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Molecular Studies on Some Emerging Pathogens in Dairy Products Retailed in Dakahlia Governorate, Egypt

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Abstract

In this study, one hundred and ten samples (25 raw buffalo milk, 25 kareish cheese, 25 small-scale yoghurt, and 35 small-scale ice cream) were collected randomly from local markets, dairy shops, and supermarkets from different localities in Dakahlia Governorate, Egypt during October 2019 to March 2020. The prevalence rates of foodborne pathogens including *E. coli*, *Salmonella* spp., and *Aeromonas hydrophila* (*A. hydrophila*) were examined. Molecular confirmation and detection of toxin-producing and drug-resistance-related genes were carried out using PCR. *E. coli* was isolated from the examined raw milk, ice cream, Kareish cheese, and yoghurt at 72%, 25.71%, 40%, and 44%, respectively, while *Salmonella* spp. was isolated at 36%, 4%, and 24% from the examined raw milk, Kareish cheese, and yoghurt samples, respectively. *Aeromonas hydrophila* was isolated at 36% from the examined raw milk samples, 8% from Kareish cheese samples, and 76% from the examined yoghurt samples. Twelve out of 98 *E. coli* isolates, 18 out of 30 *Salmonella* isolates, and 24 out of 50 *A. hydrophila* were multidrug-resistant, respectively. The most resistant antibiotics were ceftriaxone and tetracycline. All examined *E. coli*, *Salmonella* spp., and *A. hydrophila* isolates contained *blaTEM* and *TetA*(A) resistance genes. 66.7% of the examined *E. coli* isolates harbored *stx1*, while *stx2* was absent in all examined *E. coli* isolates. All examined *Salmonella* spp. isolates contained both *stn* and *avrA* virulence genes. All examined *A. hydrophila* contained the Aerolysin, but just 40% contained the hemolysin virulence genes. Therefore, it is necessary to reduce the excessive usage of antibiotics in dairy farms and to apply strict hygienic measures to inhibit microbial contamination of dairy products intended for human consumption.

KEYWORDS
Antibiotic Resistance, *E. coli*, *Aeromonas* hydrophilic, *Salmonella*e spp., Dairy products

INTRODUCTION

In recent days, the rate of food contamination rises with a noisy picture through many routes. On the other hand, antimicrobial agents are used in a repeated manner resulting in the emergence of drug-resistant foodborne pathogens. Milk can support part of the human needs of protein, fat, and minerals. Raw milk as well supports the growth of a vast array of microorganisms (Tasnim and Islam, 2015). Food Standards Agency (2019) stipulated the consumer's demands for the consumption of raw milk, and raw dairy products (Hazeleger and Beumer, 2016). Cross-contamination of milk might take place from the animal body, operators' hands, use of unclean water, and inadequate cooking (Smigic *et al*., 2016), The milking equipment plays an important role in the microbial-cross contamination of milk (Uddin *et al*., 2011).

Emerging pathogens (EPs) are new microbial hazards to which increased exposure is possible to humans (European Food Safety Authority "EFSA", 2014) that result in antibiotic resistance and have public health hazards (Koluman and Dikici, 2013). About 26 bacterial emerging and reemerging infectious diseases are regarded as zoonoses (Vouga and Greub, 2016). Children and immunocompromised persons are the people who are at high risk for exposure to these pathogens (Ahmed *et al*., 2014a; Odeyemi and Sani, 2016). Several factors facilitate the spread of Eps including climate change, microbial adaptation, development of microbial mutations, and improved disease surveillance (Nii-Trebi, 2017; Ohimian, 2017; WHO, 2017).

The most recognized emerging foodborne pathogens are non-typhoidal *Salmonella* (Lund and O'Brien, 2011), Shiga toxin-producing *E. coli* (STEC), and *Aeromonas hydrophila* (Koluman and Dikici, 2013). According to WHO, it was estimated that infectious diseases contribute to about one-third of annual deaths worldwide, of which 70% are caused by emerging pathogens (Devleesschauwer *et al*., 2014).

Antibiotics are largely used for prophylactic and therapeutic purposes in the veterinary field and as animal growth promoters. However, the massive abuse of antibiotics is a major cause for the development of multidrug resistance (Tule and Hassani, 2017; Jans *et al*., 2018), and is considered the major reason for the emergence or reemergence of pathogens (WHO, 2017). The discovery of new drugs either of natural or synthetic origin, vac-

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cines, bacteriophages, antimicrobial peptides, nanotechnology, phytochemicals, and herbal medicines are necessary for controlling this problem (Ibrahim *et al*., 2021).

