implemented at the farm level (**Motlagh and Yang, 2019).**

The heat resistant Shiga toxins (Stx1 and Stx2) cause severe E. ***coli*** virulence, especially in isolates from clinical and subclinical mastitis. Shiga toxins produce serotypes; O11, O26, O55, and O111 that are associated with bloody diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS). Several outbreaks are brought on by ***E. coli*** O157:H7 **(Lee and Tesh, 2019).** The EAE gene encodes a protein called Intimin which is responsible for the close attachment of STEC to the intestinal epithelial cells that results in attaching and effacing (A/E) lesions in the mucosa.

The introduction of the Intimin gene (EAE) into enterohemorrhagic E. coli O157 mutant restored the pathogen's full virulence **(Ndahetuye, 2019).** Complete prevention of mastitis in dairy farms could not be fulfilled so mastitis control depends on two principles: the first is to identify and protect healthy cows, and the second is to identify and minimize risk factors. The primary step in curing the ailment is the accurate identification of the causative microorganisms. However, a quick and accurate screening test for detection of E. coli can be done using gold standard techniques, including somatic cell count and microbial isolation followed by complete identification of different serotypings of *E.coli*, the polymerase chain reaction has gained a lot of attention as an accurate diagnostic tool.

The goal of the current study was to look at the incidence of pathogenic *E.coli* with serological identification and detect some of the virulence genes related to pathogenesis (stx1, stx2, eaeA, and biofilm formation adrA) in addition to the antibiotic liability patterns of isolated ***E.coli*** .

MATERIALS AND METHODS

1. Collection of samples

In sterile screw-capped bottles under aseptic conditions, 150 milk samples were taken from 10 private dairy cattle farms in the Delta region and Alexandria desert road that applied sanitary hygienic measures (as pre- and post-dipping, regular examination for detection of mastitis and segregation of mastitis-affected cows). After sterilization and examination of teats and udders, 50 quarter samples from 27 clinically mastitic cows and 100 milk bulk tank samples were collected during the period from January to October 2021.

2. The SCC

This was measured by the Lactoscan Somatic Cell counter-Milkotronic Ltd, Bulgaria, according to the manufactured method.

3. Bacteriological Examination for bulk tank milk

According to the **National Mastitis Council (2001),** under aseptic conditions, preparation of ten-fold serial dilutions from collected samples and thoroughly mixing to make a 1:10 dilution, for which decimal serial dilutions were arranged, each dilution was subjected to:

3.1. According to APHA (1992), each prepared dilution was inoculated into duplicate plates with one ml of each, then 12 to 15 ml of melted standard plate count agar medium (Oxoid, England) that had been cooled to 44-46 °C was added, uniformly mixed, allowed to solidify, and then incubated at 35 °C for 48 hours. The total colony count was counted and recorded.

3.2. Enumeration of coliforms (the Most Probable Number MPN), as recommended by **ICMSF (1986),** into each of three separate fermentation tubes of MacConkey's broth provided with upturned Durham's tubes, one ml of the previously prepared decimal dilutions of the milk sample was inoculated. All inoculated tubes as well as control tubes were incubated at 37°C for 24-48h. The tubes containing gas and acid were noted as a positive test, using common table to determine the most probable number (MPN) of coliforms /ml.

4. *E.coli* isolation and identification according to (Quinn *et al.* 2011)

Milk samples were incubated aerobically for 24 hours at 37 degrees Celsius, then centrifuged at 3000 rpm for 20 minutes, discarding the supernatant fluid and streaking a sterile loopful from the sediment onto the surface of MacConkey's agar for 18-24 hours. Eosin-Methylene blue (EMB) agar plates were streaked with lactose fermenter (pink) colonies which were incubated aerobically at 37°C for 24-48h. Suspected colonies were subjected to morphological and biochemical characters.

