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Prevalence and Antibiogram of *Escherichia coli* Isolates Recovered from Bovine Milk

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ABSTRACT

Antimicrobial resistance is considered a major threat facing humanity. It doesn't only affect public health, but also causes great losses in the dairy industry. Mastitis is a major threat to the dairy industry. The aim of this study was to monitor the antimicrobial resistance of *Escherichia coli* (*E. coli*) collected from raw milk of both healthy and mastitis-infected cows and buffaloes in Egypt. In total, 450 milk samples were collected and examined in the period from 2018 to 2021. The samples were collected from healthy cows and buffaloes (30, 58), suffering from clinical mastitis (139, 223), respectively. *E. coli* was isolated from 33 mastitis milk samples (9.1%) and from 3 (3.4%) normal milk samples. The antibiotic susceptibility testing was performed using the disc diffusion method (Kirby-Bauer method). *E. coli* isolated from mastitis milk samples showed resistance The Extended Spectrum b-Lactamases test (ESBL) performed on the *E. coli* isolates showed positive results in 9% of mastitis milk samples, but no results in normal milk samples. Out of 36 *E. coli* isolates, 34 possessed the *amp*C gene, but *bla*TEM and *bla*SHV were detected in 5 isolates with percentages of 94%, 1.4%, and 1.4%, respectively, while *Bla*IPM and *Sul*1 were found in one isolate (2.7%).

Keywords: Antibiogram, Bovine mastitis, *E. coli*, ESBLs, Milk.

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INTRODUCTION

Escherichia coli is one of the most widespread bacteria among many animal species, such as cattle (**Heba, 2011**), ducks (**Soliman *et al.*, 2018**), and chickens (**Desouky *et al.*, 2021**). *E. coli* infection causes symptoms that vary from local to severe clinical mastitis. The antimicrobial resistance is a challenging threat that faces humanity. According to many recent studies, antimicrobial resistance will cause 10 million deaths per year in 2050 (**Sugden *et al.*, 2016**). Multidrug-resistant *E. coli* occurs due to the misuse of these agents by humans and animals, which consequently threatens their lives. Multidrug-resistant *E. coli* was isolated from food samples including raw milk in Egypt (**Aly *et al.*, 2012**), and milk products (**Samy *et al.*, 2022**).

Bovine clinical mastitis is frequently caused by coliform bacteria. *E. coli* is the most prevalent species, isolated in more than 80% of coliform mastitis (**Suojala *et al.*, 2013**), subclinical mastitis cases (**Ombarak *et al.*, 2019; Abed *et al.*, 2021**), and clinical mastitis (**Ahmed *et al.*, 2021**). Mastitis costs a lot of money due to milk production losses, discarded milk, reduced milk quality, veterinary services (veterinarians, diagnostics, and drugs), the treatment cost, and culling (**Halasa *et al.*, 2007**).

The current study was done to add recent data to the prevalence and antibiotic susceptibility profiles of *E. coli* strains isolated and consumed from raw normal milk as well as mastitis milk. Additionally, to provide detailed information on the presence of multiple antibiotic-resistant (MAR) *E. coli* in such samples, submit a suggestion as a major human health concern.

MATERIALS AND METHODS

Sample collection

A total of 450 samples (169 from cows, and 281 from buffaloes) were collected from Qalyubia and Alsharqia governorates, in the period from 2018 to 2021. The samples were collected under aseptic conditions in sterile tubes and transported to the laboratory in the Microbiology Department, Faculty of Veterinary Medicine, Cairo University, in an ice box at 4°C for isolation and identification of *E. coli* **(Ghanbarpour and Oswald, 2010; Ameen *et al*., 2019**).

