ANTIBIOTIC RESISTANCE PROFILES AND PREVALENCE OF ESBL-PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* IN MASTITIS CASES ON DAIRY FARMS IN EGYPT

EL ZAHRAA A. YOUSSEF [1]; SHEREEN S. EL-MOHANDES [2] AND MONA I. EL-ENBAAWY[3]

[1-2] Department of Mastitis and Neonatal Diseases, Animal Reproduction Research Institute, Agriculture Research Center (ARC), Giza 12611, Egypt

[3] Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt

ABSTRACT

Bovine mastitis, an inflammation in dairy cattle, causes significant economic losses, often triggered by bacterial pathogens like *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). The overuse of antibiotics has led to resistance through β-lactamase enzymes, including extended-spectrum beta-lactamases (ESBLs). This study focuses on isolating and characterizing ESBL-producing *E. coli* and *K. pneumoniae* from Egyptian dairy farms using both culture and molecular methods. Among 259 subclinical and 127 clinical mastitis milk samples, *Enterobacteriaceae* was found in 79.15% and 79.53% of cases, respectively. *E. coli* was the predominant pathogen, with 141 subclinical and 77 clinical cases, while *K. pneumoniae* was less common. The study identified ESBL-producing strains in 71 *E. coli* and 25 *K. pneumoniae* from subclinical mastitis, and 36 *E. coli* and 15 *K. pneumoniae* from clinical mastitis. The blaTEM gene was found in 85.04% of *E. coli* and 100% of *K. pneumoniae* isolates, indicating its dominant role in resistance. Antibiotic sensitivity analysis revealed significant resistance, with *E. coli* showing 100% resistance to tetracycline, 77.6% to ciprofloxacin, and 46.7% to gentamycin, while *K. pneumoniae* exhibited 100% resistance to tetracycline, 70% to ciprofloxacin, and 62.5% to cefpodoxime. Both pathogens had lower resistance to amoxicillin-clavulanic acid. These results underscore the need for improved control measures to combat antibiotic resistance and highlight the importance of alternative therapeutic strategies in treating mastitis.

Keywords: Beta-lactamase, *Escherichia coli, Klebsiella pneumoniae*, Mastitis, Dairy Cattle

INTRODUCTION

Bovine mastitis is characterized by inflammation of the udder in dairy cattle, presents a considerable challenge in global dairy farming, leading to substantial economic losses due to reduced milk yield and increased culling rates (Azooz *et al*., 2020 and Sharun *et al.,* 2021). Pathogens from the *Enterobacteriaceae* family, notably *E. coli* and *K. pneumoniae*, play a critical role in the aetiology of mastitis, particularly thriving in environments of stress induced by factors such as overcrowding and suboptimal management practices (Pal *et al*., 2020 and Zhang *et al*., 2016).

The β-lactam class of antibiotics is frequently employed to manage infections caused by Gram-negative bacteria, owing

Corresponding author: El Zahraa A. Youssef
E-mail address: dr_mona_1@hotmail.com
Present address: Department of Mastitis and Neonatal Diseases, Animal Reproduction Research Institute, Agriculture Research Center, Giza 12611, Egypt

to their efficacy and relatively low incidence of adverse effects (Aguilar-Montes de Oca et al., 2015). However, excessive use of these antibiotics can result in resistance, primarily through the production of β-lactamase enzymes (King et al., 2017). These enzymes, encoded by genes located on chromosomes, plasmids, and mobile genetic elements such as transposons and insertion sequences, have facilitated the dissemination of resistance across bacterial species within the Enterobacteriaceae family, contributing to multidrug resistance. Furthermore, the global population of ESBL-producing E. coli is predominantly comprised of a highly virulent clonal lineage (Castanheira et al., 2021 and Hussain et al., 2021).

ESBLs are a subset of β-lactamases capable of hydrolyzing a wide spectrum of β-lactam antibiotics, including penicillins and third- and fourth-generation cephalosporins. They represent a significant global public health concern. The genes encoding ESBLs are frequently located on plasmids, facilitating their dissemination. The most prevalent ESBL types are CTX-M, SHV, and TEM, although others such as OXA and PER are also notable (Paterson and Bonomo, 2005 and Castanheira et al., 2021). Multi-drug resistance (MDR) is commonly observed in ESBL-producing bacteria, often accompanied by co-resistance to antibiotics such as aminoglycosides, fluoroquinolones, and tetracyclines (Chen et al., 2010 and Timofte et al., 2014). This resistance significantly complicates treatment, leading to prolonged illness, increased healthcare costs, and a heightened risk of therapeutic failure (Pitout and DeVinney, 2017). A variety of diagnostic tools are available to clinical microbiology laboratories, including both phenotypic and genotypic assays for the detection of β-lactamases (Castanheira et al., 2021). Raw milk from cows with mastitis is a recognized source of ESBL-producing E. coli, with the potential for

horizontal gene transfer of these resistance genes between different bacterial species (Giedraitienė et al., 2011 and Haque et al., 2014).This article aims to isolate and characterize ESBL-producing E. coli and K. pneumoniae from mastitic cattle at a large-scale dairy farm in Egypt, assessing their prevalence and antimicrobial resistance patterns to reduce the chance of their transmission. The used methods were culture and molecular methods.

MATERIALS AND METHODS

Study period and location:
The study was conducted from October 1, 2021, to September 20, 2023, across four Egyptian regions: Giza, El Menofia, El Beheira, and Alexandria. Specimens were analyzed at the Animal Reproduction Research Institute in Giza.

