

Prevalence of Shiga-toxin-producing *Escherichia coli* from milk samples of some cattle farms Al-Buḥayrah Governorate Egypt

Abstract

Objective: *Escherichia coli* (*E. coli*) contamination of milk 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 specimens 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 selective culture media. 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). With 84.62% (11/13) for *Stx2a*, 69.23% (9/13) for *Stx2f*, and 61.54% (8/13) for *Stx1* and *Stx2* being the most predominant. Antibiotic resistance was observed against Amikacin, Ciprofloxacin, Erythromycin, Linezolid, Penicillin G, and Trimethoprim/Sulfamethoxazole with percentages of 100%, Amoxicillin/Clavulanic acid, and Tylosin, was noticed at 92.3% and Oxytetracycline resistance was observed at 84.62% of isolates indicating multidrug-resistant profiles.

Conclusions: The present work highlights the presence of various *E. coli* strains, including potential pathogens and multidrug-resistant isolates, in milk specimens from dairy cows. Milk testing regularly and improved mastitis control measures are key to reducing public health risks and safeguarding dairy farm productivity.

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

1. Introduction

Milk is an ideal cause of severe threats and spreads a wide variety of harmful microorganisms to humans, such as pathogenic *Escherichia coli* (*E. coli*) [1,2]. Bovine mastitis is among the most public and costly diseases in dairy cows worldwide [3,4]. In particular, *E. coli* is one of the most prevalent and potential pathogens 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 from raw

milk samplings[8,9,10] and also from bovine mastitic milk[11,12,13]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[14,15,16]. STEC strains are a significant group for bovine mastitis as recorded in several studies [11,17]. Among the most important virulence genes were Shiga toxins (Stx1, Stx2) and *eae*(intimin) in *E. coli* strains isolated from milk samplings 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]. In Veterinary Medicine, the hysterical and excessive misuse of antibiotics in dairy cattle raises the AMR incidence in raw milk [21]. Some previous studies in the past have indicated in the past that there was no AMR among the causes of bovine mastitis [22,23]. Despite that, 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 easy 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 for the isolates were achieved to provide vital consideration for the management of the dairy sector in Egypt.

2. Materials and methods

2.1. Samplings

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) in complete hygienic measures. Fresh milk samples (about 100 ml) were placed in a sterile milk bottle. These bottles were kept in an ice box at 4°C after labeling. Then transported to the Faculty of Veterinary Medicine, Sadat City University for examination in the Department of Bacteriology, Mycology, and Immunology laboratory.

2.2. Isolation and identification of *E. coli*

Pre-enrichment in nutrient broth and incubation for 24 hrs at 37°C was performed for the milk samplings shortly after arrival. An incubation period of 24 hours was conducted after a loopful of the culture appeared to be growing on MacConkey Agar medium plates at 37°C. A sterile loop was used to pick up the pink lactose fermenting colonies and transferred them to Eosin Methylene Blue for 24 hours at 37°C. The semisolid medium was inoculated with isolated *E. coli* colonies with a metallic green sheen, and the inoculum was incubated for 24 hours at 37°C and maintained at 4°C. 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. The virulence genes of *E. coli* serotypes characterization

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 *stx1*, *stx2*, *eaeA*, and *hlyA* were used to check for the presence of virulence genes in all of the obtained *E. coli* serotypes.

2.4. Antimicrobial susceptibility test

Using McFarland tube 0.5, all confirmed isolates were cultured on Mueller-Hinton (Oxoid) broth for a period of 18 hours at 37°C, till the density of bacteria was set to 1.5×10^8 /1 ml. Following that, Mueller Hinton agar (Oxoid) plates were covered with 1 ml from each tube. The disk diffusion technique was used to determine susceptibility to 21 antimicrobials based on the Clinical and Laboratory Standards Institute guidelines [29]. The tested antibiotics were Amikacin 30µg, Amoxicillin/Clavulanic acid 30µg, Ampicillin 10µg, Ceftriaxone 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. Ethical Approval

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

2.6. 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 percentages (%). Using the Fisher's exact test and the Z-test in R statistical software, the significance of the differences in STEC isolation rates from various sources, serotypes, antimicrobial efficacy, and virulence gene distribution patterns was assessed at a statistical significance of $P < 0.05$.

