Received: 15 September 2023, Accepted: 11 October 2023

Original Research

Journal of Advanced Veterinary Research
(2023) Volume 13, Issue 10, 1954-1959

Isolation and Identification of Food Poisoning Bacteria from some Dairy Farms in El-Menoufia Governorate using VITEK 2

Shaimaa M. Nada[1*], Shaimaa S. Elnahriry[2], Ahmed A. Sultan[3], Mohamed H. Gaffer[1]

[1]Department of Food hygiene, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC) , Shebin El kom Branch, Egypt.

[2]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

[3]Food Safety Lead Auditor in TÜV AUSTRIA, Egypt.

***Correspondence**
Corresponding author: Shaimaa M. Nada
E-mail address: Shaimaanada80@gmail.com

Abstract

Because milk is rich in nutrients, it creates an environment conducive to the growth of bacteria that could be harmful to consumers. Therefore, assessing of its bacteriological quality and the resistance of these pathogens to several antibiotic groups is very important. VITEK 2 compact detected *E. coli*, *Salmonella*e spp., *S. aureus* and *B. cereus* in the examined samples while it failed to detect Listeria spp. The serological identification of *E. coli* showed the presence of O44: H18, O127: H6, O159, O15: H2, and O91: H21. Also, *Salmonella* serotypes as *S. enteritidis* (25%), *S. infantis* (12.5%), *S. kentucky* (12.5%), *S. montevideo* (6.25%), *S. shangani* (12.5%), *S. tsevie* (12.5%). *S. typhimurium* (18.75%). Furthermore, the entero-toxigenic strains of *S. aureus* were 31.25% of the identified strains; the percentage of entero-toxigenic strains that secrete A, A&C and D enterotoxin were 60%, 20% and (20%); respectively. Ampicillin, amoxicillin / clavulanic acid, cefpodoxime, cefovecin, ceftiofur, and trimethoprim / sulfamethoxazole were all ineffective against the isolated *E. coli* strains. Intermediate sensitive to cefalothin while they were sensitive to cefalexin, imipenem, amikacin, gentamicin, neomycin, enrofloxacin, marbofloxacin, pradofloxacin, doxycycycline, tetracycline, nitrofurantoin and chloramphenicol. Additionally, the isolated *S. aureus* exhibited resistance to tetracycline, benzylpenicillin, oxacillin, gentamycin, ciprofloxacin, levofloxacin, and moxifloxacin also; it showed intermediate resistance to rifampicin. The identified *S. aureus* strains were also susceptible to linezolid, nitrofurantoin, vancomycin, tigecycline, and trimethoprim / sulfamethoxazole.

KEYWORDS
Bacillus cereus, *E. coli*, *Staphylococcus aureus*, Raw milk, VITEK 2

INTRODUCTION

Milk contains protein, minerals, energy, hormones, and growth factors (Anema, 2020). So, milk promotes the development of bacteria that present a biological risk to consumers (Zakary *et al*., 2011). Consequently, it is imperative to evaluate the hygienic and sanitary quality of raw milk (Rechidi-Sidhoum *et al*., 2021). At any stage of the production, manufacturing, and distribution processes, milk can get contaminated with bacteria from staff, milk utensils, parlors, animals, and parlor equipment could all contribute to bacterial pollution (Garedew *et al*., 2012). Furthermore, a high microbiological load has a limited shelf life. Contamination might be caused by internal factors like nutrients, water activity, pH, or temperature, or external factors such events that happened during the production, processing, and packaging stages (Hosny *et al*., 2011).

Sever fatal diarrhea in children caused by food-borne bacteria (Enteropathogenic *E. coli*) has been reported in developing nations (Alonso *et al*., 2011). *E. coli* resists production conditions due to the continual source of contamination. Because they may be easily found in the environment and are used to living in the colons of healthy humans and animals, Shiga toxin-producing *E. coli* (STEC) are the most common. It can spread to people when they eat infected food. The prevalence of the *E. coli* toxin Shiga-like toxin (STEC) in raw milk depends on the use of effective control measures (Velázquez-Ordoñez *et al*. 2019).