Isolation and identification of *E. coli* isolates

The **s**amples were directly inoculated on MacConkey agar medium, and incubated for 24 h at 37°C. Then, the suspected *E. coli* pink colonies were streaked on Eosin Methylene Blue Agar (EMB), and incubated for 24 h at 37° (**Leininger *et al*., 2001; Soomro *et al*., 2002; Disassa *et al*., 2017**). Finally, the metallic sheen colonies were picked up and subcultured on nutrient agar for morphological and biochemical examination, including Oxidase, Indole production, methyl red, Voges-Proskauer, Citrate, Triple sugar iron agar (TSI), and urease tests (**Islam *et al*., 2014**).

Antibiotic sensitivity testing

Escherichia coli isolates were tested for their antibiotic susceptibility by using the disc diffusion method (Kirby-Bauer method), and the results were interpreted according to **CLSI (2020)**, Colistin results were interpreted according to **Galani *et al*. (2008); Gales *et al*. (2001**). The used antimicrobials were ampicillin (AMP, 10µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), cefotaxime (CTX, 30µg), ceftazidime (CAZ, 30µg), aztreonam (AT, 30µg), imipenem (IPM, 5µg), colistin (CL, 10µg), gentamicin (GEN, 10µg), tetracycline (TE, 30 µg), ciprofloxacin (CIP, 5µg), trimethoprim-sulfamethoxazole (COT, 1.25/23.75µg), and chloramphenicol (C, 30µg).

Extended Spectrum b-Lactamases (ESBL) testing

All *E. coli* isolates were tested for b-lactamase (**CLSI, 2020)** by using the disc diffusion method. Cefotaxime (CTX 30 µg), and cefotaxime-clavulanic acid (CEC 30/10 µg) were used for testing. A zone diameter of more than 5 mm between cefotaxime and cefotaxime-clavulanic acid was considered ESBL positive **(CLSI, 2020**).

Detection of antibiotic resistance genes

All *E. coli* isolates were tested for the presence of b-lactamase genes (*bla*TEM, *bla*SHV, and *amp*C), carbapenem genes (*Bla*IPM), and sulphonamide resistance genes (*Sul*1). The isolates were inoculated into brain heart infusion broth (BHIB) and incubated for 24 h at 37 °C. According to the manufacturer's instructions, DNA was extracted using the Kogene Biotech ® DNA Extraction Kit. The primers used in this study are shown in Table 1.

Table 1: The target genes, primer sequences, and Polymerase chain reaction (PCR) conditions used for genomic analysis of *E. coli* isolates

The target gene	**Primer sequence (5′–3′)**	**(bp)**	**PCR conditions**	**References**
***bla*TEM**	F: TTGCTCACCCAGAAACGCTGGTG R: TACGATACGGGAGGGCTTACC	708	Initial denaturation for 5 min. at 94 °C, followed by 30 cycles of 94 °C for 30 sec., 63 °C for 45 sec., and 72 °C for 1.5 min., and final Extension at 72 °C for 5 min.	
***bla*SHV**	F: CGCCGGGTTATTCTTATTTGTCGC R:TCTTTCCGATGCCGCCGCCAGTCA	1016		(**Mataseje *et al*., 2012**)
***amp*C**	F: GATCGTTCTGCCGCTGTG R: GGGCAGCAAATGTGGAGCAA	271	Initial denaturation for 5 min. at 94 °C, followed by 30 cycles of 94 °C for 30 sec., 56 °C for 45 sec. and 72 °C for 1.5 min. and final extension at 72 °C for 5 min.	(**Oliver *et al*., 2002**)
***bla*IPM**	F: TCGTTTGAAGAAGTTAACG R: ATGTAAGTTTCAAGAGTGATGC	568	Initial denaturation for 5 min. at 94 °C, followed by 30 cycles of 94 °C for 30 sec., 60 °C for 45 sec., and 72 °C for 1.5 min. and final extension at 72 °C for 5 min.	(**Mahmoud *et al*., 2020**)
***sul*1**	F: TGGTGACGGTGTTCGGCATTC R: GCGAGGGTTTCCGAGAAGGTG	789	Initial denaturation for 5 min. at 94 °C, followed by 30 cycles of 94 °C for 30 sec., 63 °C for 30 sec. and 72 °C for 1.5 min. and final extension at 72 °C for 5 min.	(**Costa *et al*., 2008**)

RESULTS

From the tested 450 milk samples, 36 isolates (8%) were identified as *E. coli* (33 out of 362 mastitis milk samples; and 3 out of 88 normal milk samples (representing 9.12%, and 3.4%, respectively), as shown in Table 2.