3. Results

3.1. Incidence of *E. coli* in the examined milk samplings

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* incidence (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 genes among *E. coli* isolates are presented in Table 2. The gene *Stx2a* was detected in 11/13 (84.62%), gene *Stx2f* was represented in 9/13 (69.23%), genes *Stx1* and *Stx2* were detected in 8/13 (61.54%), gene *ea* was detected in 7/13 (53.85%), genes *Stx1d* and *Stx2g* were represented in 4/13 (30.77%). While the virulence genes *Stx1c*, *Stx2c*, *Stx2d*, *Stx2e*, and *ehxA* were not detected in the milk samples 0/13 (0%).

3.3. Prevalence of antimicrobial resistance pattern of *E. coli* isolates

The positive *E. coli* milk samples were examined for antibiotic resistance and were applied antibiotics disks, Amikacin, Amoxicillin/clavulanic acid, Ampicillin, Ceftiofur, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Doxycycline, Gentamycin, Linezolid, Marbofloxacin, Nalidixic acid, Nitrofurantoin, Oxytetracycline, Penicillin, Streptomycin, Trimethoprim/Sulfamethoxazole, tulathromycin, 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 Tulathromycin, Marbofloxacin, Doxycycline, and Ceftiofur were the most sensitive antibacterial agents against *E. coli* isolates from the milk samplings.

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											
		<i>Stx</i> ₁	<i>Stx</i> _{1d}	<i>Stx</i> _{1c}	<i>Stx</i> ₂	<i>Stx</i> _{2a}	<i>Stx</i> _{2c}	<i>Stx</i> _{2d}	<i>Stx</i> _{2e}	<i>Stx</i> _{2f}	<i>Stx</i> _{2g}	<i>eae</i>	<i>ehxA</i>
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%

4. Discussions

In the current study, a total of twenty milk samplings (ten samples from mastitic milk and the other ten samples from normal milk) 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. [30]. Lower results, 11%, 28.31%, and 29.8%, from infected milk of mastitic cows were reported by Yu et al. [31], Majumder et al. [32], and Dos Santos Alves et al. [33] respectively. Other results showed the isolation of *E. coli* in cow's milk by a percentage of 50% [34]. Remarkably, several studies have conducted the incidence of *E. coli* from milk samples of mastitic dairy cattle in Egypt [35] 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, hygienic measures for cattle and the surrounding environment, health conditions of animals, the management process, 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, and *eae* were represented by percentages of 84.62%, 69.23%, 61.54%, and 53.85% respectively. While the virulence genes *Stx*_{1c}, *Stx*_{2c}, *Stx*_{2d}, *Stx*_{2e}, and *ehxA* were not detected. These results were nearly similar to several studies that were performed in Egypt [43,44,45,46]. 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 *E. coli* 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 against *E. coli*, the isolated strains in the current study exhibited varying resistance to antibiotics. Among our results, the isolate strains resistance was complete 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%), Nitrofurantoin (38.46%), Gentamicin and Marbofloxacin (30.77%), Doxycycline (23.08%), and Tulathromycin (15.38%). These findings exceeded that of Elsayed and his team [34]. Another Egyptian study recorded that the highest resistances for Tetracycline, Ampicillin, Streptomycin, and Sulfamethoxazole-Trimethoprim were (27.5%), (18.9%), (18.5%), and (11.3%) respectively, while there was no resistance for fosfomicin 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 investigated that the maximum occurrence of resistances shown against ampicillin, gentamicin, and tetracycline were (100%), (100%), and (96.87%) respectively [9]. The data demonstrated by Ahmadi et al. recorded that the maximum antimicrobial resistance was noticed against Streptomycin, Tetracycline, and Ampicillin [51]. Moreover, Momtaz et al. [11] recorded the *E. coli* isolates resistance in mastitic milk to Penicillin, Tetracycline, and 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 (68.7%), (50%), and (25%) respectively. Moreover, in the study performed in Punjab, India Jindal et al. [53] investigated that the *E. coli* isolates resistance was found for Oxytetracycline, Enrofloxacin, and Sulphadiazine (61.5%) (42.3%), and (55.7%) 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 bovine farms.