5. Serotyping E.coli by agglutination test

This was performed by using rapid diagnostic ***E.coli*** antisera sets (DENKA SEIKEN CO., Japan) at the serological department in the Animal Health Research Institute, Dokki, Giza, Egypt as described by **Kok, *et al.* (1996)**.

6. Antimicrobial susceptibility testing

The disc diffusion method was used to examine antimicrobial susceptibility patterns. The bacterial isolate suspension was aerobically incubated on Muller-Hinton agar for 24 hours at 37°C, with different antimicrobial discs added: amoxicillin (25 g), neomycin (30 g), enrofloxacin (5 g), streptomycin (10 g), ciprofloxacin (5 g), gentamycin (10 g), ampicillin (10 g), florfenicol (30 g). The diameters of the inhibition zones around the disc were measured to the nearest millimeter using calibrated rulers according to **CLSI (2015).**

7. Molecular finding of some virulence genes in isolated ***E.coli*** According to **Sambrook *et al.* (1989**). PCR assays were applied at the reference laboratory for veterinary quality control on poultry production at the Animal Health Research Institute, Dokki, Giza, Egypt, using specific primers for (stx1, stx2, eaeA, and adrA) virulence genes of (6) most pathogenic serotypes of E.coli.

DNA extraction: Adapting the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) from the manufacturer's instructions, DNA extraction from samples was carried out. In a nutshell, 200 µl of the sample suspension was treated at 56 °C for 10 min with 200 µl of lysis buffer and 10 µl of proteinase K. 200 µl of 100% ethanol were added to the lysate after incubation. After that, the sample was cleaned and centrifuged in accordance with the manufacturer's instructions. Nucleic acid elution required 100 l of elution buffer.

Oligonucleotide primers

Were listed in Table (1), and provided by Metabion (Germany).

Uniplex PCR amplification

In a biosystems 2720 thermal cycler, the reaction was done by utilized of 25µl primers in 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of each primer at 20 pmol concentration, 5.5µl of water, and 5µl of DNA template.

Analysis of the PCR Products

The PCR products were separated using 5V/cm gradient electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1 x TBE buffer at room temperature. Each gel slot had 20 µl of the PCR products put in it for the gel analysis. The sizes of the fragments were determined using the gene ruler 100 bp ladder (Fermentas, Germany) and the Gelpilot 100 bp plus DNA ladder (Qiagen, Germany, GmbH). A gel took a picture of the gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1: Primers sequences, and PCR setting program of amplification

Target gene	Primers Sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
*adr*A	F: ATGTTCCCAAAAATAATGAA R: TCATGCCGCCACTTCGGTGC	1113	94˚C 5 min.	94˚C 30 sec.	50˚C 1 min.	72˚C 1min .	72˚C 10 min.	**Bhowmick *et al.* (2011)**
*stx*1	F; ACACTGGATGATCTCAGTGG R; CTGAATCCCCCTCCATTATG	614 bp	95˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec	72˚C 10 min	**Dipineto *et al.* (2006)**
*stx*2	F; CCATGACAACGGACAGCAGTT R; CCTGTCAACTGAGCAGCACTTTG	779 bp	95˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
*eae*A	F; ATGCTTAGTGCTGGTTTAGG R; GCCTTCATCATTTCGCTTTC	248 bp	95˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	72˚C 7 min	**Bisi-Johnson *et al.* (2011)**

RESULTS

Table 2: Statistical analysis results of SCC, SPC, and coliform/ml in examined bulk tank milk.

Type of count	Number of positive samples	Minimum	Maximum	Mean	Standard deviation
Somatic cell count	100	6.9×10^4 cell/ml	5.32×10^5 cell/ml	3.67×10^5 cell/ml	1.08×10^4 cell/ml
Standard plate count	100	1.5×10^3 cfu/ ml	9.16×10^4 cfu/ ml	7.08×10^4 cfu/ml	6.25×10^4 cfu/ml
Total coliform count	100	5.2×10 cfu/	1.1×10^3 cfu	3.04×10^2 cfu	1.43×10^2 cfu

Table 3: Incidence of isolated ***E.coli***.