Salmonella is more prevalent in raw milk, which is a serious public health risk, to reduce the risk of human infections with *Salmonella*; sanitary practices must be implemented during milking (Omar *et al*., 2018). Also, both the emetic (vomiting) and diarrheal types of foodborne disease are caused by *Bacillus cereus*. Symptoms of the emetic syndrome include nausea, vomiting, and cramping in the abdomen. It has a brief incubation period of 1 to 5 hours and a quick recovery time of 6 to 24 hours, a form of diarrhea brought on by eating food contaminated with a lot of *B. cereus* spores (Senesi and Ghelardi, 2010).

Furthermore, a common pathogenic bacterium called *Staphylococcus aureus* contaminates milk-related surroundings and can lead to gastrointestinal illnesses due to thermo-stable enterotoxins (Valihrach *et al*., 2013). Due to the high nutritional value of milk, harmful bacteria such as Listeria species (El Marnissi *et al*., 2013) that can thrive there and cause listeriosis in both humans and animals (Ryser and Marth, 2007). Antimicrobial susceptibility testing (AST) is usually carried out by clinical microbiology laboratories on the predominant pathogen that was isolated. Methicillin resistance in *S. aureus* is one of the significant resistance phenotypes linked with several taxa that are regularly observed in clinical practice (Bobenchik *et al*., 2014).

So, this study goal was to assess the bacteriological quality of raw milk. and discuss the public health importance of these microorganism also use VITEK 2 Compact system for rabid biochemical examination and sensitivity testing for the most prominent microorganisms.

ISSN: 2090-6277/2090-6269/

MATERIALS AND METHODS

Collection of samples

The research study was conducted on raw milk collected from local farms in EL Menoufia Governorate in September 2022. To evaluate the bacteriological profile of 100 semi-intensively reared Holstein cows, 100 random samples of raw milk were obtained. Each sample was stored in a separate, sterile glass bottle, kept chilled in an ice box, and delivered to the lab within an hour without unnecessary delay. In order to assess the potential health risks connected with their contamination and, ultimately, their suitability for human consumption, the gathered samples were subjected to a bacteriological test.

Bacteriological examination

Preparation of samples (ISO 4833-1, 2013)

In order to create tenfold serial dilutions, 225 ml of sterile peptone water (OXOID, CM0009) was added to 25 mL of the sample and thoroughly blended for 1.5 minutes using a sterile blender. The prepared samples went through the following tests:

Screening for Enteropathogenic *E. coli*

Started by Pre-enrichment performed according to ISO 16649-2 (2001). One ml of the original dilution was used to inoculate inverted Durham's tubes together with MacConkey broth tubes (HIMEDIA, M539-500G) from the original dilution. For 24 hours, inoculated tubes were incubated at 37°C. After that, it was enriched by adding one milliliter of the positive MacConkey tube's contents to another MacConkey broth tube and incubating it there for 24 hours at 44°C. Loopfuls were individually streaked onto Eosin Methylene Blue agar medium (HIMEDIA, M022), which was then incubated at 37°C for 24 hours, from positive MacConkey broth tubes. The suspected colonies were metallic green in colour when purified, and they were subsequently injected into tubes of slope nutrition agar for additional analysis.

Identification of Enteropathogenic E. coli

Morphological identification

Microscopical examination

Gram's stain was used to color films of pure suspected cultures, which were then examined under a microscope. Coccobacilli that were medium in size, gram negative and uniformly colored were thought to be *E. coli*.

Motility test

Stably inoculating the motility medium to a depth of 5 mm, then incubating it for 24 hours at 37°C, was the procedure used. A circular extension from the stabbing line served as a sign of a successful test.

Screening for Salmonellae was done according to Harvey and Price (1981).

One ml of the original dilution was used to inoculate sterile peptone water, and the mixture was then cultured for 18 hours at 37°C. After enrichment, from the mixture 1ml was placed in a 9 ml Rappaport Vassilidis broth tube (HUMEDIA, MH1491-500G), which was then incubated for 24 hours at 43°C.

Following (HIMEDIA, MH031-500) the sample is placed on XLD (Xylose Lysine Desoxychoclate Agar). From the inoculation tubes, distinct loopfuls were streaked over XLD agar and allowed to incubate for 24 hours at 37°C. Suspected colonies showed red with or without black centers. The suspicious colonies were sub-cultured on nutrient agar plates and incubated at 37°C for 24 hours. For further identification, the various colonies were chosen and streaked onto slope nutrition agar. Biochemical and serological tests were used to identify the isolated pure samples.

