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Advanced studies on extended spectrum beta-lactamase producing *Enterobacteriaceae* in dairy cattle farms at Behaira province

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ABSTRACT

The rise of bacteria resistant to antibiotics, such as *Escherichia coli* and *Klebsiella* species, in milk poses a severe threat to public health since milk is widely regarded as a complete food and is a staple of the daily diet of people, especially those in Egypt. Beta lactamase with an extended spectrum (ESBL). Therefore, the purpose of this study was to investigate the prevalence and characteristics of *K. pneumoniae* and *E. coli* that produce ESBLs in dairy cattle and milk farms. In this investigation, a total of 150 milk samples were cultured on VBGR, MacConkey, and Hichrome to isolate these bacteria, and antibiotic resistance was evaluated by using the double disc diffusion technique. Subsequently, biochemical and serotyping testing were used to confirm and identify the suspicious microorganisms. Using a PCR test that targets bla_{CTX-M}, ESBL producers were identified. Of the samples tested, 34 (22.7%) tested positive for *K. pneumoniae* in milk, and 21 (14%), positive for *E. coli*. Different ETEC serotypes (O128:H2 and O127: H6) and EHEC serotypes (O91: H21 and O26: H11) were found contaminated in milk. Additionally, 21 samples (67.7%) had *K. pneumoniae* serotype B1 as the prevalent serotype. Most isolates had the β-lactamase bla_{CTX-M} gene, which has become more significant among bacteria that produce ESBL globally. bla_{CTX-M} gene was detected in 65% of *E. coli* and 80% of *K. pneumoniae*. Thus, given the trend of farmer-to-consumer direct marketing, raw milk is a possible source of exposure for the consumer—something that is becoming increasingly important. Lastly, calves may potentially acquire ESBL-producing bacteria via waste milk, which would increase the prevalence of antibiotic resistance in the agricultural setting.

Introduction

Dairy products and milk are high-nutrient meals that provide energy, high-quality protein, and a variety of readily absorbed important micronutrients (Górska-Warsewicz *et al*., 2019). For two reasons, the demand for dairy products and technologies will increase during the next fifty years. First, as global per capita wealth rises, there will be a greater demand for dairy and other animal-based foods, which will increasingly supply emerging nations with vital nutrients. Second, while considering agricultural methods, dairy products effectively satisfy human nutritional needs. The production of milk requires less space to generate 1 g of easily consumable protein (Britt *et al*., 2018). Global milk production roughly 81% from cows, 15% from buffalo, and 4% from goats, sheep, and camels combined is expected to increase at a rate of 1.5% per year over the next ten years, to reach 1 039 Mt in 2032, faster than the majority of other major agricultural commodities (OECD and FAO, 2023). Given its ability to generate money for African nations, the dairy sector ought to take the lead in advancing the transformation agenda.

Animals and humans are both affected by the complex public health problem known as multidrug resistance (MDR) (Tigabie *et al*., 2023). Because of their excellent selective toxicity, strong killing effects, and high specificity, beta-lactam antibiotics are often utilized in veterinary medicine (Zeng and Lin, 2013). Antibiotics belonging to the β-lactam group can cause resistance in Gram-negative bacteria through several ways. The primary resistance mechanism is the production of β-lactamase enzyme (King *et al*., 2014). Genes in chromosomes, plasmids, or transposons of microbes encode the β-lactamase enzymes; these plasmids and mobile elements are easily transmitted across the species within the *Enterobacteriaceae* family (Hussain *et al*., 2021). Enzymes known as extended spectrum beta-lactamases (ESBL) have the ability to hydrolyze several β-lactam antibiotics, hence contributing to penicillin resistance, the 3rd and 4th generation cephalosporins (Mariana Castanheira *et al*., 2021). Because of this, *Enterobacteriaceae* especially *E. coli* and *Klebsiella* species have begun to develop resistance to ESBLs (Montso *et al*., 2019). According to Pishtiwan and Khadija 2019), bla_{CTX-M}, *bla*TEM, bla_{SHV}, and bla_{OXA} are the most prevalent ESBL-encoding genes.

Data on ESBL-producing bacteria in dairy cattle from Egypt are few. Thus, this study aimed to identify, classify, and assess the pattern of antibiotic sensitivity for *Enterobacteriaceae* (*E. coli* and *K. pneumoniae*).