Collection of milk samples
The study examined dairy herds in Giza, El Menofia, El Beheira, and Alexandria, Egypt. These medium-sized herds, comprising 60 to 160 cattle aged between 2 and 10 years, were evaluated for clinical and subclinical mastitis through visual and physical examinations, supplemented by the California Mastitis Test (Hussein et al., 2018).

A total of 386 milk samples were collected, with 259 samples from subclinical cases and 127 from clinical cases. Milk samples were collected aseptically following a teat preparation procedure that included wet cleaning and disinfection with 70% ethyl alcohol. The first three squirts of milk were discarded, after which 25-ml samples were obtained from each udder quarter into sterile containers. The samples were kept on ice, transported to the laboratory within 24 hours, stored at 4°C, and subjected to bacteriological examination within 3-4 hours of arrival (Amer et al., 2018).

Isolation

All quarter milk samples that yielded positive results in the field tests were subjected to microbiological examination. The samples were inoculated into nutrient broth and incubated at 37°C for 18 to 24 hours. Following incubation, a loopful of each inoculated broth was cultured on both MacConkey agar and blood agar media. All culture plates were then incubated at 37°C for 24 to 48 hours. Smears from suspected colonies were prepared and subjected to Gram staining. Colonies identified as Gram-negative, non-sporulating, medium-sized rods were further characterized biochemically using tests such as indole, methyl red, Voges-Proskauer, citrate, urease, and triple sugar iron tests (Quinn *et al.,* 2011). The isolates were subsequently preserved in 20% glycerol for further identification.

Phenotypic ESBL screening tests

For the phenotypic identification of ESBL-producing *E. coli* and *K. pneumoniae*, MacConkey agar supplemented with 1 mg/L cefotaxime (CFX) that was employed to distinguish between ESBL-positive and ESBL-negative isolates (Wilson and McCabe, 2007).

Additionally, ESBL-producing bacterial isolates were detected and differentiated using ChromID ESBL agar (bioMérieux, France), in accordance with the manufacturer's instructions. The bacterial isolates were cultured aerobically on ChromID ESBL agar at 37°C for 18 to 24 hours. Suspected ESBL-producing *E. coli* strains produced colonies exhibiting a pink to burgundy color on the agar, while ESBL-producing *Klebsiella* spp. presented with green colonies (Färber *et al.,* 2008).

ESBL confirmatory test

The confirmatory test was performed using the double-disc synergy test (DDST) in accordance with the guidelines for the detection of resistance mechanisms and specific resistance of clinical and/or epidemiological importance (EUCAST, 2017).

For this test, the following antibiotic discs were employed: cephalosporins [cefotaxime (30 μg), ceftazidime (30 μg)] and amoxicillin with clavulanic acid (30 μg). The cephalosporin discs were placed 20 mm apart from the amoxicillin-clavulanic acid disc on Mueller–Hinton agar. Following an incubation period of 18 to 24 hours, a strain was considered ESBL-positive if an increase in the inhibition zone around any cephalosporin disc, or an enlargement-commonly referred to as a "ghost zone"-towards the clavulanic acid disc was observed.

Antibiotic susceptibility testing

Antibiotic susceptibility testing of ESBL-producing *E. coli* isolates was carried out on Mueller-Hinton agar (HiMedia, Mumbai, India) using nine different commercially available antibiotic discs: ciprofloxacin [CIP] (5 μg), levofloxacin [LEV] (5 μg), amoxicillin-clavulanic acid [AMC] (30 μg), cefpodoxime [CPD] (10 μg), gentamicin [GN] (10 μg), meropenem [MEM] (10 μg), chloramphenicol [C] (30 μg), tetracycline [TE] (30 μg), and rifampicin [RD] (5 μg). The disk diffusion method was employed, and susceptibility was evaluated by measuring the inhibition zones, with classification as either sensitive or resistant according to the recommended guidelines (CLSI, 2019).

Analysis of ESBL Genes

Phenotypically confirmed ESBL isolates underwent genotypic analysis to identify the bla gene group. DNA extraction was performed using the QIAamp DNA Mini Kit (Catalogue No. 51304).

Screening of DNA from ESBL-producing bacteria was carried out using standard PCR with primers targeting genes associated with ESBL production (Table 1), specifically blaTEM, blaSHV, and blaOXA-1, as described by Colom *et al.*

(2003). The PCR assays were conducted using One PCR Master Mix™ (Emerald Amp GT PCR Mastermix, Takara, Code No. RR310A). The temperature and time conditions for the primers during PCR are detailed in Table 2, in accordance with the recommendations from specific authors and the Emerald Amp GT PCR Master mix (Takara) kit.

Statistical analysis

The statistical analysis was conducted using the Statistical Analysis System (SAS, 2014). The chi-square test was employed to analyze the enumeration data, following the methods outlined by Snedecor and Cochran (1989). This non-parametric test allowed for the evaluation of relationships between categorical variables, assessing whether the observed frequencies significantly deviated from the expected values. By applying the chi-square procedure, the study ensured a robust approach to understanding the distribution and association of the data, providing valuable insights into the patterns and relationships within the dataset.