5- Conclusion

According to this study's results, *E. coli* remains to be one of the main reasons for severe economic losses all over the world. The gene; *Stx_{2a}*, is the most prevalent virulence gene associated with STEC followed by *Stx_{2f}*, *Stx₁*, *Stx₂*, *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, Tulathromycin, Marbofloxacin,

Doxycycline, and Ceftiofur are the most effective antibacterial agents against isolated *E. coli*. Our results should inform veterinarians about the probable prognosis of *E. coli* infections in dairy cattle thereby reducing the indiscriminate usage of antibacterial agents.

References

1. Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodborne pathogens and disease*, 2(2), 115–129. <https://doi.org/10.1089/fpd.2005.2.115>
2. Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS microbiology reviews*, 37(5), 664–698. <https://doi.org/10.1111/1574-6976.12030>
3. Mazimpaka, E., Mbuza, F., Michael, T., Gatari, E. N., Bukenya, E. M., & James, O. A. (2017). Current status of cattle production system in Nyagatare District-Rwanda. *Tropical animal health and production*, 49(8), 1645–1656. <https://doi.org/10.1007/s11250-017-1372-y>
4. Yu, Z. N., Wang, J., Ho, H., Wang, Y. T., Huang, S. N., & Han, R. W. (2020). Prevalence and antimicrobial-resistance phenotypes and genotypes of *Escherichia coli* isolated from raw milk samples from mastitis cases in four regions of China. *Journal of global antimicrobial resistance*, 22, 94–101. <https://doi.org/10.1016/j.jgar.2019.12.016>
5. Lejeune, J. T., & Rajala-Schultz, P. J. (2009). Food safety: unpasteurized milk: a continued public health threat. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, 48(1), 93–100. <https://doi.org/10.1086/595007>
6. Belachew, T. (2016). Bovine mastitis: prevalence, isolation of bacterial species involved and its antimicrobial susceptibility test around Debrezeit, Ethiopia. *J Vet Sci Technol*, 7(06), 2.
7. Wenz, J. R., Barrington, G. M., Garry, F. B., Ellis, R. P., & Magnuson, R. J. (2006). *Escherichia coli* isolates' serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. *Journal of dairy science*, 89(9), 3408–3412. [https://doi.org/10.3168/jds.S0022-0302\(06\)72377-3](https://doi.org/10.3168/jds.S0022-0302(06)72377-3)
8. Ombarak, R. A., Hinenoya, A., Awasthi, S. P., Iguchi, A., Shima, A., Elbagory, A. M., & Yamasaki, S. (2016). Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. *International journal of food microbiology*, 221, 69–76. <https://doi.org/10.1016/j.ijfoodmicro.2016.01.009>
9. Ranjbar, R., SafarpourDehkordi, F., SakhaeiShahreza, M. H., & Rahimi, E. (2018). Prevalence, identification of virulence factors, O-serogroups and antibiotic resistance properties of Shiga-toxin producing *Escherichia coli* strains isolated

from raw milk and traditional dairy products. Antimicrobial resistance and infection control, 7, 53. <https://doi.org/10.1186/s13756-018-0345-x>

