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Antimicrobial Resistance and Virulence of Shiga-Toxin-Producing *Escherichia coli* from Milk Samples of Some Cattle Farms Al-Buḥayrah Governorate Egypt

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objective: Contamination of milk with *Escherichia coli* (*E. coli*) can pose significant public health and economic concerns. The current study was conducted to explore the prevalence and antibiotic resistance profiles of Shiga-toxin-producing *Escherichia coli* in milk samples of dairy cows in Egypt.
Study Design: Twenty milk samples were gathered from dairy cattle (ten from healthy cows and ten from mastitic cows) and examined for the presence of Shiga-toxin-producing *E. coli* using Eosin

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Methylene Blue agar. Molecular detection of virulence genes and phenotypic antimicrobial resistance were performed.

Results: *E. coli* was isolated from 65% (13/20) of the total milk samples (90% from mastitic cows and 40% from normal healthy cows). The virulence gene profiling of *E.coli* by polymerase chain reaction revealed that 84.62% (11/13) were *Stx*2a, 69.23% (9/13) were *Stx*2f, and 61.54% (8/13) were positive for *Stx*1 and *Stx*2 similarly. All the isolates showed resistance to Amikacin, Ciprofloxacin, Erythromycin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole, while 92.3% expressed resistance to Amoxicillin/ Clavulanic acid, and Tylosin, and 84.62% of the isolates were resistant to Oxytetracycline indicating multidrug-resistant profiles.

Conclusion: The present work highlights the presence of various *E. coli* strains, including potential pathogens and multidrug-resistant isolates, in milk samples from dairy cows. Regular milk testing and improved mastitis control measures are the key to reduce the public health risks and to safeguarde dairy farm productivity.

Keywords: E. coli; milk; dairy cows; virulence genes; antibiotic resistance.

1. INTRODUCTION

Milk is regarded as the nature's most complete food. However, it could also act as an ideal cause of severe threats and spreads a wide variety of harmful micro-organisms to humans, such as pathogenic *Escherichia coli* (*E. coli*) [1,2]. Bovine mastitis is one among the most prevalent and costly diseases in dairy cows worldwide [3,4]. In particular, *E. coli* is one of the most prevalent and potent pathogen that causes mastitis in dairy cattle [5,6]. The organism is a common inhabitant of the ruminant's gastrointestinal tract which can invade and damage the mammary gland tissues through the teat canal [7]. Isolation of the pathogenic *E. coli* strains has been reported from raw milk samples [8-10] and also from bovine mastitic milk samples [11-13] in Egypt and all over the world. Enteropathogenic *E. coli,* enterotoxigenic *E. coli*, and enterohemorrhagic *E. coli* which are known as lethal Shiga toxin *or* verotoxin-producing *E. coli* strains are categorized based on their virulence factors [14-16]. STEC strains are a significant group of bovine mastitis pathogens, as recorded in several studies [11,17]. Among the most important virulence genes were shiga toxins (*stx*1, *stx*2) and *eae* (intimin) in *E. coli* strains isolated from milk samples of bovine mastitis [18,19].

In the last few decades, antimicrobial resistance (AMR) has become a significant threat to public health around the world [20]. The hysterical and excessive use of antibiotics in dairy cattle raises the AMR incidence in mastitis pathogens [21]. Previous studies have indicated that there was no AMR among mastitis pathogens [22,23]. However, in recent years, pathogens such as *E. coli* that are resistant to several antibacterials have been discovered, which may be spread from cattle to humans through milk. [24,25]. AMR investigation studies have selected *E. coli* as a sentinel pathogen in investigation studies related to antibiotic resistance because of its ease acquisition of resistance and its presence in the intestinal tract of humans and animals [26]. Consequently, this work aimed to study the prevalence of *E. coli* in milk samples from mastitic and normal dairy cattle in Egypt. Additionally, the determination of their virulence factors and antibacterial sensitivity tests forwere achieved to provide vital consideration for the management of the dairy sector in Egypt.