Table 2: Isolation of *E. coli* from the examined milk samples

Samples	Sample type	Total	*E. coli* isolates	%
Normal milk	Cows' raw milk	30	1	3.33
	Buffaloes' raw milk	58	2	3.45
Mastitis milk	Cows' Mastitis milk	139	18	12.95
	Buffaloes' Mastitis milk	223	15	6.73
	Total	450	36	8

Escherichia coli isolates collected from mastitis milk samples showed resistance to amoxicillin-clavulanic acid (75.8%), ceftazidime (75.8%), colistin (69.8%), ampicillin (39.4%), imipenem (24.2%), trimethoprim-sulfamethoxazole (24.2%), aztreonam (15.2%), cefotaxime (15.2%), tetracycline (15.2%), ciprofloxacin (12.2%), chloramphenicol (6%), and gentamicin (3%). While the isolates from normal raw milk samples showed resistance to ampicillin, and imipenem in a percentage of 66.7%, each, and amoxicillin-clavulanic acid, tetracycline, and trimethoprim-sulfamethoxazole in a percentage of 33.3%, each (Table 3)

Table 3: Antibiotic sensitivity results for *E. coli* isolated from mastitis milk,and normal milk samples

Type of isolates	Mastitis milk isolates						Normal milk isolates					
Antibiotics	R		S		I		R		S		I	
	n=	%	n=	%	n=	%	n=	%	n=	%	n=	%
Amoxicillin-clavulanic acid	25	75.8	8	24.2	0	0	1	33.3	2	66.7	0	0
Ceftazidime	25	75.8	7	21.2	1	3	0	0	1	33.3	2	66.7
Colistin	23	69.8	7	21.2	3	9	0	0	3	100	0	0
Ampicillin	13	39.4	14	42.4	6	18.2	2	66.7	1	33.3	0	0
Imipenem	8	24.2	24	72.8	1	3	2	66.7	1	33.3	0	0
Trimethoprim-Sulfamethoxazole	8	24.2	25	75.8	0	0	1	33.3	2	66.7	0	0
Aztreonam	5	15.2	28	84.8	0	0	0	0	3	100	0	0
Cefotaxime	5	15.2	28	84.8	0	0	0	0	3	100	0	0
Tetracycline	5	15.2	28	84.8	0	0	1	33.3	2	66.7	0	0
Ciprofloxacin	4	12.2	28	84.8	1	3	0	0	2	66.7	1	33.3
Chloramphenicol	2	6	31	94	0	0	0	0	3	100	0	0
Gentamicin	1	3	32	97	0	0	0	0	3	100	0	0

R: Resistant, S: Sensitive, I: Intermediate

Escherichia coli isolates from normal raw milk were negative for the ESBL test. However, 3 isolates (9%) from mastitis milk samples were positive for the ESBL test, as shown in Table 4.

PCR results for detection of antimicrobial resistance genes are not compatible with the antibiotic sensitivity results. Out of 36 *E. coli* isolates, 34 of them were possessed *amp*C gene (94.4%); *bla*TEM, *bla*SHV were detected in 5 (14%) isolates, each. While, *bla*IMP and *Sul*1 were found in one (2.8%) isolate, for each (Figs. 1, 2, 3 and 4 showed the gel electrophoresis results).