10. Dell'Orco, F., Gusmara, C., Loiacono, M., Gugliotta, T., Albonico, F., Mortarino, M., & Zecconi, A. (2019). Evaluation of virulence factors profiles and antimicrobials resistance of *Escherichia coli* isolated from bulk tank milk and raw milk filters. *Research in veterinary science*, 123, 77–83. <https://doi.org/10.1016/j.rvsc.2018.12.011>
11. Momtaz, H., SafarpoorDehkordi, F., Taktaz, T., Rezvani, A., & Yarali, S. (2012). Shiga toxin-producing *Escherichia coli* isolated from bovine mastitic milk: serogroups, virulence factors, and antibiotic resistance properties. *The Scientific World Journal*, 2012, 618709. <https://doi.org/10.1100/2012/618709>
12. Hinthong, W., Pumipuntu, N., Santajit, S., Kulpeanprasit, S., Buranasinsup, S., Sookrung, N., Chaicumpa, W., Aiumurai, P., & Indrawattana, N. (2017). Detection and drug resistance profile of *Escherichia coli* from subclinical mastitis cows and water supply in dairy farms in Saraburi Province, Thailand. *PeerJ*, 5, e3431. <https://doi.org/10.7717/peerj.3431>
13. Tahar, S., Nabil, M. M., Safia, T., Ngaiganam, E. P., Omar, A., Hafidha, C., Hanane, Z., Rolain, J. M., & Diene, S. M. (2020). Molecular Characterization of Multidrug-Resistant *Escherichia coli* Isolated from Milk of Dairy Cows with Clinical Mastitis in Algeria. *Journal of Food Protection*, 83(12), 2173–2178. <https://doi.org/10.4315/JFP-20-198>
14. Karch, H., Tarr, P. I., & Bielaszewska, M. (2005). Enterohaemorrhagic *Escherichia coli* in human medicine. *International journal of medical microbiology: IJMM*, 295(6-7), 405–418. <https://doi.org/10.1016/j.ijmm.2005.06.009>
15. Solomakos, N., Govaris, A., Angelidis, A. S., Pournaras, S., Burriel, A. R., Kritas, S. K., & Papageorgiou, D. K. (2009). Occurrence, virulence genes and antibiotic resistance of *Escherichia coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. *Food microbiology*, 26(8), 865–871. <https://doi.org/10.1016/j.fm.2009.06.002>
16. Wang, Q., Ruan, X., Wei, D., Hu, Z., Wu, L., Yu, T., Feng, L., & Wang, L. (2010). Development of a serogroup-specific multiplex PCR assay to detect a set of *Escherichia coli* serogroups based on the identification of their O-antigen gene clusters. *Molecular and cellular probes*, 24(5), 286–290. <https://doi.org/10.1016/j.mcp.2010.06.002>
17. Murinda, S. E., Ibekwe, A. M., Rodriguez, N. G., Quiroz, K. L., Mujica, A. P., & Osmon, K. (2019). Shiga Toxin-Producing *Escherichia coli* in Mastitis: An International Perspective. *Foodborne pathogens and disease*, 16(4), 229–243. <https://doi.org/10.1089/fpd.2018.2491>
18. Wieler, L. H., Vieler, E., Erpenstein, C., Schlapp, T., Steinrück, H., Bauerfeind, R., Byomi, A., & Baljer, G. (1996). Shiga toxin-producing *Escherichia coli* strains from bovines: association of adhesion with carriage of *eae* and other genes.

Journal of clinical microbiology, 34(12), 2980–2984.
<https://doi.org/10.1128/jcm.34.12.2980-2984.1996>