In sight of the previous facts, this study aimed to investigate the prevalence of three important foodborne pathogens, namely *A. hydrophila*, *E. coli*, and *Salmonella* spp. in the most commonly consumed dairy products (raw buffalo milk, kareish cheese, small-scale yoghurt, and small-scale ice cream) in Dakahlia Governorate, Egypt. Molecular confirmation and detection of toxin-producing genes in the recovered isolates were carried out using PCR.

MATERIALS AND METHODS

Collection of samples

One hundred and ten samples (25 buffalo raw milk, 25 kareish cheese, 25 small-scale yoghurt, and 35 small-scale ice cream) were collected randomly from markets, dairy shops, and supermarkets from different localities in Dakahlia Governorate, Egypt from October 2019 to March 2020. Samples were directly transferred to the laboratory in an ice box to be examined.

Preparation of samples

Samples (25 ml from buffalo raw milk, and 25 g of the other dairy products (kareish cheese, small-scale yoghurt, and small-scale ice cream) were aseptically homogenized into a sterile, wide-mouth, screw-capped jar containing 225 ml of tryptone soya broth (2% sodium citrate was added to kareish cheese to accelerate its homogenization). This mixture was used to prepare decimal dilutions by using sterile distilled water (Salfinger and Tortorello, 2015).

Bacteriological examination

Enumeration of *E. coli*

Enumeration of *E. coli* was done through a direct method by the spread surface technique on selective agar media, eosin methylene blue (EMB) agar, and incubation for 24 h at 37°C. The countable plates with suspected colonies of *E. coli* were recognized based on colonial morphology that appeared as distinctive metallic green shiny colonies (FDA, 2012).

Isolation of *Salmonella* spp.

Through plating on Xylose Lysine Desoxycholate (XLD) agar and followed by incubation at 37ºC for 24 h. The growing colonies were morphologically described including size, shape, consistency, lactose fermentation, and pigment production. Each colony showing a typical appearance was subcultured on *Salmonella* selective solid agar to obtain a pure culture (ISO-6579, 2002).

Biochemical analysis of *E. coli* and *Salmonella* spp. Were done by using the Vitek 2 Compact system (bioMérieux. Marcy l'Etoile, France) (Chang *et al*., 2002)

Enumeration of *Aeromonas hydrophila*

Using *Aeromonas* isolation medium base with the addition of *Aeromonas* Selective Supplement (FD039). Cultured plates were incubated at 35-37°C for 18-24 h. Dark green, and opaque with a dark center were considered *A. hydrophila* (Salfinger and Tortorello, 2015). The suspected *A. hydrophila* isolates were exposed to Gram's staining and biochemical tests (Cheesbrough, 2006). Further confirmation of *A. hydrophila* isolates was done using MicrobactTM GNB 24E identification kit (Burke *et al*., 1982).

Antimicrobial susceptibility testing of the recovered isolates

Antimicrobial susceptibility testing of the recovered isolates was done by the disc diffusion method using Mueller-Hinton agar (Merck, Germany) following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015, 2017). The following antibiotics were tested: C (Chloramphenicol 30 µg), CN (Gentamycin 10 µg), CRO (Ceftriaxone 30 µg), CIP (Ciprofloxacin 5 µg), GAT (Gatifloxacin 5 µg), AK (Amikacin 30 µg), TE (Tetracycline 30 µg). All antibiotics standards were purchased from HiMedia Laboratories (Mumbai, India).

Molecular confirmation of the recovered isolates

Bacterial DNA was extracted using QIAamp DNA Mini Kit (Germany) following the instructions of the manufacturer. PCR was used for amplification of the specific bacterial targets using the Emerald Amp GT PCR master mix (TaKaRa, Japan) (Sambrook *et al*., 1989; Oliveira *et al*., 2003; Gordon *et al*., 2007; Hu *et al*., 2011) (Table 1).

Statistical analysis

Data were analyzed using SPSS using analysis of variance (ANOVA) followed by Tukey's Kramer HSD test, where $p < 0.05$ is considered to be significant.