Table 4: Antibiotic sensitivity results for *E. coli* isolates that show positive results of the ESBL test

Antibiotic disks	R		S		I	
	n=	%	n=	%	n=	%
Amoxicillin- clavulanic acid	3	100	0	0	0	0
Cefotaxime	3	100	0	0	0	0
Ceftazidime	3	100	0	0	0	0
Ampicillin	2	66.7	0	0	1	33.3
Aztreonam	2	66.7	1	33.3	0	0
Colistin	2	66.7	0	0	1	33.3
Trimethoprim-Sulfamethoxazole	2	66.7	1	33.3	0	0
Ciprofloxacin	0	0	3	100	0	0
Chloramphenicol	0	0	3	100	0	0
Gentamicin	0	0	3	100	0	0
Imipenem	0	0	3	100	0	0
Tetracycline	0	0	3	100	0	0

R: Resistant, S: Sensitive, I: Intermediate

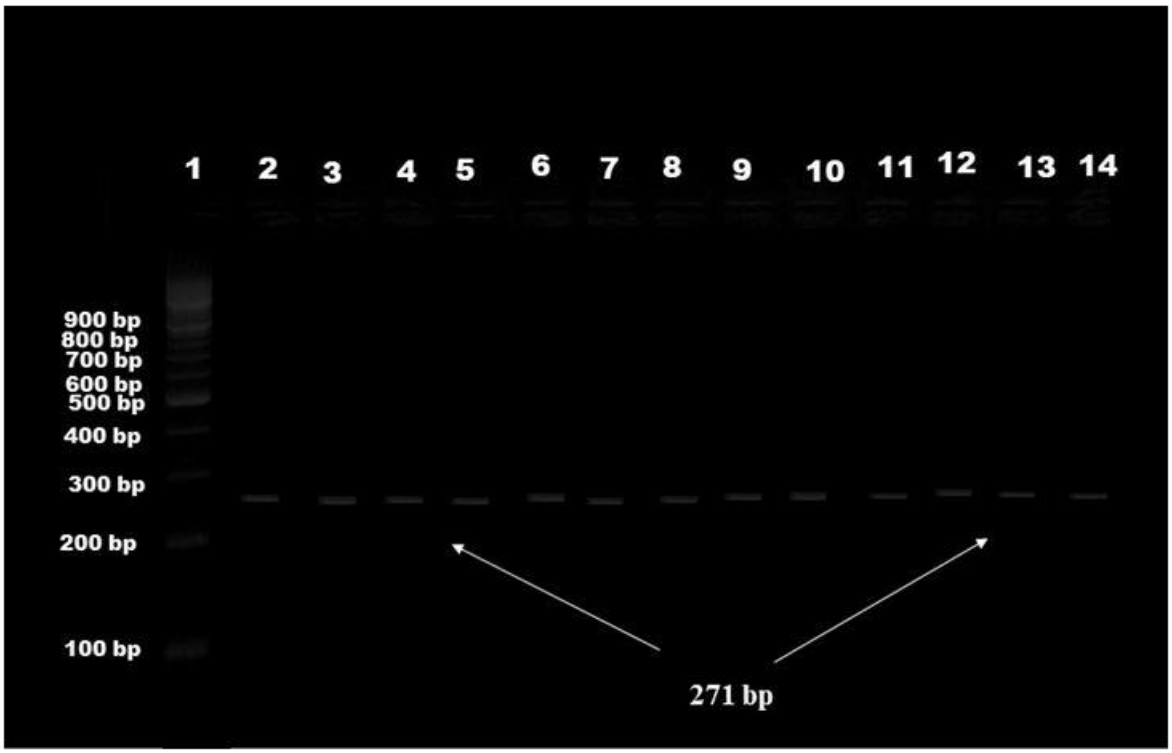

Fig. 1: Agarose gel electrophoresis shows amplification of *amp*C gene using a specific primer. Lane 1: shows 1 kb Ladder; Lanes 2-14 show a positive 271 bp fragment for *amp*C gene.