Table 1: Oligonucleotide primers sequences

Gene	Primer Sequence	Amplified product	Reference
blaTEM	5'-ATCAGCAATAAACCAGC-3'	516 bp	Colom *et al.* (2003)
	5'-CCCCGAAGAACGTTTTC-3'		
blaSHV	5'-AGGATTGACTGCCTTTTTG-3'	392 bp	
	5'-ATTTGCTGATTTCGCTCG-3'		
blaOXA-1	5'-TCAACTTTCAAGATCGCA-3'	609 bp	
	5'-GTGTGTTTAGAATGGTGA-3'		

Table 2: Cycling conditions of the different primers during PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
blaTEM	94˚C 5 min.	94˚C 30 sec	54˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.
blaSHV	94˚C 5 min.	94˚C 30 sec	54˚C 40 sec	72˚C 40 sec	35	72˚C 10 min.
blaOXA-1	94˚C 5 min.	94˚C 30 sec	54˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.

The primary denaturation was carried out for one cycle, followed by secondary denaturation, annealing, and extension for 35 cycles, with a final extension performed for one cycle.

The PCR products were separated by electrophoresis in 1×TBE buffer at room temperature, with a 5 V/cm gradient applied to a 1.5% agarose gel. Following electrophoresis, the gel was transferred to a UV cabinet and visualized using a gel documentation system. Data from the gel image were analyzed using computer software for further interpretation and documentation, as outlined by Sambrook *et al.* (1989).

RESULTS

Out of a total of 259 milk samples from subclinical mastitis cases, 205 (79.15%) were positive for *Enterobacteriaceae*, while 54 (20.85%) were negative. Similarly, among the 127 milk samples from clinical mastitis cases, 101 (79.53%) tested positive for *Enterobacteriaceae*, and 26 (20.47%) were negative (Table 3 and Fig. 1).
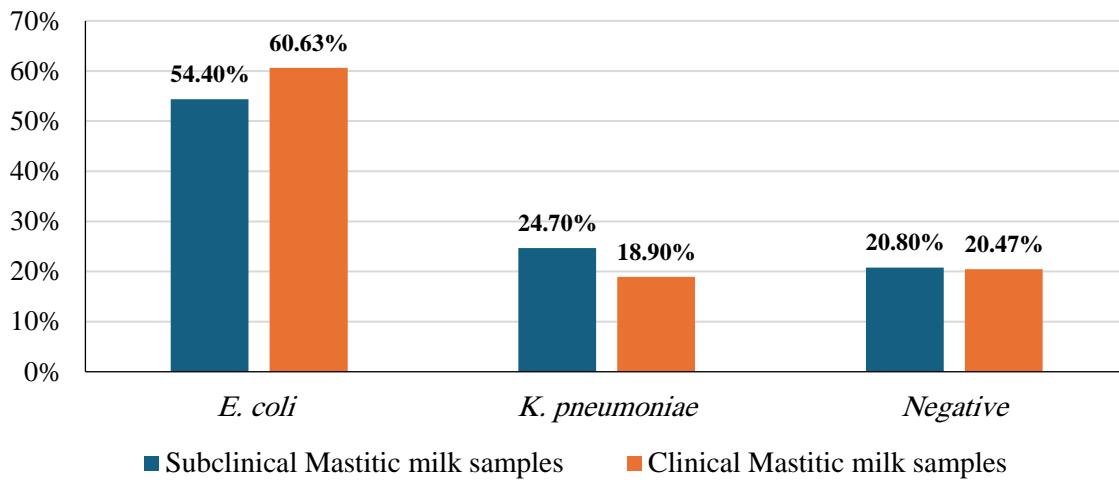
Fig. 1: Incidence of *E. coli* and *K. pneumoniae* in the examined subclinical and clinical mastitic milk samples.

Furthermore, among the 386 total milk samples, *E. coli* prevalence was higher compared to *K. pneumoniae* particularly in subclinical cases. *E. coli* was isolated from 218 samples (141 were from subclinical cases and 77 from clinical cases). Conversely, *K. pneumoniae* was identified in 88 samples overall, with 64 occurrences in subclinical mastitis and 24 in clinical mastitis. From 205 positive subclinical mastitis cases of which 141 samples were *E. coli* positive and 64 samples were *K. pneumoniae* positive and from 101 positive clinical mastitis cases, 77 samples were *E.*

coli positive and 24 samples were *K. pneumoniae positive* (Table 3 and Fig. 1).

The chi-square statistic (30.4336) was highly significant (p < 0.0001), indicating a strong association between the type of mastitis and the test results. Subclinical cases had a higher proportion of positive results compared to clinical cases. The Cramer's V value of 0.1985 suggests a weak to moderate association, underscoring that the type of mastitis influences test outcomes but to a limited extent.

Table 3: Incidence of *E. coli* and *K. pneumoniae* in the examined subclinical and clinical mastitic milk samples

Bacteria	Subclinical Mastitic milk samples (259)	Clinical Mastitic milk samples (127)	Total milk samples (386)	X^2		
				df	*Value*	*prop*
E. coli	141 (54.4%)	77 (60.63 %)	218 (56.4%)			
K. pneumoniae	64 (24.7 %)	24 (18.90%)	88 (22.7%)			
Total positive	205 (79.1 %)	101(79.5%)	306 (79.3%)	3	30.4336	p<0.0001
Negative	54 (20.8 %)	26 (20.47 %)	80 (20.7%)			

Percentages were calculated according to the total number of each group (259, 127 and 386)
The chi-square at p-value (p<0.0001) confirmed a highly significant