RESULTS AND DISCUSSION

Prevalence and characterization of E. coli isolates recovered from the examined dairy products

E. coli is associated with several cases of human illnesses including hemorrhagic colitis, hemolytic uremic syndrome, bloody diarrhea, thrombotic thrombocytopenic purpura, (Wang *et al*., 2016), meningitis in neonates (Logue *et al*., 2012) that may reach to critical illness and death (Ibrahim *et al*., 2020). In this study, 72% of the examined raw milk samples were positive for *E. coli* with a mean *E. coli* count of $1.48x10^8$ cfu/ml, and 25.71% of the examined ice cream samples were positive for *E. coli* with a mean count of $0.21x10^8$ cfu/g, 40% of the examined Kareish cheese samples were positive for *E. coli* with a mean count of $0.26 x10^8$ cfu/g and 44% of the examined yoghurt samples were positive for *E. coli* with a mean count of $8x10^6$ cfu/g (Table 2). In agreement with the obtained results of the present study, Mohamed *et al*. (2019) reported that the incidence of *E. coli* in raw Kareish cheese was 86.7% while Abd-Alla *et al*. (2020) found that *E. coli* was detected in all kareish cheese samples with a count range from 3.21 to 5.52 log cfu/g. Likely, Hosseini-Naveh *et al*. (2019) found that 15% of the examined traditional ice cream samples contained *E. coli*, Das *et al*. (2020) isolated *E. coli* from ice cream with a count ranging between $221×10^3$ and $245×10^3$ cfu/g. Such result indicates poor hygienic practices during manufacture, post-process contamination, poor sanitary conditions during frozen storage, and unsatisfactory transportation. Similarly, Fadihl *et al*. (2019) noticed that 47% of the collected ice cream samples contained *E. coli* which indicates the bad microbiological quality of the ice cream samples and renders them unfit for human consumption. On contrary, GadAllah *et al*. (2020) reported that all ice cream samples were free from *E. coli*. Regarding raw milk, Kadyan *et al*.

Table 1. Oligonucleotide sequences of the primers used in the present study.

Bacteria	Gene	Sequence	Amplified product	Reference
Salmonella	*invA*	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284 bp	Oliveira *et al*. (2003)
	Stn	F: TTG TGT CGC TAT CAC TGG CAA CC R: ATT CGT AAC CCG CTC TCG TCC	617 bp	Murugkar *et al*. (2003)
	avrA	F: CCT GTA TTG TTG AGC GTC TGG R: AGA AGA GCT TCG TTG AAT GTC C	422 bp	Huehn *et al*. (2010)
E. coli	*phoA*	F: CGATTCTGGAAATGGCAAAAG R: CGTGATCAGCGGTGACTATGAC	720 bp	Hu *et al. (*2011)
	stx1	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATG	614 bp	Dipineto *et al.* (2006)
	stx2	F: CCATGACAACGGACAGCAGTT R: CCTGTCAACTGAGCAGCACTTTG	779 bp	
A. hydrophila	16S rRNA	F: GAAAGGTTGATGCCTAATACGTA R: CGTGCTGGCAACAAAGGACAG	685 bp	Gordon *et al. (*2007)
	aerolysin	F: CACAGCCAATATGTCGGTGAAG R: GTCACCTTCTCGCTCAGGC	326 bp	Singh *et al.* (2008)
	Hemolysin	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGGG	592 bp	Rozi *et al.* (2017)
All tested bacteria	*blaTEM*	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	516 bp	Colom *et al*. (2003)
	TetA(A)	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	576 bp	Randall *et al.* (2004)

Table 2. Prevalence (%), and counts of *E. coli* among the examined samples in the present study

Type of examined Samples	No of examined samples	Positive samples		Min.	Max.	Mean	± S.E
		No	%				
Buffalo raw milk	25	18	72	$3x10^4$	$6 x10^8$	$1.48x10^{8a}$	$0.39x10^8$
Ice cream	35	9	25.71	$1x10^6$	$3 x10^8$	$0.21x10^{8a}$	$0.66 x10^8$
Kareish cheese	25	10	40	$25 x10^4$	$36 x10^7$	$0.26 x10^{8a}$	$0.18 x10^8$
Yoghurt	25	11	44	$35x10^5$	$38x10^6$	$8.0x10^{6b}$	$0.02x10^8$

Means within the same column carrying different superscript letters are statistically different at $p < 0.05$.