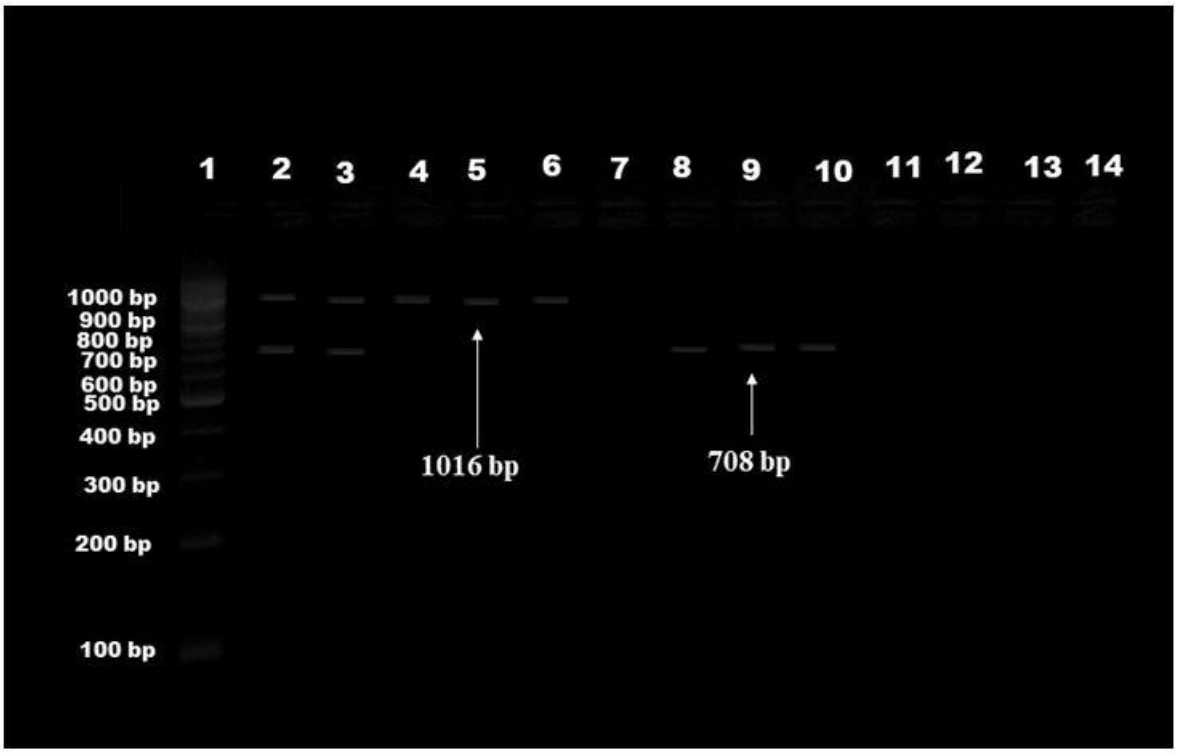

Fig. 2: Agarose gel electrophoresis shows amplification of *bla*SHV and *bla*TEM genes using specific primers. Lane 1: shows 1 kb Ladder, Lanes (2, 3): positive 1016 bp and 708 bp fragments for both *bla*SHV and *bla*TEM genes, respectively. Lanes (4-6): show a positive 1016 bp for *bla*SHV gene. Lanes (8-10): show a positive 708 bp for the *bla*TEM gene.