To distinguish between ESBL-positive and ESBL-negative isolates, MacConkey agar supplemented with 1 mg/L cefotaxime (CFX) and ChromID ESBL agar were utilized. Among the subclinical mastitis

cases, a total of 81 (31.64%) samples were initially suspected to be ESBL-producing *E. coli*, and 30 (11.72%) samples were suspected to be ESBL-producing *K. pneumoniae*. Subsequent confirmation

using the double disc synergy (DDS) method revealed that 71 (27.4%) samples were indeed ESBL-producing *E. coli* and 25 (9.65%) were confirmed as ESBL-producing *K. pneumoniae*. For the clinical mastitis cases, using MacConkey agar supplemented with 1 mg/L cefotaxime (CFX) and ChromID ESBL agar, 41 (32.28%) samples were suspected of being ESBL-producing *E. coli*, while 16 (12.60 %) samples were suspected of being ESBL-producing *K. pneumoniae*. The DDS method confirmed 36 (28.35%) samples as ESBL-producing *E. coli* and 15 (11.81%) as ESBL-producing *K. pneumoniae* (Table, 4 and Fig. 2- 5).

Table 4: Incidence of ESBL-producing *E. coli* and *K. pneumoniae* after phenotypic identification

Isolate	Subclinical Mastitic milk samples (259)	Clinical Mastitic milk samples (127)	Total milk samples (386)
ESBL *E. Coli*	71 (27.4%)	36 (28.35%)	107 (27.72%)
ESBL *K. pneumoniae*	25 (9.65%)	15 (11.81%)	40 (10.36%)
Total Positive ESBL	96 (37.07%)	51 (40.16%)	147 (38.08%)

Percentages were calculated according to the total number of each group (259, 127 and 386)
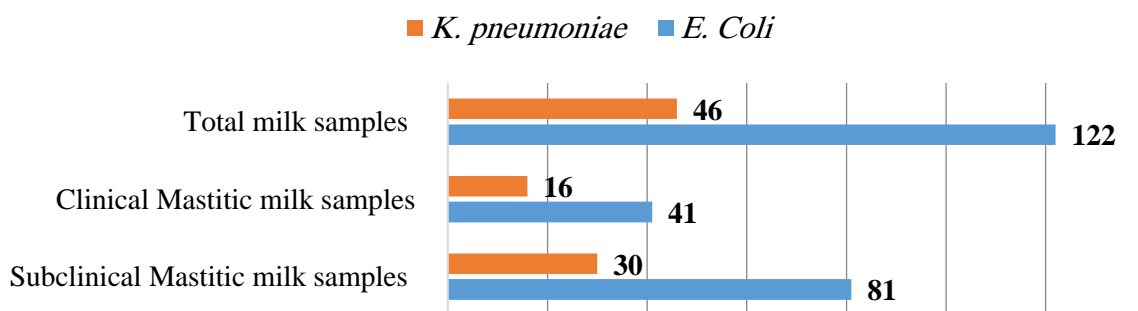*The chi-square at p-value (p<0.0001) confirmed a highly significant.*

Fig. 2: Incidence of suspected ESBL-producing *E. coli* and *K. pneumoniae* after screening on MacConkey agar.
NB: Percentage was calculated according to the total number of each group
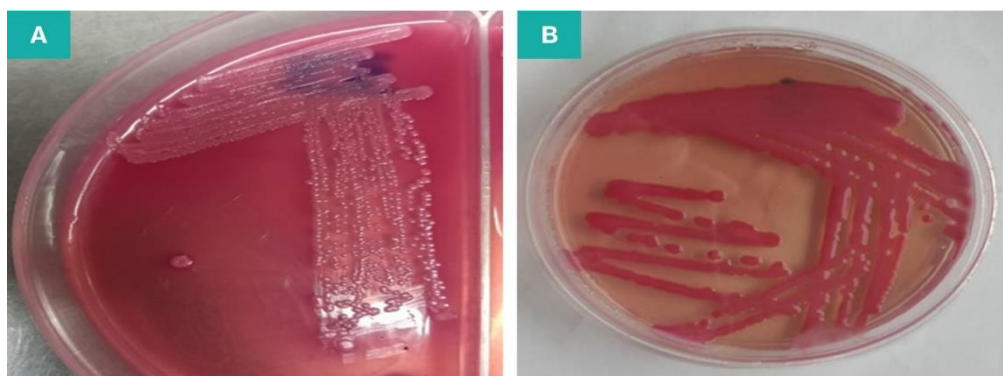

Fig. 3: *E. coli* and *K. pneumoniae* after screening on MacConkey agar supplemented with 1 mg/L cefotaxime (CFX). (A) *E. coli*, (B) *K. pneumoniae*
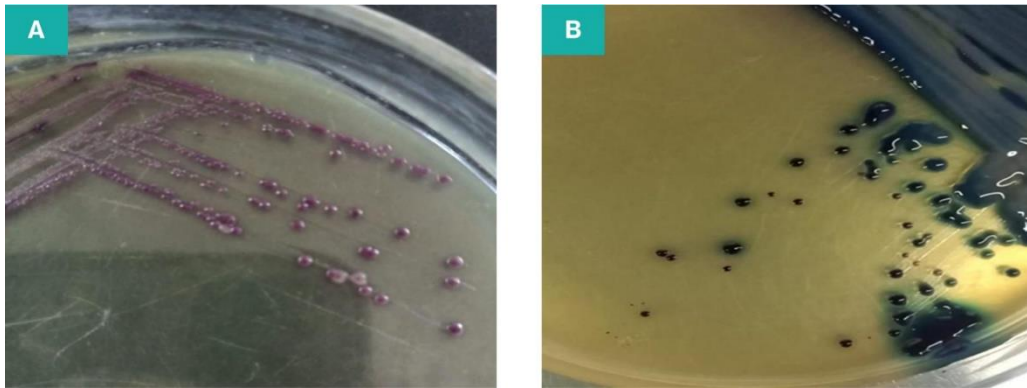
Fig. 4: *E. coli* and *K. pneumoniae* after screening on ChromID ESBL *agar* (A) *E. coli* ,(B) *K. pneumoniae*
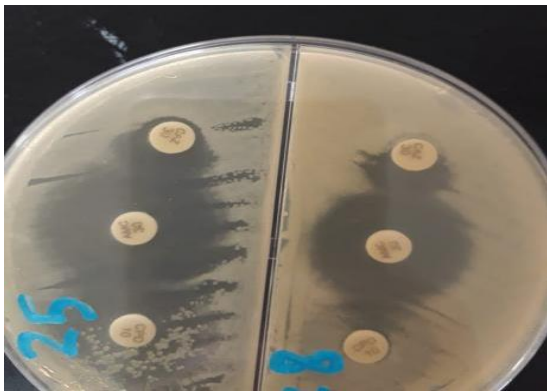