Table 3: MAR index analysis of *E. coli*, *Salmonella* spp., and *A. hydrophila* isolates in the present study

No. of multi-resistant Antibiotics	MAR bacterial isolates		MAR index value
	No.	%	
E. coli (MAR isolates = 12)			
7	4	33.33%	1
4	4	33.33%	0.57
3	4	33.33%	0.43
Salmonella spp. (MAR isolates = 18)			
5	6	33.33%	0.71
4	5	27.78%	0.57
3	7	38.89%	0.43
A. hydrophila (MAR isolates = 24)			
5	3	12.50%	0.71
4	3	12.50%	0.57
3	18	75%	0.43

No. of antibiotics to which the isolates were subjected = 7

(2020) isolated *E. coli* at 57.29% from 96 examined raw milk, while Elbehiry *et al*. (2021) reported that thirty-three E.
coli isolates were recovered from 254 milk samples. Younis *et al*. (2021) reported that 10 out of 100 milk samples were contaminated with *E. coli* and Alam *et al*. (2021) isolated *E. coli* at 26.2% from raw milk.

Foods of animal origin are considered an important source for the spread of multiple antibiotic-resistant pathogens (Copur-Cicek *et al*., 2014). The emergence of the antimi¬crobial-resistant *E. coli* is alarming manner causing public health hazards all over the world. In this study, 12.24% of the isolates were resistant to C, 8.16% were resistant to CN, 12.24% of the isolates were resistant to CRO, 12.24% were resistant to CIP, 4% were resistant to GAT, 10.2% were resistant to AK, 38.78% were resistant to TE. The study showed that 12 isolates out of 98 isolates showed multidrug resistance, 4 isolates recovered from kareish cheese were resistant to all tested 7 antibiotics (C, CN, CRO, CIP, GAT, AK, and TE). MAR (Multiple antibiotic resistance) indexes were calculated. In this study, 33.33% of *E. coli* isolates were resistant to the 7 tested antibiotics, 33.33% to 4 antibiotics, and 33.33% to 3 antibiotics (Table 3). Similrly, El-Seedy *et al*. (2020) found that most isolates of *E. coli* were multidrug-resistant (MDR). About 41.5% of *E. coli* isolates were resistant to tetracycline. Six isolates harbored the *TetA* gene (35.3%), while the *blaTEM* gene was found in 76.5% of the isolates.

In this study, all recovered *E. coli* isolates were positive for *phoA* gene (a confirmatory target for *E. coli*) (data are not shown), while 66.7% of the examined *E. coli* strains contained *stx1* virulence gene, while *stx2* virulence gene was not detected in all examined *E. coli* isolates. The most resistant antibiotics were ceftriaxone and tetracycline, therefore, *blaTEM* and *TetA* genes were detected in the recovered isolates. The results showed that all examined *E. coli* strains contained *blaTEM* and *TetA*(A) resistance genes (Fig. 1). In agreement with the obtained results of the present study, Momtaz *et al*. (2013a) found that *stx1* and *stx2* were the most significant virulence genes in isolated *E. coli* with public health hazards, while Ismail and Abutarbush (2020) reported that out of 216 milk samples, 14 samples yielded *E. coli* isolates. All isolates (100%) were sensitive to ciprofloxacin. 93% of the isolates carried the *TetA*, gene but bla1 was detected in 17% and *blaTEM* (12%), and 93% of the isolates carried *stx1*, and *stx2* was detected only in 36% of the recovered isolates.

Prevalence and characterization of Salmonella spp. isolates recovered from the examined dairy products

Salmonella spp. is one of the foodborne pathohens listed in the WHO priority pathogen list. Dairy cattle is the major reservoir for *Salmonella* spp. that causes human salmonellosis (Halimi *et al*., 2014). In the present study, 36%, 24%, and 4% of the examined raw milk, yoghurt, and kareish cheese samples were contaminated with *Salmonella*, while it was absent in the examined ice cream samples. It notes worthy to mention that 18 out of 30 isolates showed multi-drug resistance: 61.1% (raw milk), 27.8% (youghurt), and 11.11% (Kareish cheese). As 33.3% of the isolates were resistant to C, 33.3% to CN, 56.67% to CRO, 50% to CIP, 16.67% to GAT, 10% to AK, and 90% of the isolates were resistant to TE (Table 4). The calculated MAR index for the recovered *Salmonella* isolates was 33.33% for 5 antibiotics, 27.78% for 4 antibiotics and 38.89% for 3 antibiotics (Table 3). Pathogenicity of *Salmonella* spp. was mainly attributed to their possession to some virulence attributes such as the invasion protein gene (*invA*) that facilitate adhesion and invasion of the host intestinal epithelial cells, and the *Salmonella* enterotoxin gene (*stn*) that causes severe diarrhea (Omar *et al*., 2018). All examined *Salmonella* isolates harbored *invA*, and *stn*, and the drug resistance related genes, *TetA*, and *blaTEM* (Fig. 1). In agreement with the obtained results of the present study, Sohair and Eman (2008) reported that 20% from 150 buffalo raw milk samples were contaminated with *Salmonella* spp. While Alam *et al*. (2021) found that *Salmonella* spp. was isolated from raw milk at just 3.75%. Adzitey *et al*. (2020) reported that *Salmonella enterica* isolates recovered from raw milk were susceptible to ciprofloxacin (100%), chloramphenicol (91%), ceftriaxone (91%), and tetracycline (86%). Regarding the cheese samples, Abd-Alla *et al*. (2020) found that *Salmonella* was detected in all examined kareish cheese samples with a range from 3.22 to 5.42 log cfu/g. Moreover, Fadihl *et al*. (2019) and GadAllah *et al*. (2020) found that all of examined ice cream samples were free from *Salmonella*.