Materials and methods

Sample collection, isolation and identification of Enterobacteriaceae

A total of 150 milk samples were collected in Behaira province from September 2022 to March 2023, comprising 50 bulk tank milk and 100 raw milk samples from cattle farms. Within an hour, collected samples were delivered to Animal Health Research Institute, Damanhour,s lab in a chilled box at 4°C, where they were processed right away. According to (MacFaddin, 2000), suspected isolates of *K. pneumoniae* and *E. coli* were detected on VRBG media.

Morphological identification

Gram's stain was used to stain films of pure suspected cultures, which were then examined under a microscope. It was assumed that the medium-sized, Gram-negative, evenly stained coccobacilli were *K. pneumoniae* and *E. coli* (ISO, 2013).

Biochemical identification

The triple sugar iron agar (TSIA) test and IMViC techniques were

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used in the subsequent biochemical tests to determine the presence of *K. pneumoniae* and *E. coli* in each isolate. To generate enough surface growth on Triple Sugar Iron (TSI) agar, isolated organisms were inserted with a needle into the butt's bottom and then pulled over the slant. The tubes that were injected were kept at 37°C for a full day of incubation. Blacking the medium revealed the presence of hydrogen sulphide. A helpful collection of reactions known as IMViC was frequently used to identify members of the *Enterobacteriaceae* family. The four tests included the citrate utilization test, the Voges-Proskauer (VP) test, the methyl red (MR) test, and the indole test (Sridhar Rao, 2019). In addition to other test such as Urease test, Gelatin hydrolysis test, Detection of Ornithine decarboxylase (ODC), Detection of L- lysine decarboxylase (LDC), Detection of Arginine decarboxylase (ADH), Detection of β- galactosidase (ONPG) and Fermentation of sugars.

Table 1. Biochemical tests for identification of *E. coli*

Biochemical test	*E. coli*
Motility	V
Indole	+
Methyle red	+
Voges Proskuaer	-
Citrate utilization	-
Urease	-
H2S	-
Nitrate reduction	+
Gelatin liquefaction	-
ODC	V
LDC	V
Arginine dihydrolase	V
ONPG	+
Sugar fermentation	
Lactose	+
Sucrose	V
Dulcitol	V
Salicin	V
Arabinose	+
Inositol	-
Xylose	V

Nitrate reduction test (only for E. coli)

One milliliter of a solution containing eight grams of sulphanilic acid in 100 milliliters of 5 N acetic acid was added and mixed, followed by the dropwise addition of five grams of alpha-naphthylamine in 100 milliliters of 5 N acetic acid. The culture to be examined was inoculated into five milliliters of peptone broth containing 0.1% potassium nitrate, and the mixture was then incubated at 37°C for four days. The test resulted in a positive result when the color became red.

Serological identification of E. coli

The isolates were identified serologically by the use of rapidly diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) in accordance with the manufacturer's instructions for enteropathogenic type detection (Kok *et al*., 1996).

String test for detection of mucoviscosity for identification of K. pneumoniae (HVKP)

On nutrient agar plates, a loopful of the suspicious culture was extended. Hypermucoviscous *K. pneumoniae* (HVKP) was the term given to any produced viscous string longer than 5 mm, which was deemed a positive result of the test. On the other hand, the remaining negative isolates were classified as classic *K. pneumoniae* (CKP) as reported by Shon *et al*. (2013)

Serological identification of K. pneumoniae (Quellung test)

The antigens K1 and K2 of *Klebsiella pneumoniae* were found serologically in the suspected isolates. We acquired these particular antigens from the Statens Serum Institute in Copenhagen, Denmark. As a result, the Quellung test was conducted in compliance with the producer's instructions. Microscopically, the antigen-antibody responses are monitored. When the typing antiserum's type-specific antibody binds to the capsular polysaccharide, a positive quellung response occurs, and the capsule surrounds the dark blue-stained cell like a clearly defined halo. Conversely, the absence of a distinct, expanded halo encircling the stained cell indicates a negative quelling reaction (Edmondson and Cooke, 1979).