Fig. 5: DDST for conformation of positive ESBL-producing strains involves observing an increase or distortion in the inhibition zone of one of the antibiotics (Cefotaxime, Ceftazidime) towards the amoxicillin-clavulanate disk in an E. coli isolate

The prevalence of specific antibiotic resistance genes in ESBL-producing *E. coli* and *K. pneumoniae* isolates were presented in Table 5 and Fig. 6, 7 and 8. Among the 107 *E. coli* isolates, the *blaTEM* gene was identified in 91 cases, representing a high prevalence of 85.04%, additionally; the *blaSHV* gene was present in 15 isolates (14.01%), while the *blaOXA-1* gene was found in 46 isolates (42.9%). In contrast, all 40 *K. pneumoniae* isolates tested positive for the *blaTEM* gene (100%), while the *blaSHV* gene was present in 15 samples (37.5%) and *blaOXA-1* was not detected in any of the *K. pneumoniae* isolates. The antibiotic resistance patterns of ESBL-producing *E. coli* isolates from a total of 107 samples indicate varying levels of resistance and sensitivity to different antibiotics (Table 6). Notably, 46.7% of isolates were resistant to gentamycin, while 53.3% were sensitive. Ciprofloxacin showed a resistance rate of 77.6%, with 22.6% classified as intermediate, highlighting significant concerns regarding its efficacy. For cefpodoxime, 20.5% of isolates were resistant, but 27.1% remained sensitive. Resistance to amoxicillin-clavulanic acid was relatively low at 14.9%, with a high sensitivity rate of 65.4%. Other antibiotics, including rifampicin and tetracycline, showed resistance in 29.1% and 100% of isolates, respectively. The antibiotic resistance patterns of ESBL-producing *K. pneumoniae* isolates from a total of 40 samples outlined in Table (7). The data reveal significant resistance rates for various antibiotics. Specifically, 55% of the isolates were resistant to gentamycin, while only 45% were sensitive. Ciprofloxacin exhibited a notable resistance rate of 70%, with 12.5% of isolates remaining sensitive. For cefpodoxime, 62.5% were resistant, indicating limited effectiveness. Resistance to amoxicillin-clavulanic acid was lower, with only 7.5% of isolates resistant and a high sensitivity rate of 72.5%. Rifampicin resistance was found in 22.5% of cases, while response to tetracycline showed universal resistance, with no sensitive isolate.

Table 5: Prevalence of different resistant genes in ESBL-producing *E. coli* and *K. pneumoniae* isolates

Type of resistance gene	Positive *E. coli* isolates (107)		X^2			Positive *K. pneumoniae* isolates (40)		X^2			Total positive (147)	
	No.	%	df	Value	p	No.	%	df	value	p	No	%
blaTEM	91	85.04%	2	109.1	<.0001	40	100%	2	82.24	p<0.0001	131	89.12%
blaSHV	15	14.01%				15	37.5%				30	20.41%
blaOXA-1	46	42.9%				0	0 %				46	31.29%

Percentages were calculated according to the total number of each group (259, 127 and 386)
The chi-square at p-value (p<0.0001) confirmed a highly significant

Table 6: Antibiotic resistance pattern of ESBL-producing *E. coli* producers (n = 107)

Antibiotic used	Susceptibility pattern					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Gentamycin	50	46.7 %	0	0	57	53.3%
Ciprofloxacin	83	77.6%	24	22.4%	0	0
Cefpodoxime	56	52.3%	22	20.6%	29	27.1%
Levofloxacin	73	68.2%	0	0	34	31.8%
Amoxicillin-Clavulanic Acid	23	21.5%	25	23.4%	59	55.1%
Meropenem	16	14.9%	21	19.7%	70	65.4%
Rifampicin	107	100%	0	0	0	0
Chloramphenicol	64	59.8%	11	10.3%	32	29.9%
Tetracycline	107	100%	0	0	0	0

Table 7: Antibiotic resistance pattern of ESBL-producing *K. pneumoniae* isolates (n = 40)

Antibiotic used	Susceptibility pattern					
	Resistant		Intermediate		Sensitive	
	No	%	No	%	NO	%
Gentamycin	22	55%	0	0	18	45%
Ciprofloxacin	28	70%	7	17.5%	5	12.5%
Cefpodoxime	24	50%	9	22.5%	7	17.5%
Levofloxacin	26	65%	3	7.5%	11	27.5%
Amoxicillin-clavulanic acid	13	32.5%	6	15%	21	52.5%
Meropenem	8	20 %	3	7.5%	29	72.5%
Rifampicin	40	100%	0	0	0	0
Chloramphenicol	40	100%	0	0%	0	0%
Tetracycline	40	100%	0	0	0	0

The chi-square statistics (109.0980) and the p-value (p<0.0001) confirmed a highly significant relationship between gene type and infection status. Cramer's V value of 0.5830 indicates a strong association, implying that the presence of specific genes significantly predicts the likelihood of infection, moreover, the chi-square value (82.2378) was highly significant (p< 0.0001), confirming a strong association between gene type and infection status. Cramer's V of 0.8278 indicated a powerful relationship, suggesting that the type of resistance gene is a highly predictive factor for infection in *K. pneumoniae* cases.