Types of samples	Number of samples	Number of isolates	Percentage
Bulk tank milk samples	100	12	12
Mastitic milk samples	50	9	18

Table 4: Serotyping of isolated ***E. coli*** (n=21).

Number of isolates	Antigenic structure of ***E. coli*** serotypes		
	E.coli serotypes	Numbers	Total number of serotypes
Bulk tank milk samples (12 isolates)	O158:H10	2	8
	O124	2	
	O86	1	
	O78	1	
	O55	1	
	O1	1	
	Untyped	4	4
Clinical mastitic milk samples (9isolates)	O157:H7	1	7
	O119	1	
	O111	1	
	O55:K99	1	
	O44	1	
	O26	2	
	Untyped	2	2

Table 5: Antibiotic sensitivity test of ***E. coli*** isolates (n=21)

Antibiotics	Sensitive		Resistant	
	Number	%	Number	%
Ciprofloxacin (CIP5)	20	95.24	1	4.76
Enrofloxacin (EN5)	19	90.48	2	9.52
Neomycin (N30)	17	80.95	4	19.05
Gentamycin (CN10)	16	76.19	5	23.81
Florfenicol (FFC30)	14	66.67	7	33.33
Flucloxacillin (FL5)	8	38.10	13	61.90
Ampicillin (AM10)	6	28.57	15	71.43
Streptomycin (S10)	4	19.05	17	80.95
Amoxicillin (AX25)	3	14.29	18	85.71

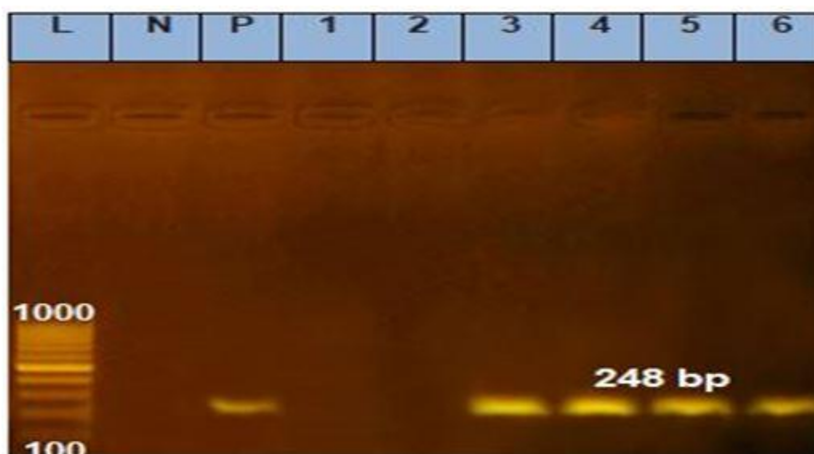

Fig.1: Agarose gel electrophoresis photo of PCR assay amplified 248bp product of eaeA gene from ***E.coli*** isolates. L: DNA ladder (100-1000bp), N: negative control, P: positive control, lanes (3-6) of ***E.coli*** samples showing the presence of the eaeA gene.