19. Paton, J. C., & Paton, A. W. (1998). Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clinical microbiology reviews*, 11(3), 450–479. <https://doi.org/10.1128/CMR.11.3.450>
20. Hassani S, Moosavy MH, Gharajalar SN, Khatibi SA, Hajibemani A, Barabadi Z. High prevalence of antibiotic resistance in pathogenic foodborne bacteria isolated from bovine milk. *Sci Rep* 2022; 12(1):3878; <https://doi.org/10.1038/s41598-022-07845-6>
21. Kamaruzzaman EA, Abdul Aziz S, Bitrus AA, Zakaria Z, Hassan L. Occurrence and characteristics of extended-spectrum β -Lactamase-producing *Escherichia coli* from dairy cattle, milk, and farm environments in Peninsular Malaysia. *Pathogens* 2020; 9(12):1007; <https://doi.org/10.3390/pathogens9121007>
22. Erskine, R. J., Walker, R. D., Bolin, C. A., Bartlett, P. C., & White, D. G. (2002). Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. *Journal of dairy science*, 85(5), 1111–1118. [https://doi.org/10.3168/jds.S0022-0302\(02\)74172-6](https://doi.org/10.3168/jds.S0022-0302(02)74172-6)
23. Oliver, S. P., Murinda, S. E., & Jayarao, B. M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. *Foodborne pathogens and disease*, 8(3), 337–355. <https://doi.org/10.1089/fpd.2010.0730>
24. Massé, J., Lardé, H., Fairbrother, J. M., Roy, J. P., Francoz, D., Dufour, S., & Archambault, M. (2021). Prevalence of Antimicrobial Resistance and Characteristics of *Escherichia coli* Isolates From Fecal and Manure Pit Samples on Dairy Farms in the Province of Québec, Canada. *Frontiers in veterinary science*, 8, 654125. <https://doi.org/10.3389/fvets.2021.654125>
25. DeGo, O.K. Current status of antimicrobial resistance and prospect for new vaccines against major bacterial bovine mastitis pathogens. In *Animal Reproduction in Veterinary Medicine*; IntechOpen: London, UK, 2020.
26. von Baum, H., & Marre, R. (2005). Antimicrobial resistance of *Escherichia coli* and therapeutic implications. *International journal of medical microbiology: IJMM*, 295(6-7), 503–511. <https://doi.org/10.1016/j.ijmm.2005.07.002>
27. Quinn PJ, Markey BK. concise review of veterinary microbiology. Second Edition, Blackwell Publishing. Oxford, United Kingdom; 2003.
28. Osman, K. M., Mustafa, A. M., Elhariri, M., & Abdelhamed, G. S. (2013). The distribution of *Escherichia coli* serovars, virulence genes, gene association and combinations and virulence genes encoding serotypes in pathogenic *E. coli* recovered from diarrhoeic calves, sheep and goat. *Transboundary and emerging diseases*, 60(1), 69–78. <https://doi.org/10.1111/j.1865-1682.2012.01319.x>
29. CLSI, I. "Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals." CLSI supplement VET08 (2018).