2. MATERIALS AND METHODS

2.1 Sample Collection

This study was performed during the period from January 2019 to July 2020. A total of twenty milk samples were assembled from Egyptian dairy cattle. Ten samples were from mastitic cows and ten samples were from healthy cows. These samples were collected during hand milking in the early morning (6.00-7.00 am) under aseptic conditions. Fresh milk samples (about 100 ml) were placed in a sterile milk collection vials and transported to the Department of Bacteriology, Mycology, and Immunology laboratory, Faculty of Veterinary Medicine, Sadat City University for examination in the

2.2 Isolation and Identification of *E. coli*

Pre-enrichment in nutrient broth and incubation for 24 hrs at 37°C was performedshortly after arrivaland the milksamples was initially inoculated on to MacConkey Agar and incubated at 37°C. A sterile loop was used to transfer the pink lactose fermenting colonies to Eosin Methylene Blue agar which produced the

characteristic bluish green colonies with a metallic green sheen. Additional morphological, biochemical, and molecular characterization was done on the suspicious colonies. Different biochemical tests, like cytochrome oxidase, indole test, triple sugar iron agar, and urease, were used to confirm the isolated *E. coli* strains at the species level [27,28].

2.3 Characterisation of Virulence Genes

E. coli serotypes were inoculated on MacConkey agar medium plates after it had been preserved on semisolid medium. Following an overnight incubation period at 37°C, a small number of colonies were chosen using a sterile toothpick in order to extract DNA using the QI Aamp Miniprep kit, following the manufacturer's recommendations. Primers that target *stx*1, *stx*2, *eae*A, and *hly*A were used to check for the presence of virulence genes in all of the obtained *E. coli* isolate. These primers and protocols were used after the recent report from Egypt during 2021 [29]

2.4 Antimicrobial Susceptibility Test

Using 0.5 McFarland tube, all confirmed isolates were cultured on Mueller-Hinton (Oxoid) broth and incubated at37°C for a period of 18 hours at, till the density of bacteria was set to $1.5x10^8$/ml. The disk diffusion technique was used to determine susceptibility to 21 antimicrobials based on the Clinical and Laboratory Standards Institute guidelines [30]. The tested antibiotics were Amikacin 30µg, Amoxicillin/Clavulanic acid 30µg, Ampicillin 10µg, Ceftiofur 30µg, Ceftriaxone 30µg, Chloramphenicol 15µg, Ciprofloxacin, Doxycycline 20µg, Gentamicin, linezolid 30µg, marbofloxacin 5µg, Nalidixic acid 30µg, Nitrofurantoin 200µg, Oxytetracycline 20µg, Penicillin G 10µg, Streptomycin 5µg, Trimethoprim/Sulfamethoxazole 25µg, Tulathromycin 30µg, and Tylosin 30µg.

2.5 Statistical Analysis

The STEC rates of isolation, the distribution patterns of virulence genes and the susceptibility and resistance of isolates to antimicrobials are denoted as percentage (%).

3. RESULTS

3.1 Isolation of *E. coli* from Milk Samples

The results of *E. coli* isolation from twenty milk samples (ten samples from mastitis cows and ten from healthy cows) were illustrated in Table 1. Out of the twenty samples, thirteen samples with a total percentage of 65% were positive for *E. coli* (nine from mastitic milk with a percentage of 90% and four from normal and healthy milk with a percentage of 40%).

3.2 Percentage of Virulence Genes among the Obtained Isolates

The results of virulence gene profiling *E. coli* isolates are presented in Table 2. The gene *stx2*a was detected in 11/13 (84.62%), gene *stx2*f was represented in 9/13 (69.23%), genes stx1 and *stx*2 were detected in 8/13 (61.54%), gene *eae* was detected in 7/13 (53.85%), genes *stx*1d and stx2g were represented in 4/13 (30.77%). While the virulence genes *stx1*c, *stx*2c, *stx*2d, *stx*2e, and *ehx*A were not detected in any of the milk samples 0/13 (0%).