Molecular Diagnostic Methods

Using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) and making certain adjustments based on the manufacturer's instructions, DNA was extracted from the samples. By using a direct PCR that targets the *bla*TEM gene, *E. coli* and *K. pneumoniae* were identified. The PCR experiment was conducted using the following primer sequences: In forward direction, 5′ -ATCAGCAATAAACCAGC-3′; in reverse, 5′ is CCCCGAAGAACGTTTTC-3′. A 25 µl PCR reaction was conducted with 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template. According to Colom *et al*. (2003), the PCR for bla_{CTX-M} was conducted as follows: denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, annealing at 54°C for 40 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. Following ethidium bromide staining, the amplified products were visualized using 1.5% agarose gel electrophoresis. A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel, and computer software was used to analyse the data.

Determination of antimicrobial susceptibility

Using commercially available discs, the disc diffusion technique was used to assess the antimicrobial susceptibility of the ESBL isolates against 16 antimicrobials from 7 different antimicrobial classes. The CLSI recommendations (CLSI, 2018) were followed while considering the various types of antibiotics. Penicillin, aminoglycosides, tetracycline, cefatoxine, flurophenicol, quinolone, and fluoroquinolone were among them. According to Cantón and Ruiz-Garbajosa (2011), resistance to at least one antimicrobial agent from three or more antimicrobial classes is known as multidrug resistance (MDR).

Results

Of the 55 samples (36.7%) that included *Enterobacteriaceae*, 21 (38%) had colonies similar to *E. coli*. isolated and identified, while 34 (62%) had colonies similar to *K. pneumoniae*. Using a 24-hour culture method and the Gram staining approach, the cellular morphology was examined under a microscope. Gram-negative *E. coli* was a motile, Gram-negative bacterium that looked like a rod with a little tail under a microscope. Moreover, macConkey has a strong pink hue, EMB displays blue-black with green metallic gleam, and ESBL Hicrome displays violet colonies (Table 1). Conversely, *Klebsiella* was a rod-shaped, encapsulated, non-motile, Gram-negative bacteria. On MacConkey agar, it showed up as a mucoid lactose fermenter. On EMB it produced large, mucoid, pink to purple col-

onies with no metallic green sheen. On ESBL Hicrome it appeared as blue or green colonies (Table 2).

Table 2. Biochemical tests for identification of *K. pneumoniae*.

Biochemical test	Biotypes				
	B1	B2	B3	B4	B5
Motility	-	-	-	-	-
Indole	-	-	-	-	-
Methyle red (MR)	+	+	+	+	+
Voges Proskuaer (VP)	-	-	-	-	-
Citrate utilization	+	+	-	+	-
Urease	+	+	+	-	+
H2S	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-
ODC	-	-	-	-	-
LDC	-	-	+	+	+
Arginine dihydrolase	-	-	-	-	-
ONPG	+	+	+	+	+
Sugar fermentation					
Lactose	+	+	+	+	+
Glucose	+	+	+	+	+
Mannitol	+	+	+	+	+
Inositol	+	+	+	+	-
Sorbitol	+	+	+	+	+
Rhamnose	+	-	+	+	+
Arabinose	+	+	+	+	+

Table 3. Serological identification of the isolated *E. coli* strains.

Sero-diagnosis	Strain characterization
O128: H2	ETEC
O128: H2	ETEC
O159	EIEC
O17: H18	EPEC
O128: H2	ETEC
O91: H21	EHEC
O127: H6	ETEC
O26: H11	EHEC
O91: H21	EHEC
O128: H2	ETEC
O128: H2	ETEC
O91: H21	EHEC
O17: H18	EPEC
O128: H2	ETEC
O128: H2	ETEC
O91: H21	EHEC
O127: H6	ETEC
O26: H11	EHEC
O128: H2	ETEC
O91: H21	EHEC

Twenty samples were verified by serological analysis as *E. coli* colonies from milk, out of the twenty-one suspected colonies. The 20 positive samples included six distinct serotypes of *E. coli.* Table 3 displays the outcomes of the latex agglutination serotyping of *E. coli.* Out of all the six *E. coli* serotypes that were identified, *E. coli* O128:H2 had the greatest proportion of incidence (40%), followed by *E. coli* O91 (25%) and *E. coli* O159 (5%). Thirteen of the thirty-four milk samples that were thought to be *K. pneumoniae* colonies had serological confirmation. Two mixed cultures and one *Serratia marscesens* were found in the other three samples. Table 4 displays the findings from the serotyping of *K. pneumoniae.*

Table 4. Serological identification of the *Klebsiella* pneumonia isolated strains.