These statistical analyses illustrate significant relationships between mastitis type, resistance genes, and infection outcomes, providing valuable insights into disease patterns and the predictive value of specific genetic markers.
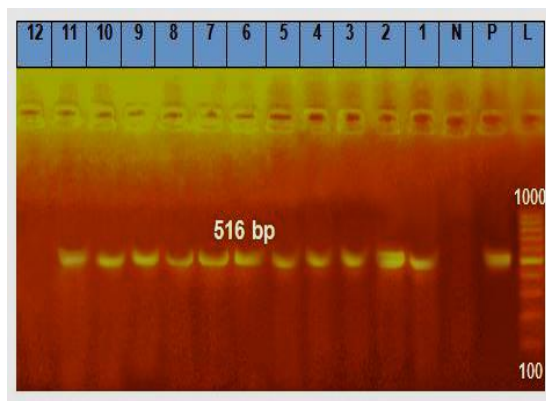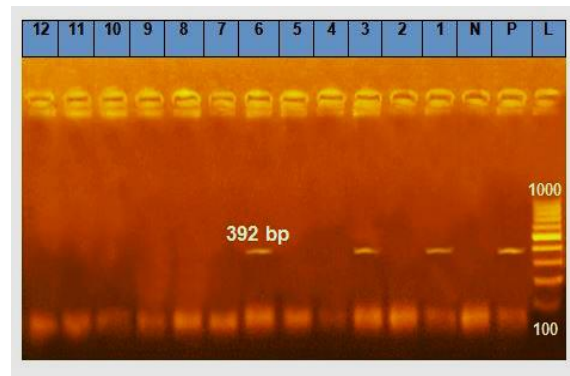

Fig. 7: Gel Electrophoresis image showing amplification of 392 bp product corresponding to the blaSHV gene of E. coli and K. pneumoniae isolated from milk. Lane (L): 100 bp DNA ladder, lane (P): positive control, lane (N): Negative control and lanes corresponding to milk samples 1, 3, 6 were positive while samples in lanes 2,4,5,7,8,9,10,11 and 12 were negative.
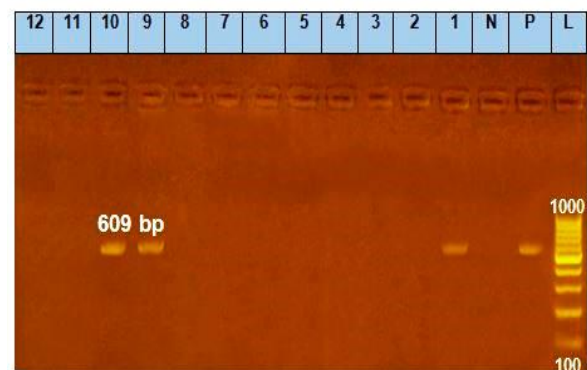

Fig. 6: Gel Electrophoresis image showing amplification of 516 bp product corresponding to the blaTEM gene of E. coli and K. pneumoniae isolated from milk. Lane (L):100 bp DNA ladder, lane (P): positive control, lane (N): Negative control and lanes corresponding to milk samples 1,2,3,4,5,6,7,8, 9, 10 and 11 were positive while sample in lane 12 was negative.

Fig. 8: Gel electrophoresis image showing amplification of 609 bp product corresponding to the *bla*OXA-1 gene of *E. coli* and *K. pneumoniae* isolated from milk. Lane (L): 100 bp DNA ladder, lane (P): positive control, lane (N): Negative control and lanes corresponding to milk samples 1, 9, 10 were positive while samples in lanes 2,3.4,5,6,7,8 ,11 and 12 were negative.

DISCUSSION

This study addresses the significant threat posed by antibiotic-resistant bacteria, with a specific emphasis on ESBL-producing *E. coli* and *K. pneumoniae*, which undermine the efficacy of numerous antibiotics. Within the context of mastitic dairy cattle in Egypt, these resistant bacteria present a substantial risk to the nation's dairy industry and, consequently, to its food security. The research aims to detect and characterize these pathogens to elucidate their prevalence and resistance profiles, thereby informing the development of improved control strategies and optimizing antibiotic usage.

Mastitis continues to represent a major infection challenge in dairy cows, constituting a critical issue for dairy farmers and the broader dairy industry. This condition is caused by a diverse array of bacterial species (Roussel *et al.,* 2017), with members of the *Enterobacteriaceae* family, including *E. coli* and *K. pneumoniae*, being particularly concerning as they are recognized significant etiological agents of bovine mastitis (Zhang *et al.,* 2016). Coliform mastitis may present with a range of symptoms, from mild udder inflammation and subtle changes in milk appearance to severe clinical manifestations and marked reduction in milk yield (Suojala *et al.,* 2013).