3.3 Antimicrobial Resistance Pattern of *E. coli* Isolates

The *E. coli* isolates were examined for antibiotic resistance against the following ntibioticsie., Amikacin, Amoxicillin/clavulanic acid, Ampicillin, Ceftiofur, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Doxycycline, Gentamycin, Linezolid, Marbofloxacin, Nalidixic acid, Nitrofurantoin, Oxytetracycline, Penicillin, Streptomycin, Trimethoprim/Sulfamethoxazole, tulathithromycin, and tylosin (Table 3). The current study detected complete resistance to Amikacin, Ciprofloxacin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole (100%). Moreover, resistance to Amoxicillin/ Clavulanic acid, and Tylosin were noticed at 92.3%. Oxytetracycline resistance was observed at 84.62%. On the other hand, resistance to Streptomycin (69.23%), Ceftiofur, Ceftriaxone, Chloramphenicol, and Nalidixic acid (53.85%). Whereas, the lowest percentages of resistance were recorded for ampicillin (46.15%), Nitrofurantoin (38.46%), Gentamicin and Marbofloxacin (30.77%), Doxycycline (23.08%), and Tulathromycin (15.38%). Moreover, our findings demonstrated that Tulathithromycin, Marbofloxacin, Doxycycline, and Ceftiofur were the most sensitive antibacterial agents against *E. coli* isolated s from bovine mastitis.

4. DISCUSSION

In the current study, a total of twenty milk samples (ten samples from mastitic milk and the other ten samples from healthy cows) were investigated for the isolation of *E. coli*. The total

prevalence was 65% (13/20) with a percentage of 90% (9/10) for the milk samples from mastitic cows and 40% (4/10) for the milk samples from the healthy cows. These total results of *E. coli* isolation were nearly similar (66%) to Rasheed et al. [31]. Lower results, 11%, 28.31 %, and 29.8%, from infected milk of mastitic cows were reported from China [32], Canada [33], and from Brazil [34] respectively. Other results showed the isolation of *E. coli* in cow's milk by a percentage of 50% [35]. Remarkably, several studies have analysed the incidence of *E. coli* from milk samples of mastitic dairy cattle in Egypt [29] and various areas of the world, such as Algeria [13], Brazil [36], China [37], Ethiopia [38,39], Mexico [40], Turkey [41], and Uruguay [42]. These differences in the incidence of *E. coli* between the different studies could be attributed to different geography, farm hygiene health status of animals, the management practices, and the different laboratories that used various isolation techniques.

Concerning the obtained results of the virulence genes of *E. coli* isolates from milk samples, the *Stx*$_{2a}$, *Stx*$_{2f}$, (*Stx*$_1$ and *Stx*$_2$, similar percentage for each), and *eae* represented 84.62%, 69.23%, 61.54%, and 53.85% respectively. While the virulence genes *Stx*$_{1c}$, *Stx*$_{2c}$, *Stx*$_{2d}$, *Stx*$_{2e}$, and *ehx*A were not detected. These results were nearly similar to several studies that were performed in Egypt [43-46]. Similarly, Wang et al. identified the genes *Stx*$_2$ and *eae* in 25% of the *E. coli* isolates [47]. In the same line, in Switzerland, *Stx*$_1$ and *Stx*$_2$ genes were positive in samples from bovine coliform mastitis [48]. In contrast to our findings, *Stx*$_1$ and *Stx*$_2$ were not found in Southern Finland [49]. Also, genes *stx* were not identified in the *E. coli* isolates from clinical bovine mastitis in Turkey [41]. Additionally, the *eae* gene was negative in the study of Stephan and Kuhn [48].