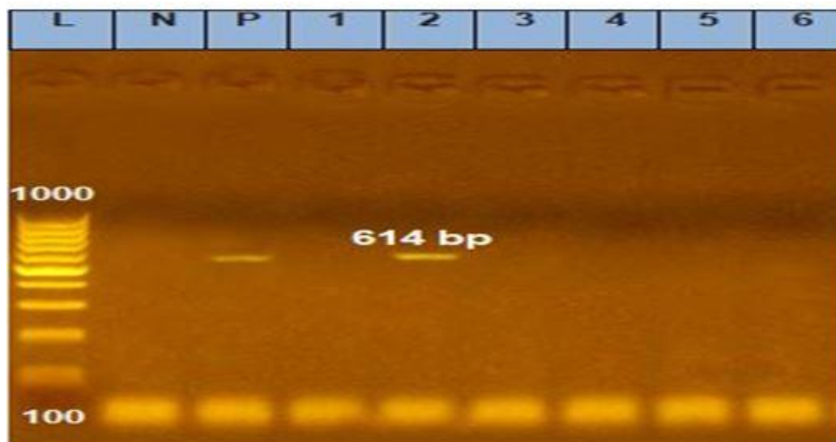

Fig. 2: PCR assay, agarose gel electrophoresis photo amplified 614bp product of stx1 gene in ***E.coli*** isolates. L: DNA ladder (100-1000bp), N: negative control, P: positive control, lane (2) ***E.coli*** sample showing the presence of stx1 gene.

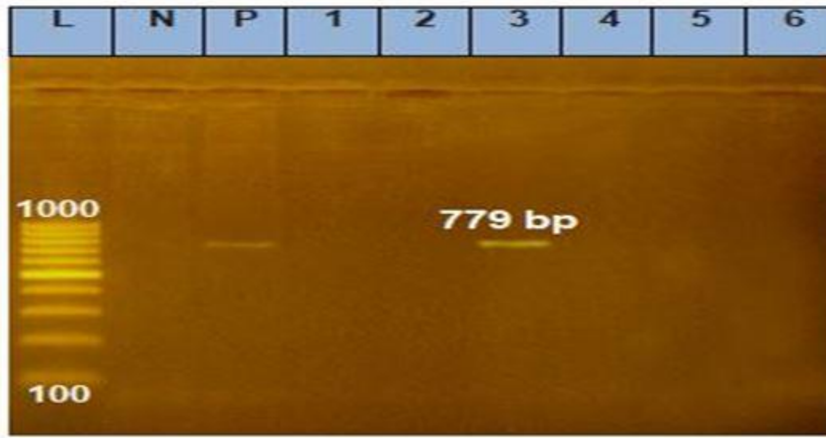

Fig. 3: PCR assay agarose gel electrophoresis photo amplified 779bp product of stx2 gene in ***E.coli*** isolates. L: DNA ladder (100-1000bp), N: negative control, P: positive control, lane (3) ***E.coli*** sample showing the presence of stx2 gene.

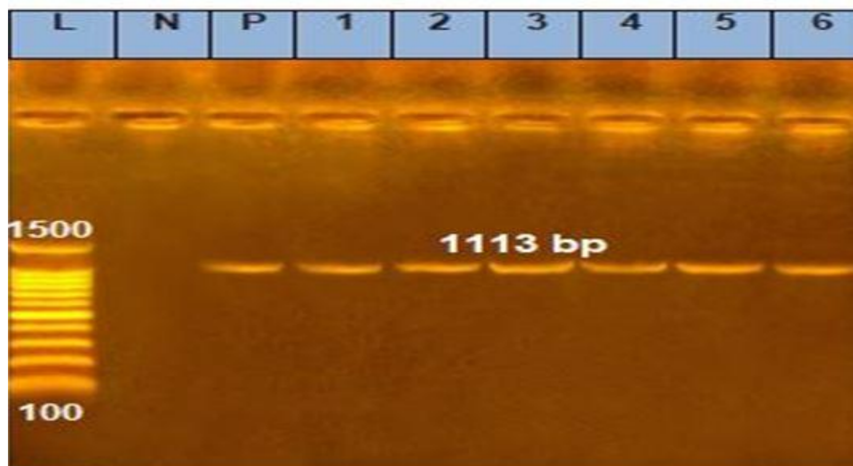

Fig. 4: PCR assay, agarose gel electrophoresis photo amplified 1113bp product of adrA gene in ***E.coli*** isolates. L: DNA ladder (100-1500bp), N: negative control, P: positive control, lanes (1-6) ***E.coli*** samples showing the presence of adrA gene,