Estimates indicate that clinical mastitis accounts for 33%-38% of health-related expenses for dairy herds and is the primary reason for antibiotic administration in lactating dairy cattle, representing approximately one-third of all antibiotics used (Ahmed and Shimamoto, 2011). Subclinical mastitis, responsible for nearly 70% of the economic losses associated with mastitis, poses an even greater challenge on dairy farms. Its prevalence is estimated to be approximately 15 to 40 times higher than that of clinical mastitis (El-Attar *et al.,* 2002; Losinger, 2005 and El-Awady and Oudah, 2011).

In this study, *E. coli* was isolated from both subclinical and clinical mastitis cases at rates of 54% and 61%, respectively. These rates are significantly higher compared to those reported in Egypt by Ibrahim *et al.* (2018), who observed *E. coli* isolation rates of 14% for subclinical and 31% for clinical mastitis, and by Ahmed *et al.* (2021), who reported rates of 9.1% for clinical cases and 40% for subclinical mastitis. In comparison, *E. coli* was isolated from 16.98% of dairy cattle in Nepal (Bhandari *et al.,* 2021) and 33.8% in Ethiopia (Sarba *et al.,* 2023). The variation in prevalence may be attributed to factors such as hygiene and sanitation practices, udder cleanliness, proper teat end management, and appropriate stall conditions (Neculai-Valeanu and Ariton, 2022). However, the prevalence observed in this study was lower than that reported by El-Mohandes *et al.* (2022), who isolated *E. coli* from 80.5% of subclinical and 85.7% of clinical mastitis cases, as well as by Botrel *et al.* (2010) and Al-Emon *et al.* (2024), who found *E. coli* in more than 80% of coliform mastitis cases, with a prevalence of 81.99%.

K. pneumoniae was isolated from 25% of subclinical and 19% of clinical mastitis cases. Comparable findings were reported in China by Wu *et al.* (2022) and Cheng *et al.* (2021), with prevalence rates of 26.94% and 23%, respectively. However, these rates are lower than those reported by Al-Emon *et al.* (2024), who found prevalence of 66.82%, and by Fu *et al.* (2022) and Badri *et al.* (2017), who detected prevalence rates of 35.91% and 51.42% in raw milk samples from cows in Sudan.

Over recent decades, numerous studies have highlighted the role of production animals as reservoirs and carriers of ESBL-producing bacteria. These animals are directly involved in the food chain, potentially facilitating the spread of ESBLs through the consumption of animal-origin foods, especially when these foods are

consumed raw or inadequately processed, such as in raw milk production (Madec, 2017 and Liu *et al.,* 2018).

ESBL detection methods have been previously described. In this study, MacConkey agar supplemented with 1 mg/L cefotaxime (CFX) and ChromID ESBL agar were used to distinguish between ESBL-positive and ESBL-negative isolates. Phenotypically confirmed ESBL isolates were further analyzed using DDST to assess the bla gene group.

Swarna *et al.* (2015) utilized ESBL Screen Agar (ESA) comprising MacConkey agar I with ceftazidime (1.0 mg/L) and MacConkey agar II with cefotaxime (1.0 mg/L), cloxacillin (400 mg/L), and vancomycin (64 mg/L). Cefotaxime (CFX) suppresses the growth of non-ESBL-producing bacteria while permitting the growth of ESBL strains.

The chromogenic agar medium ChromID ESBL was compared with ESBL agar medium for selective isolation and presumptive identification of ESBL-producing *Enterobacteriaceae* from clinical samples (Réglier-Poupet *et al.,* 2008).

ESBL production was confirmed using DDST according to EUCAST guidelines. This method, which involves third-generation cephalosporins (3GC), is a straightforward and reliable technique. However, the presence of AmpC β-lactamases may lead to false-negative results (Kaur *et al.,* 2013). In this study, phenotypic confirmation revealed that 27.4% of *E. coli* isolates from subclinical mastitic milk samples and 28.35% from clinical mastitic milk samples were ESBL producers. These figures are lower than the 38.2% of subclinical and 39.3% of clinical mastitic milk cases reported by El-Mohandes *et al.* (2022), but higher than the 22.96% detection rate reported by Yang *et*

al. (2018). Similarly, ESBL production was confirmed in 9.65% of *K. pneumoniae* isolates from subclinical mastitic milk samples and 11.81% from clinical mastitic milk samples, a higher rate than the 1.4% reported by Taniguchi *et al.* (2021).

β-lactamase-producing organisms exhibit resistance not only to extended-spectrum cephalosporins but also to other antimicrobials (Hemalatha *et al.,* 2007). The antibiotic resistance patterns of ESBL-producing *E. coli* isolates revealed complete resistance to rifampicin (100%) and tetracycline (100%), followed by high resistance rates to ciprofloxacin (77.6%), levofloxacin (68.2%), chloramphenicol (59.8%), and cefpodoxime (52.3%). Most ESBL producers showed greater sensitivity to meropenem (73.8%) and amoxicillin-clavulanic acid (55.1%), with gentamicin showing a sensitivity of 53.3%. In contrast, *K. pneumoniae* isolates exhibited 100% resistance to rifampicin, tetracycline, and chloramphenicol, with further resistance to ciprofloxacin (70%), levofloxacin (68.2%), and cefpodoxime (52.3%). These isolates demonstrated higher sensitivity to meropenem (72.5%) and amoxicillin-clavulanic acid (52.5%), with gentamicin sensitivity at 45%. These findings are consistent with a study in Pakistan, where *K. pneumoniae* showed high resistance to vancomycin, fusidic acid, amoxicillin, sulfamethazine, and chloramphenicol, while being highly sensitive to ceftazidime, ciprofloxacin, levofloxacin, amikacin, gentamicin, tetracycline, and imipenem (Saddam *et al.,* 2023).