Regarding the findings of antibacterial resistance of *E. coli*, the isolates in the current study exhibited varying resistance to antibiotics. Among our results, all the strains isolated were resistant to Amikacin, Ciprofloxacin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole (100%), Amoxicillin/ Clavulanic acid, and Tylosin was (92.3%), Oxytetracycline (84.62%), Streptomycin (69.23%), Ceftiofur, Ceftriaxone, Chloramphenicol, and Nalidixic acid (53.85%), Ampicillin (46.15%), Nitrofuranation (38.46%), Gentamicin and Marbofloxacin (30.77%), Doxycycline (23.08%), and Tulathithromycin (15.38%). These findings exceeded that of Elsayed and his team [34]. Another Egyptian study recorded that the maximum isolates were resistant to Tetracycline, Ampicillin, Streptomycin, and Sulfamethoxazole-Trimethoprim while there was no resistance towards fosfomycin and imipenem [50]. Moreover, Majumder et al. recorded a smaller extent of resistance to streptomycin, tetracycline, and ampicillin by percentages of (17.7 %), (15.93 %), and (11.5 %) respectively [32]. Another study reported the maximum isolates were being resistant to ampicillin, gentamicin, and tetracycline [9]. The data demonstrated by Ahmadi et al. recorded that the maximum number of isolates were resistant against Streptomycin, Tetracycline, and Ampicillin [51]. Moreover, Momtaz et al. [11] recorded the *E. coli* isolates resistance in mastitic milk to Penicillin, Tetracyclineand Cephalothin by percentages of (100%), (57.44%), and (6.38%) respectively. In central Ethiopia, Messele et al. [52] stated the resistance of the *E. coli* isolates to ampicillin, sulphamethoxazole-trimethoprim, and streptomycin were 68.7%, 50%, and 25% respectively. Moreover, in the study performed in Punjab, India Jindal et al. [53] reported that 61.5%) (42.3%), and (55.7%) of *E. coli* isolates were resistant towards Oxytetracycline, Enrofloxacin, and Sulphadiazine (respectively. Younis et al. [54] reported that the antibiotic resistance of all *E. coli* isolates was nearly complete for β-lactams, Clindamycin, and Rifampin from milk samples taken from Qena, Egypt. The differences in the antibacterial resistance of the *E. coli* isolates in different areas in Egypt and different countries all over the world might be attributed to the difference in the hygienic measures and also to the misuse of veterinary antibiotic drugs in dairy farms.

Table 1. Isolation of *E. coli*

Samples	No. of collected samples	No. and percentage of isolates
Mastitis	10	9 (90%)
Normal milk	10	4 (40%)
Total Samples	20	13 (65%)

Table 2. Occurrence of virulence genes of *E. coli*

Isolate code	Source	Virulence factors											
		***Stx*$_1$**	***Stx*$_{1d}$**	***Stx*$_{1c}$**	***Stx*$_2$**	***Stx*$_{2a}$**	***Stx*$_{2c}$**	***Stx*$_{2d}$**	***Stx*$_{2e}$**	***Stx*$_{2f}$**	***Stx*$_{2g}$**	***eae***	***ehx*A**
1	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
2	Milk	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
3	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
4	Milk	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
5	Milk	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
6	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
7	Milk	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	Milk	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
9	Milk	+ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
10	Milk	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
11	Milk	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
12	Milk	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve
13	Milk	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Total		**8/13 61.54%**	**4/13 30.77%**	**0/13 0.0%**	**8/13 61.54%**	**11/13 84.62%**	**0/13 0.0%**	**0/13 0.0%**	**0/13 0.0%**	**9/13 69.23%**	**4/13 30.77%**	**7/13 53.85%**	**0/13 0.0%**