Prevalence and characterization of A. hydrophila isolates recovered from the examined dairy products

A. hydrophila is transmitted to human through the consumption of contaminated food and water (Hoel *et al*., 2017). It is re-

Table 4. Prevalence (%) of *Salmonella* spp. among the examined samples in the present study

Type of examined product	No of examined samples	Positive samples	
		No	%
Buffalo raw milk	25	9	36
Ice cream	35	0	0
Kareish cheese	25	1	4
Yoghurt	25	6	24

Table 5. Prevalence (%), and counts of *A. hydrophila* among the examined samples in the present study

Type of examined Samples	No. of examined samples	Positive samples		Min.	Max.	Mean	± SE
		No	%				
Buffalo raw milk	25	9	36	13×10^5	3×10^8	28×10^{6b}	0.13×10^8
Ice cream	35	0	0	-	-	-	-
Kareish cheese	25	2	8	5×10^8	0.55×10^8	0.04×10^{8a}	0.03×10^8
Yoghurt	25	19	76	1×10^6	0.34×10^8	0.08×10^{8a}	0.02×10^8

Means within the same column carrying different superscript letters are statistically different at $p < 0.05$.

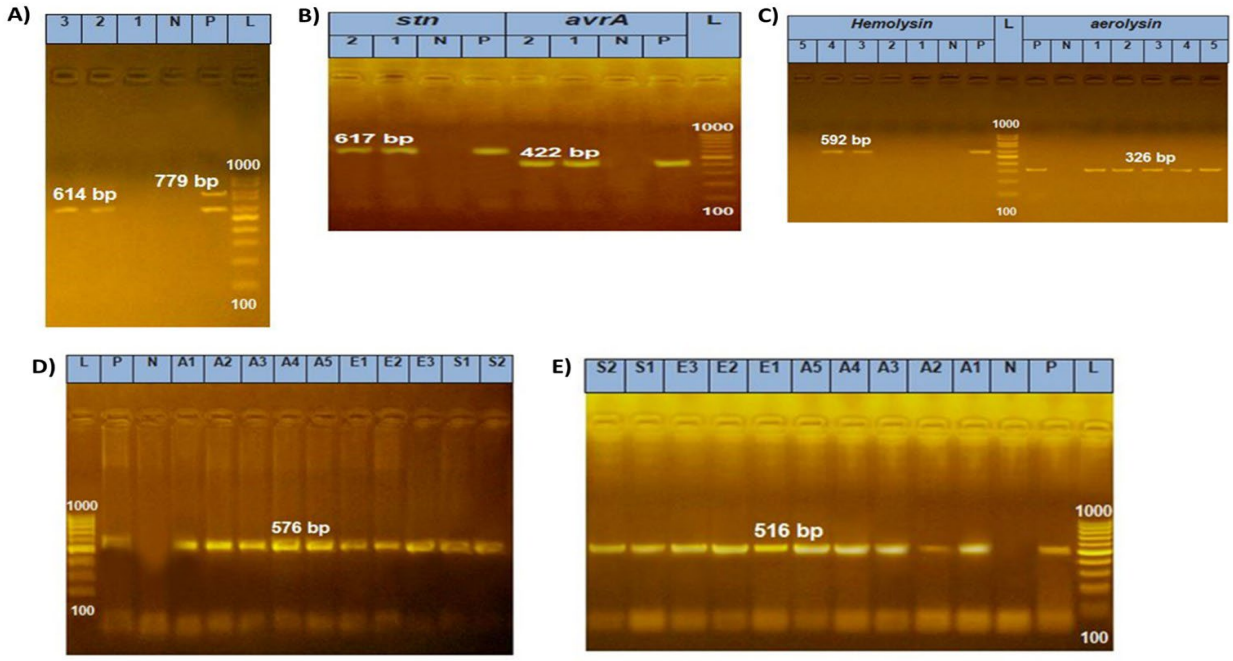

Figure 1. Molecular characterization of the recovered *E. coli*, *Salmonella* spp., and *A. hydrophila* representative isolates. A) *E. coli* Shiga toxin-coding genes (*stx1*, and *stx2*), where lanes 2, and 3 show positive results; while lane 1 shows no expression. B) *Salmonella* virulence genes (*stn*, and *avrA*) where lanes 1, and 2 show positive results. C) *A. hydrophila* virulence genes (hemolysin, and aerolysin) where lanes 1-5 show positive results for hemolysin, and lanes 3, and 4 show positive results for aerolysin. D) *TetA*(A) resistance gene. E) *blaTEM* resistance gene. N: control negative, P: control positive, L: Ladder (100-1000 bp), A: *A. hydrophila,* E: *E. coli*, S: *Salmonella* spp.

sponsible for the motile *Aeromonas* septicemia (MAS) epidemic outbreaks (Yang *et al*., 2018). In this study, 36%, 8%, and 76% of examined raw milk, kareish, and yoghurt samples were contaminated *A. hydrophila* with mean counts of 28 x106, 0.042 x108, and 0.08x108 cfu/g, respectively, but it was absent in the examined ice cream samples (Table 5). The presence of *Aeromonas* in milk and dairy products had been reported in in raw milk, local plain yoghurt, and cheese from Egypt (Ahmed *et al*., 2014b), raw milk samples from Switzerland (Alnakip *et al*., 2019), and from handlers' hands, water and utensils (Cereser *et al*., 2013).