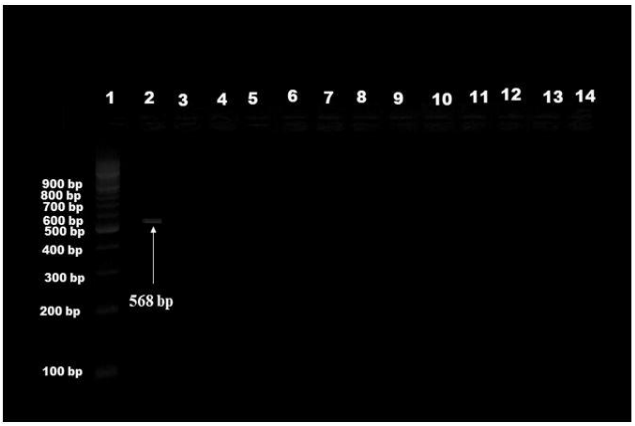

Fig. 3: Agarose gel electrophoresis shows amplification of *Bla*IPM gene using specific primer. Lane 1: shows 1kb Ladder, Lanes 2: shows positive 568 bp fragment for *Bla*IPM gene.

Fig. 4: Agarose gel electrophoresis shows amplification of *Sul*1 gene using specific primer. Lane 1: shows 1 kb Ladder, Lane 2: shows positive 789 bp fragment for *Sul*1 gene.

DISCUSION

In the present study, resistant *E. coli* isolates from both clinical and subclinical mastitis milk samples could be isolated, and these results are similar to those of **Ahmed *et al.*, (2021**) who isolated resistant *E. coli* isolates from both clinical and subclinical mastitis milk samples. The presence of *E. coli* in normal raw milk in this study was 3.4%. This finding is lower than what was previously detected by **Altalhi and Hassan, (2009**) in Saudi Arabia (66%), **Heba (2011)** in Egypt (14.5%), **Skočková *et al.* (2015**) in the Czech Republic (92.4%); **Ombarak *et al.*, (2016**) in Egypt (76.4%), **Batabyal *et al.*, (2018**) in India (12.08%), and **Samy *et al.*, (2022**) in Egypt (42.3%).

In the present study, the isolation rate of *E. coli* from bovine mastitis milk samples was 9.1%, which is similar to the findings of **Heba, (2011)** in Egypt (10.5%), **Ombarak *et al*., (2019**), and **Ahmed *et al*., (2021)** with a rate of 9.3%, each. The isolation rate was close to the finding found by **Ali *et al*., (2017)** (12.5%), while this was lower than what was detected by **Ameen *et al*., (2019)** (20%).

The isolated *E. coli* were examined using PCR which is one of the identification techniques used worldwide for *E. coli* diagnosis, as mentioned by **Heba, (2011)**. In the present study, the genes used for *E. coli* identification were *bla*TEM, *bla*SHV, *amp*C, *Bla*IPM, and *Sul*1, but the gene used by **Heba, (2011)** was stx_2.

Escherichia coli isolated from normal raw milk samples showed resistance to more than one antimicrobial agent. It showed resistance to ampicillin, amoxicillin-clavulanic acid, imipenem, tetracycline, and trimethoprim-sulfamethoxazole. These results were similar to the results of **Skočková *et al*., (2015)** which showed resistance of *E. coli* isolates to ampicillin, amoxicillin-clavulanic acid, tetracycline, and trimethoprim-sulfamethoxazole, and those of **Samy *et al*., (2022)** who detected resistance of *E. coli* isolates to oxytetracycline, amoxicillin, and ampicillin, and **Ombarak *et al*., (2018)** who found resistance to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole among *E. coli* isolates.

Escherichia coli, which is similar to the results of **Ameen *et al*., (2019)** who recorded resistance to streptomycin (40%), ampicillin (33%), trimethoprim-sulphamethoxazole (23%), ceftriaxone (20%), cefepime (16%) and tetracycline (16%), more than 60% of the isolates were sensitive to amoxicillin, clavulanic acid, and ciprofloxacin. These results are similar to those of **Ahmed *et al*., (2021**) who isolated resistant *E. coli* isolates: 100% of these isolates were resistant to chloramphenicol and trimethoprim-sulfamethoxazole, each, 85.7% of them were resistant to ceftazidim, ciprofloxacin, gentamicin, and tetracycline, each, 42.9% were resistant to amoxicillin-clavulanic acid,, and the results are also lower than t those of **Bag *et al*., (2021**) who recorded resistant *E. coli* isolates to amoxicillin-clavulanic acid (94.5%), followed by ampicillin (89.5%), and tetracycline (89.5%), each. *Escherichia coli* isolates from mastitis milk samples showed 24.2% resistance to imipenem (carbapenem) which was lower than that detected by **Ahmed *et al*., (2021**) (42.9%).