Table 3. Antimicrobial susceptibility pattern of *E. coli* isolates

No.	Amikacin	Amoxicillin/ clavulanic acid	Ampicillin	Ceftiofur 30	Ceftriaxone	Chloramphenicol	Ciprofloxacin	Doxycycline	Gentamicin	Linezolid	Marbofloxacin 5	Nalidixic acid	Nitrofurantoin	Oxytetracycline	Penicillin G	Streptomycin	Trimethoprim/ sulfamethoxazole	Tulathithromycin 30	Tylosin 30
1	R	R	S	S	S	R	R	R	R	R	S	I	I	S	R	I	R	S	I
2	R	R	R	R	I	R	R	I	I	R	S	R	I	R	R	R	R	S	R
3	R	R	S	S	I	S	R	R	I	R	S	S	I	R	R	R	R	S	R
4	R	R	R	I	I	I	R	R	S	R	S	R	R	R	R	S	R	S	R
5	R	R	I	R	R	I	R	S	I	R	R	I	I	R	R	R	R	I	R
6	R	R	R	R	S	I	R	S	R	R	I	R	R	R	R	R	R	S	R
7	R	R	R	I	R	R	R	S	I	R	R	R	I	R	R	R	R	S	R
8	R	R	S	I	R	R	R	S	R	R	S	S	R	R	R	R	R	R	R
9	R	R	R	R	R	R	R	S	R	R	I	R	R	R	R	I	R	I	R
10	R	R	R	R	R	R	R	I	S	R	R	R	I	R	R	R	R	I	R
11	R	S	S	I	R	S	R	S	I	R	S	I	I	S	R	I	R	S	R
12	R	R	S	R	R	R	R	I	I	R	R	I	I	R	R	R	R	R	R
13	R	R	S	R	I	S	R	I	I	R	S	R	R	R	R	R	R	S	R
S	0/13 0%	1/13 7.7%	6/13 46.15%	2/13 15.38%	2/13 15.38%	3/13 23.08%	0/13 0%	6/13 46.15%	2/13 15.38%	0/13 0%	7/13 53.85%	2/13 15.38%	0/13 0%	2/13 15.38%	0/13 0%	1/13 7.69%	0/13 0%	8/13 61.54%	0/13 0%
R	13/13 100%	12/13 92.3%	6/13 46.15%	7/13 53.85%	7/13 53.85%	7/13 53.85%	13/13 100%	3/13 23.08%	4/13 30.77%	13/13 100%	4/13 30.77%	7/13 53.85%	5/13 38.46%	11/13 84.62%	13/13 100%	9/13 69.23%	13/13 100%	2/13 15.38%	12/13 92.3%
I	0/13 0%	0/13 0%	1/13 7.7%	4/13 30.77%	4/13 30.77%	3/13 23.08%	0/13 0%	4/13 30.77%	7/13 53.85%	0/13 0%	2/13 15.38%	4/13 30.77%	8/13 61.54%	0/13 0%	0/13 0%	3/13 23.08%	0/13 0%	3/13 23.08%	1/13 7.7%

5. CONCLUSION

According to this study, *E. coli* remains to be one of the predominant pathogen responsible for bovine mastitis. The gene; *Stx*$_{2a}$, is the most prevalent virulence gene associated with STEC followed by *stx*$_{2f}$, *stx*$_{1}$, *stx*$_{2}$, *eae*, *stx*$_{1d}$, and *stx*$_{2g}$. The majority of the isolated *E. coli* have resistance patterns to Amikacin, Ciprofloxacin, Linezolid, Penicillin G, Trimethoprim/ Sulfamethoxazole, Amoxicillin/ Clavulanic acid, Tylosin, and Oxytetracycline. Moreover, Tulathithromycin, Marbofloxacin, Doxycycline, and Ceftiofurare the most effective antibacterial agents against isolated *E. coli*. Our results are new in the chain of detection of antimicrobial resistance of *E. coli* in dairy farms. The veterinerians shall lower the indiscriminate usage of antibacterial agents.

ETHICAL APPROVAL

The current work was revised and approved by the Animal Use Ethics Committee of the Faculty of Veterinary Medicine, Sadat City University.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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