30. Rasheed, M. U., Thajuddin, N., Ahamed, P., Teklemariam, Z., & Jamil, K. (2014). Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources. *Revista do Instituto de Medicina Tropical de Sao Paulo*, 56(4), 341–346. <https://doi.org/10.1590/s0036-46652014000400012>
31. Yu, Z. N., Wang, J., Ho, H., Wang, Y. T., Huang, S. N., & Han, R. W. (2020). Prevalence and antimicrobial-resistance phenotypes and genotypes of *Escherichia coli* isolated from raw milk samples from mastitis cases in four regions of China. *Journal of global antimicrobial resistance*, 22, 94–101. <https://doi.org/10.1016/j.jgar.2019.12.016>
32. Majumder, S., Jung, D., Ronholm, J., & George, S. (2021). Prevalence and mechanisms of antibiotic resistance in *Escherichia coli* isolated from mastitic dairy cattle in Canada. *BMC microbiology*, 21(1), 222. <https://doi.org/10.1186/s12866-021-02280-5>
33. Dos Santos Alves, T., Rosa, V. S., da Silva Leite, D., Guerra, S. T., Joaquim, S. F., Guimarães, F. F., de Figueiredo Pantoja, J. C., Lucheis, S. B., Rall, V. L. M., Hernandez, R. T., Langoni, H., & Ribeiro, M. G. (2023). Genome-Based Characterization of Multidrug-Resistant *Escherichia coli* Isolated from Clinical Bovine Mastitis. *Current microbiology*, 80(3), 89. <https://doi.org/10.1007/s00284-023-03191-6>
34. Elsayed, M. S. A. E., Eldsouky, S. M., Roshdy, T., Bayoume, A. M. A., Nasr, G. M., Salama, A. S. A., Akl, B. A., Hasan, A. S., Shahat, A. K., Khashaba, R. A., Abdelhalim, W. A., Nasr, H. E., Mohammed, L. A., & Salah, A. (2021). Genetic and antimicrobial resistance profiles of non-O157 Shiga toxin-producing *Escherichia coli* from different sources in Egypt. *BMC microbiology*, 21(1), 257. <https://doi.org/10.1186/s12866-021-02308-w>
35. Osman, K. M., Mustafa, A. M., Aly, M. A., & AbdElhamed, G. S. (2012). Serotypes, virulence genes, and intimin types of shiga toxin-producing *Escherichia coli* and enteropathogenic *Escherichia coli* isolated from mastitic milk relevant to human health in Egypt. *Vector borne and zoonotic diseases (Larchmont, N.Y.)*, 12(4), 297–305. <https://doi.org/10.1089/vbz.2010.0257>
36. Fernandes, J. B., Zanardo, L. G., Galvão, N. N., Carvalho, I. A., Nero, L. A., & Moreira, M. A. (2011). *Escherichia coli* from clinical mastitis: serotypes and virulence factors. *Journal of veterinary diagnostic investigation: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc.*, 23(6), 1146–1152. <https://doi.org/10.1177/1040638711425581>
37. Wang L, Yang F, Wei X, Luo Y, Zhou X, Gua W, Niu J, Guo Z. 2015. Investigation of bovine mastitis pathogen in two northwestern provinces of China from 2012–2014. *Journal of Animal and Veterinary Advances* 14:237–243 DOI 10.3923/javaa.2015.237.243
38. Abera, M., Habte, T., Aragaw, K., Asmare, K., & Sheferaw, D. (2012). Major causes of mastitis and associated risk factors in smallholder dairy farms in and

around Hawassa, Southern Ethiopia. *Tropical animal health and production*, 44(6), 1175–1179. <https://doi.org/10.1007/s11250-011-0055-3>