DISCUSSION

The excellence and safety of milk and its products are important for milk consumers to reduce the hazards of foodborne diseases. Table 2 results show that the SCC and aerobic bacterial count were within the limit, while the coliform count was slightly higher due to E. coli isolated from samples. Our result was in agreement with the national standards for bulk tank SCC which vary from < 400,000 cells/ml (EU, New Zealand, Australia, and Canada) to < 1,000,000 cells/ml (Brazil) **(USDA, 2013).** Furthermore, these findings were consistent with the findings of **Rysanek, *et al.,* (2007),** who discovered that the average SCC in milk without pathogen was 2.4 105 cells/mL, 3.3 105 cells/mL with a single pathogen, and 3.67 105 cells/mL with two pathogens. On the contrary, higher results were recorded by **Azooz *et al.,* (2020)** (556.7×10^3 cells/ml) in Egypt.

In addition the results of the aerobic bacterial count were within the limitations recommended by **Egyptian Standards (ES, 2010),** as the Egyptian Organization for Standardization and Quality (EOSQ) reported that the SPC of raw milk must not exceed 1×10^5 CFU/ml milk, and nearly agreed with **Godinho, *et al.,* (2020)** (9×10^4 cfu/ml). Additional, higher findings were reported by **Azooz, *et al.,* (2020)** (3.2×10^8cfu/ml). Moreover, data revealed that, all samples proved to have coliforms with a mean value of 3.04×10^2 $\pm1.43\times10^2$ MPN/ml which was fairly lower than that reported by **El-Kholy, *et al.,* (2018)**

(7.5×10^5/ml). Regarding the results of the bacteriological examination of the present study in Table (3), The ***E.coli*** incidence, in bulk tank milk, in some dairy farms in Egypt, was closely in agreement with that obtained by **Younis, *et al.,* (2018)** (14.65%). Despite the fact that the incidence of E. coli in this study was slightly lower due to farms using some sanitary hygienic measures during milking, the presence of E. coli in milk is primarily assumed to be an effective marker of faecal contamination, pointing to inefficient sanitary and hygienic conditions during milking and milk handling that may lead to the high possibility of intramammary infection with pathogenic E. coli strains as reported by **Metz *et al.,* (2020).**

On the other hand, in mastitic milk samples, the incidence of ***E.coli*** coli was 14.9% in different Egyptian governorates, as obtained by **AbdEl-Tawab, *et al.,* (2017).** Depending on serological typing of E. coli isolates as shown in Table 4, these results showed that, the most significant virulent serotypes existed in mastitic milk samples, which are frequently associated with human infections as reported by **EL Mahmoudy, *et al. (2021)* , Ahmed, *et al*., (2020),** and **AbdEl-Tawab, *et al.,* (2017),** who identified O111 , O157, and O26 as the most prevalent STEC isolated from mastitic milk. The majority of researchers worldwide focused on the O157:H7 STEC serotype due to its high infectivity and widespread distribution in the environment, which has been linked to large epidemics and severe diseases such as hemorrhagic colitis (HC) and the potentially lethal hemolytic uremic syndrome (HUS) **(Filho *et al.,* 2014).**

While other serotypes, particularly O111 and O26, were involved as the most important non O157 STEC strains implicated. Significantly O26, O55, O78, O111, and O158, serotypes isolated from milk proved to be an important cause of persistent watery bloody diarrhoea in humans due to their capability to colonize the intestinal epithelium **(Ahmed, *et al.,* 2020).** Shiga toxin-producing ***E. coli*** (STEC) is now one of the causes of pathogenesis worldwide, causing damage in udder tissue during mastitis infection. The severity of STEC strains originates from their ability to produce potent bacterial toxins virulence factors, mainly stx1 and stx2, which are sufficient to kill a host cell. The intimin encoded by the eaeA gene is responsible for STEC's intimate attachment to intestinal epithelia, and these genes are the most important virulence genes in E. coli strains that cause clinical mastitis in cattle **(Momtaz, *et al.,* 2012).**