Resistance genes were characterized using PCR with specific primers for blaTEM, blaSHV, and blaOXA-1. Genetic analysis of 107 phenotypically ESBL-positive *E. coli* isolates revealed that the blaTEM gene was the most prevalent, detected in 85.04% (91/107) of the isolates. This is consistent with Aflakian *et al.* (2023), who also identified blaTEM as the most frequent resistance gene, although it is higher than

the 29.4% detection rate reported by Ibrahim *et al.* (2018) and the 60.7% prevalence reported by Al Emon *et al.* (2024). Additionally, blaSHV was detected in 14.01% (15/107) of the isolates, aligning with Ibrahim *et al.* (2018) and Ali *et al.* (2016) who reported rates of 11.8% and 16.67%, respectively. blaOXA-1 was found in 42.9% (46/107) of *E. coli* isolates.

In *K. pneumoniae* isolates, blaTEM was present in 100% (40/40) of the cases, a rate higher than the 42.5% reported by Al Emon *et al*. (2024). Additionally, blaSHV and blaOXA-1 were identified in 37.5% (15/40) and 0%(0/40) of the isolates, respectively**.**

CONCLUSION

This study highlights 1- The significant presence of ESBL producing bacteria, particularly *E. coli and K. pneumoniae.* 2- It is clear that the prevalence of these bacterial isolates in clinical and subclinical mastitic milk samples, with *E. coli* being the predominant pathogen, followed by *K. pneumoniae*, and a notable portion of the samples showing no bacterial growth. 3- The widespread presence of blaTEM in both bacterial species, raises concerns regarding multidrug resistance in infections caused by these pathogens. 4- These findings underscore the need for effective control measures to address antibiotic-resistant pathogens and stress the importance of ongoing monitoring and targeted interventions on dairy farms.

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أنماط مقاومة المضادات الحيوية وانتشار البكتيريا *E. coli* و*K. pneumoniae* المنتجة لـ ESBL في حالات التهاب الضرع في مزارع الألبان في مصر

الزهراء أحمد عبد الحليم يوسف ، شيرين سمير ابراهيم المهندس ، منى اسماعيل الانبعاوى

Email: dr_mona_1@hotmail.com   Assiut University web-site: www.aun.edu.eg

التهاب الضرع البقري، وهو التهاب يصيب الأبقار الحلوب، يسبب خسائر اقتصادية كبيرة، وغالبًا ما يحدث بسبب مسببات الأمراض البكتيرية مثل الإشريكية القولونية (*E. coli*) والكلبسيلا الرئوية (*K. pneumoniae*). أدى الإفراط في استخدام المضادات الحيوية إلى ظهور مقاومة من خلال إنزيمات بيتا لاكتاماز، بما في ذلك بيتا لاكتاماز ذات الطيف الموسع (ESBLs). تركز هذه الدراسة على عزل ووصف البكتيريا (*E. coli*) المنتجة لإنزيمات بيتا لاكتاماز ذات الطيف الموسع (ESBLs) و*K. pneumoniae* من مزارع الألبان المصرية باستخدام كل من طرق الزرع والطرق الجزيئية. من بين ٢٥٩ عينة حليب تحت السريري و١٢٧ عينة سريرية مصابة بالتهاب الضرع ، تم العثور على *Enterobacteriaceae* في ٧٩,١٥٪ و٧٩,٥٣٪ من الحالات على التوالي. كانت *E. coli* هي العامل الممرض السائد، مع ١٤١ حالة تحت سريرية و٧٧ حالة سريرية، في حين كانت *K. pneumoniae* أقل شيوعًا. حددت الدراسة سلالات منتجة لإنزيمات بيتا لاكتاماز ذات الطيف الموسع في ٧١ حالة من *E. coli* و٢٥ حالة من *K. pneumoniae* من التهاب الضرع تحت السريري، و٣٦ حالة من *E. coli* و١٥ حالة من *K. pneumoniae* من التهاب الضرع السريري. تم العثور على جين blaTEM في ٨٥,٠٤٪ من عزلات *E. coli* و١٠٠٪ من عزلات *K. pneumoniae*، مما يشير إلى دوره المهيمن في المقاومة.

أظهر تحليل حساسية المضادات الحيوية مقاومة كبيرة، حيث أظهرت *E. coli* مقاومة بنسبة ١٠٠٪ للتتراسيكلين، و٧٧,٦٪ للسيبروفلوكساسين، و٤٦,٧٪ للجنتاميسين، في حين أظهرت *K. pneumoniae* مقاومة بنسبة ١٠٠٪ للتتراسيكلين، و٧٠٪ للسيبروفلوكساسين، و٦٢,٥٪ للسيفبودوكسيم. كان لدى كلا الممرضين مقاومة أقل لحمض أموكسيسيلين-كلافولانيك. تؤكد هذه النتائج على الحاجة إلى تحسين تدابير التحكم لمكافحة مقاومة المضادات الحيوية وتسلط الضوء على أهمية الاستراتيجيات العلاجية البديلة في علاج التهاب الضرع.

الكلمات المفتاحية: بيتا لاكتاماز ، *E. coli* ، *K. pneumoniae* ، التهاب الضرع، الأبقار الحلوب