39. Haftu, R., Taddele, H., Gugsu, G., & Kalayou, S. (2012). Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia. *Tropical animal health and production*, 44(7), 1765–1771. <https://doi.org/10.1007/s11250-012-0135-z>
40. Olivares-Pérez, J., Kholif, A. E., Rojas-Hernández, S., Elghandour, M. M., Salem, A. Z., Bastida, A. Z., Velázquez-Reynoso, D., Cipriano-Salazar, M., Camacho-Díaz, L. M., Alonso-Fresán, M. U., & DiLorenzo, N. (2015). Prevalence of bovine subclinical mastitis, its etiology and diagnosis of antibiotic resistance of dairy farms in four municipalities of a tropical region of Mexico. *Tropical animal health and production*, 47(8), 1497–1504. <https://doi.org/10.1007/s11250-015-0890-8>
41. Guler L, Gunduz K. 2007. Virulence properties of *Escherichia coli* isolated from clinical bovine mastitis. *Turkish Journal of Veterinary and Animal Science* 31:361–365.
42. Giannechini, R., Concha, C., Rivero, R., Delucci, I., & Moreno López, J. (2002). Occurrence of clinical and sub-clinical mastitis in dairy herds in the West Littoral Region in Uruguay. *Acta veterinaria Scandinavica*, 43(4), 221–230. <https://doi.org/10.1186/1751-0147-43-221>
43. Galal HM, Hakim AS, Dorgham SM. Phenotypic and virulence genes screening of *Escherichia coli* strains isolated from different sources in delta Egypt. *Life Sci J* 2013;10:352–361.
44. Lamey AE, Ammar AM, Zaki ERA, Khairy N, Moshref BS, Refai MK. Virulence factors of *Escherichia coli* isolated from recurrent cases of clinical and subclinical mastitis in buffaloes. *Int J Microb Res* 2013;4:86–94.
45. Sayed SM. A contribution on coliforms causing mastitis in cows with reference to serotypes and virulence factors of *E. coli* isolates. *Ass Univ Bull Environ Res* 2014;17:85–95
46. Radwan MEI, Abo-Zaid KF. Molecular studies on *E. coli* isolate from milk of mastitic cattle with special reference to associated biochemical changes in Kaliobea Governorate. *IJRDO J Health Sci Nurs* 2017;7:59–68
47. Wang, S., Yu, Z., Wang, J., Ho, H., Yang, Y., Fan, R., Du, Q., Jiang, H., & Han, R. (2021). Prevalence, Drug Resistance, and Virulence Genes of Potential Pathogenic Bacteria in Pasteurized Milk of Chinese Fresh Milk Bar. *Journal of food protection*, 84(11), 1863–1867. <https://doi.org/10.4315/JFP-21-094>
48. Stephan, R., & Kühn, K. (1999). Prevalence of verotoxin-producing *Escherichia coli* (VTEC) in bovine coli mastitis and their antibiotic resistance patterns. *Zentralblatt für Veterinärmedizin. Reihe B. Journal of veterinary medicine. Series B*, 46(6), 423–427. <https://doi.org/10.1046/j.1439-0450.1999.00244.x>

49. Suojala, L., Pohjanvirta, T., Simojoki, H., Myllyniemi, A. L., Pitkälä, A., Pelkonen, S., & Pyörälä, S. (2011). Phylogeny, virulence factors and antimicrobial susceptibility of *Escherichia coli* isolated in clinical bovine mastitis. *Veterinary microbiology*, 147(3-4), 383–388. <https://doi.org/10.1016/j.vetmic.2010.07.011>
50. Ombarak, R. A., Hinenoya, A., Awasthi, S. P., Iguchi, A., Shima, A., Elbagory, A. M., & Yamasaki, S. (2016). Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. *International journal of food microbiology*, 221, 69–76. <https://doi.org/10.1016/j.ijfoodmicro.2016.01.009>
51. Ahmadi, E., Mardani, K., & Amiri, A. (2020). Molecular Detection and Antimicrobial Resistance Patterns of Shiga Toxigenic *Escherichia coli* Isolated from Bovine Subclinical Mastitis Milk Samples in Kurdistan, Iran. *Archives of Razi Institute*, 75(2), 169–177. <https://doi.org/10.22092/ari.2019.124238.1278>
52. Messele, Y. E., Abdi, R. D., Tegegne, D. T., Bora, S. K., Babura, M. D., Emeru, B. A., & Werid, G. M. (2019). Analysis of milk-derived isolates of *E. coli* indicating drug resistance in central Ethiopia. *Tropical animal health and production*, 51(3), 661–667. <https://doi.org/10.1007/s11250-018-1737-x>
53. Jindal, P., Bedi, J., Singh, R., Aulakh, R., & Gill, J. (2021). Phenotypic and genotypic antimicrobial resistance patterns of *Escherichia coli* and *Klebsiella* isolated from dairy farm milk, farm slurry and water in Punjab, India. *Environmental science and pollution research international*, 28(22), 28556–28570. <https://doi.org/10.1007/s11356-021-12514-8>
54. Younis, W., Hassan, S., & Mohamed, H. M. A. (2021). Molecular characterization of *Escherichia coli* isolated from milk samples with regard to virulence factors and antibiotic resistance. *Veterinary world*, 14(9), 2410–2418. <https://doi.org/10.14202/vetworld.2021.2410-2418>