Biotyping	String test	Serodiagnosis
B1	HVKP	K1
B5	CKP	K2
B1	CKP	K1
B1	CKP	K1
B1	CKP	K2
B3	HVKP	K1
B1	CKP	Untypable
B1	HVKP	K1
B1	HVKP	K1
B4	HVKP	K1
B3	CKP	K2
B1	HVKP	K1
B5	CKP	K2
B1	CKP	K1
B1	CKP	K1
B1	CKP	K2
B3	HVKP	K1
B1	HVKP	K1
B1	HVKP	K1
B4	HVKP	K1
B3	CKP	K2
B1	CKP	K1
B1	CKP	K1
B1	CKP	K2
B5	CKP	K2
B1	CKP	K1
B1	CKP	K1
B1	CKP	K2
B1	CKP	Untypable
B1	HVKP	K1
B4	HVKP	K1

The isolates of ESBL (*E. coli* and *K. pneumoniae*) obtained from milk resistance were presented in Table 5.

Table 5. Antimicrobial resistance patterns of the recovered isolates.

Antibiotic	*E. coli* (Resistance %)	*K. pneumoniae* (Resistance %)
CTX	23.8	67.6
CAZ	36	79.4
AMC	22.2	100
CL	42.9	64.7
LE	33.3	47
IPM	42.9	50
CPM	23.8	100

CTX: Cefotaxime; CAZ: Ceftazidime; AMC: Amoxicillin-clavulanic acid; CL: Clindamycin; LE: Levofloxacin; IPM: imipenem; CPM: Cefpodoxime

One PCR primer pair was used to test each isolated bacterium for the presence of an ESBL-producing gene. Based on PCR analysis, bla_{CTX-M} gene was detected in 65% of *E. coli* and 80% of K. pneumonia. Totally, 38 isolates were shown to be β-lactamase producers (Figure 1).

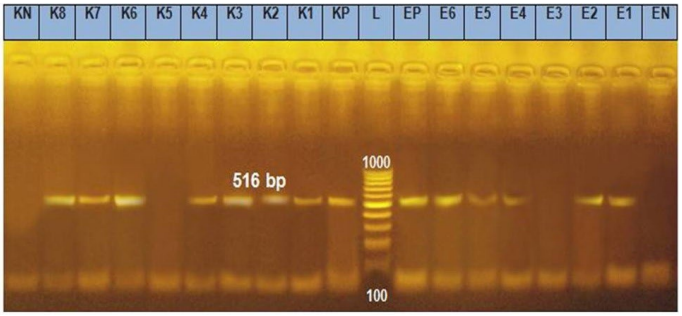

Fig. 1. Gel image showing amplification of 516 bp product corresponding to the β-lactamase gene of *E. coli* and *K. pneumoniae* serogroups isolated from milk. Lane (L): 100 bp DNA ladder; lane (EP): positive control for *E. coli*; lane (EN): Negative control for *E. coli*; lanes corresponding to milk samples E1-2, 4, 5, and 6 were positive while samples in lanes E3 was Negative. Lane (KP): positive control for *K. pneumoniae*; lane (KN): Negative control for *K. pneumoniae*; lanes corresponding to milk samples K1-2,3, 4, 6,7 and 8 were positive while samples in lanes K5 was Negative.

Discussion

The execution of Egypt's national antimicrobial stewardship program is not strictly enforced by regulations, despite its establishment (WHO, 2018; El-Sokkary *et al*., 2021). According to Jajarmi *et al*. (2017) and Abdus Sobur *et al*. (2019), veterinarians in Egypt continue to treat and prevent zoonotic infections using antimicrobials such tetracycline, quinolones, and beta lactams. They are also used to promote animal feed growth. Penicillin, aztreonam, cephalosporins, related oxyimino-β-lactams, and other antibiotics are among those to which resistance to ESBLs is caused. Plasmid-mediated resistance to ampicillin, oxacillin, and cefotaxime is among the many types of ESBLs that exist (Shaikh *et al*., 2015).