The current study found no results in the ESBL test for *E. coli* isolates from normal raw milk samples, and these results were lower than those of **Skočková *et al*., (2015)** (0.7%), while 9% of the isolates from mastitis milk were ESBL. That was close to the results of **Filioussis *et al*., (2020)** (6.7%). However, this result was higher than the findings of **Dahmen *et al*., (2013); Ali *et al*., (2017**), whose results were 0.3%, and 0.25%, respectively.

Molecular detection methods such as PCR and multiplex polymerase chain reaction (Multiplex PCR) are used for the detection of mastitis causing pathogens such as *E. coli*, and are considered to be the gold standard diagnosis method for mastitis. It is also used for the detection of virulence and antimicrobial resistance genes **(El-Sayed *et al*., 2017**). Antimicrobial resistance genes such as β-Lactamase genes (*bla*TEM, *bla*SHV, *amp*C), Imipenemase genes (*bla*IMP), and sulfonamide resistance genes (*Sul*1) were used in the present study for the detection of *E. coli* resistance genes. These genes help *E. coli* to resist a broad spectrum of antimicrobial agents, for example, the β-Lactamase genes help *E. coli* to hydrolyze β-Lactam ring found in β-lactam antibiotics, which inactivates the antibiotic effect (**Brinas *et al*., 2002**), imipenemase gene helps *E. coli* to resist carbapenems such as imipenem; and sulfonamide resistance genes such as *Sul*1 help *E. coli* to produce dihydropteorate synthetases that are insensitive to sulfonamides, which make *E. coli* undergo folic acid synthesis (**Poirel *et al*.., 2018**).

Escherichia coli isolates showed the presence of the *amp*C gene (94.4%). That was nearly similar to the results of **Fazel *et al*., (2019**) in Iran (92.8%), but was higher than those of **Ismail and Abutarbush, (2020**) in Jorden (86%). Additionally, the isolates showed the presence of the *Sul*1 gene in 2.8% of the isolates. This result is less than that studied by **Younis *et al*., (2017**) in Egypt (80%), and that of **Messele *et al*., (2019**) in Ethiopia (12.5%). In the present study, the isolates showed the presence of the *bla*SHV gene (14%) which was higher than what was mentioned by **Yang *et al*., (2018**)(2.7%), but less than what was recorded by **Xu *et al*., (2023**) in China (20.5%). The isolates of the current study, showed the presence of *bla*TEM (14%), which was lower when compared with the results of **Bag *et al*., (2021**) in Bangladesh (39.9%); and **Yu *et al*., (2020**), in China (83.1%). However, *E. coli* isolates showed the presence of the *Bla*IPM gene in 2.7% of the isolates, which couldn't be found in any other study related to bovine mastitis.

CONCLUSION

E. coli were isolated from 9.12% of the mastitis milk samples and 3.4% of normal milk

samples. All the *E. coli* isolates showed the highest antimicrobial resistance to amoxicillin-clavulanic acid, ceftazidime, and colistin. Only 3 isolates (9%) were positive for the ESBL test. *E. coli* isolates showed the presence of the *amp*C gene (94.4%), *bla*TEM, and *bla*SHV, which were detected in 5 (14%) isolates for each. While *Bla*IPM and *Sul*1 were found in one isolate (2.8%). The consumption of raw milk before any heat treatment increases the risk of *E. coli* infection.

Conflict of interest

The authors declare that no prospective conflicts of interest exist.

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