Globally, ***E.coli*** strains, especially those of animal origin, have acquired resistance against many antibiotics due to misuse of antibiotics in treatments or overuse usage outside of veterinary supervision without applying the sensitivity test which plays an important role in the sensitivity effect of choosing drugs. Antimicrobial susceptibility testing of ***E. coli*** strains isolated from milk (Table 5) revealed that, isolates were highly resistant to amoxicillin, streptomycin, ampicillin, and flucloxacillin. Meanwhile, the highest susceptibility was to ciprofloxacin followed by enrofloxacin, neomycin, gentamycin and florfenicol. These results are in line with a previous study by **Ismail and Abutarbush, (2020),** who recorded that, all isolates were resistant to ampicillin, amoxicillin, and streptomycin and all isolates were sensitive to enrofloxacin and ciprofloxacin. These findings are also consistent with those of **EL-Mahmoudy, *et al,*. (2021).**

In our study, PCR was used for the recognition of four virulence genes in six isolated E. coli. The results illustrated in Fig. 1 demonstrated that, ***E.coli*** attaching and effacing gene or intimin (eaeA) virulence gene was amplified in (O44, O111, O119, and O157) These results were in fairly close to those of **Oporto, *et al.,* (2008)** who identified eaeA in 95% of ***E.coli*** isolated from milk. Regarding the occurrence of stx genes in ***E.coli*** isolates, results were in Fig 2 and 3 revealed that, O26 was positive for stx1gene and O157 was positive to for stx2. Our results are coincidence with **Oporto, *et al.,* (2008)** who observed that, all ***E.coli*** O157:H7 were stx2 gene-positive. All EHEC strains particularly O26, O111, and O157 isolated from mastitic milk samples had stx1 and eaeA virulent genes, while **AbdEl-Tawab, *et al.,* (2017)** detected stx1 and stx2 virulence genes in O157 serovar. All EHEC strains particularly O26, O111, and O157 isolated from mastitic milk samples had stx1 and eaeA virulent genes while 13.88% for the stx2 gene reported by **Momtaz, *et al.,* (2012).**

According to several studies, the virulence gene stx2 is the most clinically important Stx type, and a higher prevalence of stx2-positive isolates from milk may pose public health risks and zoonotic implications, as STEC strains that express stx2 have been linked to the development of HUS in humans **(Farrokh, *et al.,* 2013).**Also, **Ahmed, *et al.,* (2020)** recorded a wide spread of virulent genes in STEC strains, stx2 gene in particular had a high frequency (64.10%) furthermore, the eAE gene was detected in eight isolates (20.51%). While **Ahmadi, *et al.,* (2020)** detected eaeA (39.1%) and stx1 (34.8%) from milk and milk product samples.

Fig (4) showed that, all ***E.coli*** strains were had adrA gene, high prevalence of this gene point to the wide spread biofilm strains of ***E.coli*** in raw milk, where cellulose is the core element of the biofilm matrix, it was assumed that, adrA gene involved in cellulose regulation in ***E.coli,*** the biofilm matrix offers

a constantly hydrated viscous layer shielding embedded bacteria from dryness or host defenses immune system by avoiding recognition of biofilm bacteria and play a role in antibiotic resistance **(Beloin, *et al.*, 2008)**

CONCLUSION

The presence of coliforms in milk indicates either the cows had bacterial infections or there was unhygienic performance in the process of milking, milk handling, and storage of milk which is unsafe for human consumption as it reduces the milk's keeping quality. Thus, farmers need education to receive technical guidance and professional training to adhere to an adequate management system with the correct adoption of good practices in milking hygiene along the value chain to minimize unnecessary contaminations that can be of public health significance.

Conflicts of interest

The authors acknowledge that there is no conflict of interest regarding the research data and tools used with this study.

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