In this investigation, samples of bulk tank milk and cattle's milk were tested for the presence of *Enterobacteriaceae*, including K. pneumonia and *E. coli*. In all, 36.7% of the samples tested positive for *Enterobacteriaceae*. In 14% of all samples analyzed, *E. coli* was found. In a comparable manner, *E. coli* has been recovered by many studies from raw cattle's milk (Caine *et al*., 2014). In line with the current findings, studies by Disassa *et al*. (2017) and Caine *et al*. (2014) reported higher incidences of *E. coli* in milk, with percentages of 33.9% and 32.5%, respectively.

According to our data, E.coli isolates could be classified into six serotypes; the most common serotypes were O128: H2 and O91. By using serological identification, all *E. coli* isolates were categorized into distinct pathotypes that corresponded to their unique pathogenesis features. With the exception of EAEC, DAEC, CDEC, and AIEC, which were previously described, all pathotypes were found (Canizalez-Roman *et al*., 2013). It was found that among milk isolates, ETEC and EHEC were the most common. This is concerning. Several previous studies found that food isolates have the ETEC pathotype (Gomez-Aldapa *et al*., 2014). A distinct serotype, O128:H2 constitutes 40% of the ETEC population. These results are consistent with the findings of Stenutz *et al*. (2006), who found that among ETEC isolates, O128:H2 was one of the most frequently observed serogroups.

Bovine pneumonia, metritis, and mastitis are frequently caused by *Klebsiella pneumoniae*. It is a given that while milking, milk can quickly get contaminated. Both in the food chain and dairy cows, the frequency is rising (Yang *et al*., 2019). The current investigation shows that *K. pneumoniae* was detected in 22.7% of the samples that were examined. The prevalence of *K. pneumoniae* in mastitis milk samples in West Bengal, Jharkhand, and Mizoram, India, was 45.29% (Koovapra *et al*., 2016). This prevalence is lower than other studies. Iran showed that 40% of the population tested positive for *K. pneumoniae* (Enferad and Mahdavi, 2021).

Although research on this topic is still in its infancy, four essential elements of *K. pneumoniae* pathogenesis have long been identified: lipopolysaccharides, adhesion factors, siderophores, and *K. pneumoniae* capsule antigens. *K. pneumoniae*'s virulence factor was capsular polysaccharide (K antigen). This antigen causes *K. pneumoniae* settlementsagar plates to be shimmering and mucoid, which forms a thick hydrophilic case. Too far, at least 77 K antigen serotypes—K1, K2, etc.—have been identified. According to Choi *et al*. (2020), the K antigens play a vital role in protecting cells against serum death and opsonophagocytosis. The Quellung method was used to identify K1 and K2, with K1 being the predominant serotype in 64.5% of samples.

Globally, *Enterobacteriaceae* drug resistance has sharply grown. According to Kuzucu *et al*. (2011), this rise is mostly the consequence of a rise in the prevalence of *Enterobacteriaceae* that produce ESBLs and an increase in the usage of antibiotics as a last option. *E. coli* showed the greatest resistance to levofloxacin and imipenem (42.9% for each), whereas the isolates showed the lowest resistance to amoxicillin-clavulanic acid, cefotaxime, and cefpodoxime (22.2, 23.8, and 23.8, respectively); this is likely due to the antibiotic's less frequent usage. Conversely, every isolate of *K. pneumoniae* tested positive for both cefpodoxime and amoxicillin-clavulanic acid. The main cause of this resistance was ESBLs, which also cause resistance to more recent β-lactam medications including imipenem, levofloxacin, amoxicillin-clavulanic acid, cefotaxime, and cefpodoxime. This result similar to Badri *et al*. (2017). Based on PCR analysis, bla_{CTX-M} gene was detected in 65% of *E. coli* and 80% of *K. pneumoniae*. Totally, 38 isolates were shown to be β-lactamase producers.

Conclusion

The production, handling, transportation, and marketing processes for milk and milk products are wholly dependent on the conventional system. A setting like that might be conducive to bacterial infection. Significant therapeutic ramifications might result from the animal origin isolates' rising incidence of resistance. Because it not only aids in the transmission of pathogenic bacteria but also acts as a conduit for the spread of antibiotic resistance in Egypt, surveillance of enter bacteria that produce ESBLs should be maintained at all levels and reexamined at molecular level for phylogenetic analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

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