In this study, 24 out of 50 isolates showed multi-drug resistance: 41.7% (raw milk), 25% (youghurt), and 33.3% (Kareish cheese). As 48% of the isolates were resistant to C, 14% to CN, 54% to CRO, 10% to CIP, 2% to GAT, 42% to AK, and 68% to TE. MAR index was 12.5% for 5 antibiotics, 12.5% for 4 antibiotics and 75% for 3 antibiotics (Table 3). *A. hydrophila* is a highly pathogenic organism; detection of virulence and drug resistance-related genes is of importance to determine the virulence capacity of the recovered strains (Janda and Abbott, 2010). In this study, the examined *A. hydrophila* isolates harbored hemolysin, aerlusin, *TetA*, and *blaTEM* (Fig. 1). Similar to the obtained findings, Yang *et al.* (2018) reported that the isolated *A. hydrophila* had developed resistance to tetracycline. Besides, Elbehiry *et al.* (2019) found that 50% of *A. hydrophila* isolates were resistant to third-generation cephalosporins. On the other hand, Zhou *et al.* (2019) reported that ≥ 80% of *Aeromonas* isolates were susceptible to chloramphenicol, ciprofloxacin, and ceftriaxone. Wickramanayake *et al.* (2020) found that all isolated *A. hydrophila* were susceptible to ceftriaxone, chloramphenicol, and ciprofloxacin.

CONCLUSION

The obtained results of the present study indicated isolation of multidrug resistant *E. coli*, *Salmonella* spp., and *A. hydrophila* from the examined dairy products at variable rates. Particularly, milk had the highest prevalence for *E. coli* and *Salmonella* spp.., while yoghurt had the highest prevalence rate of *A. hydrophila*. The most resistant antibiotics were ceftriaxone and tetracycline Several virulence-associated genes and drug resistance coding genes were detected in the recovered isolates. Therefore, obtaining raw milk of healthy animals from dairy farms that apply strict hygienic measures, efficient heat treatment of milk, personnel, and production hygiene are the most important critical points for minimizing the growth of emerging pathogens during the manufacturing, production, handling, and processing, distribution, and storage of dairy products. In addition, minimizing the unnecessary usage of antibiotics, application of Good Manufacturing Practices (GMP), Hazard Analysis Critical Control Points (HACCP), Good Agricultural Practices (GAP), and Good Production Practices (GPP) on dairy farms are vital procedures for reducing the spread of foodborne pathogens.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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