Isolation of Staph. aureus according to FDA (2001)

One ml from the dilution were put on 5% egg yolk and tellurite-supplemented Baird-Parker agar (HIMEDIA, M043-500G). At 37°C, the plates were incubated for 24–48 hours. *S. aureus* colonies with the typical black colour and a distinct halo zone were counted. The colonies were also picked up for further identification after being isolated on nutrient agar slopes.

Bacillus cereus isolation

A volume of 0.1 ml from the serial dilution was cultured on *B. cereus* agar media and incubated at 37 °C for 24 hours. Typical *B. cereus* colonies were positioned on nutrient agar slope and incubated at 37°C for 24 hours in order to conduct further identification. These colonies change the media's colour from green to bluish and have a rosette structure.

Detection of Listeria monocytogenes

L. monocytogenes was examined in each sample. For primary enrichment, 225 mL of Half Fraser Broth (Sigma-Aldrich, 1000250500, Germany) was homogenized with a 25 mL sample, and the mixture was then incubated at 30°C for 24 hours. Then, 10 mL of Fraser Broth and 0.1 mL of the pre-enrichment culture were combined, and the mixture was incubated at 35°C for 48 hours. Drops of the culture were then streaked onto Listeria Oxford Agar base plates (HIMEDIA, M1145F), where they were cultured for 48 hours at 35°C. Three to four typical colonies were chosen from the media, streaked over trypticase soya agar containing yeast extract (Sigma-Aldrich, 22091), and then cultured for 48 hours at 35°C. Gram stain was used to colour suspected Listeria colonies.

Biochemical identification for isolated bacteria was performed by using VITEK 2 compact system.

Serological identification of E. coli.

The isolates were identified by serology utilizing quick diagnostic *E. coli* antisera sets (DENKA SEIKEN Co., Japan) for the diagnosis of the enteropathogenic kinds.

Serological identification of Salmonellae species

Using *Salmonella*e antiserum, serological identification of *Salmonella*e was performed using the Kauffman-White technique to determine the somatic (O) and flagellar (H) antigens (DENKA SEIKEN Co., Japan).
Somatic (O) antigen was identified using the Slide agglutination test.
Flagellar (H) antigen was identified using Tube agglutination test.

Serological identification of S. aureus enterotoxin

The enterotoxin has been identified using enzyme-linked immunosorbent assay (ELISA) method.

Sensitivity testing of the most isolated bacteria

Using VITEK 2 compact (bioMérieux) AST-GN73 for *E. coli* and AST-GP71 for *S. aureus*.

RESULTS

The data present in Figure 1 showed that VITEK 2 compact detected *E. coli*, *Salmonella* serotypes., *S. aureus* and *B. cereus* in 40%, 16%, 32%, and 6% from the examined samples while it failed to detect Listeria spp.

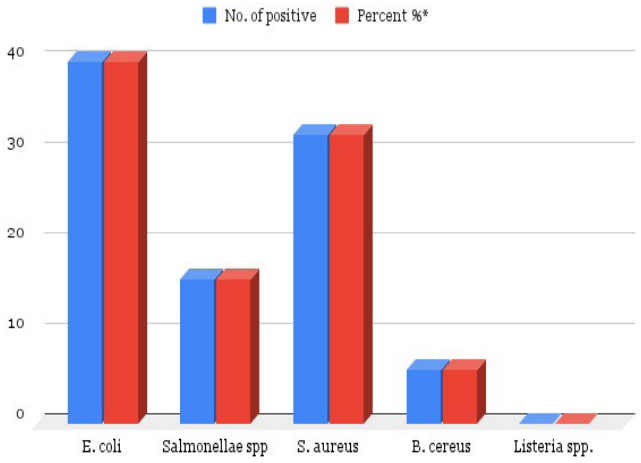

Figure 1. The prevalence of *E. coli*, *Salmonella* spp., *S. aureus*, *B. cereus* and Listeria spp. in the raw milk samples using VITEK 2 COMPACT System (n=100).

The serological identification of *E. coli* illustrated in Table 1 showed the presence of O44: H18 EPEC (18.2%), O127: H6 ETEC (45.5%), O159 EIEC (18.2%), O15: H2 EPEC (9.1%) and O91: H21 EHEC (9.1%). *Salmonella* strains that isolated from raw milk samples are mentioned in Table 2, as *S. enteritidis* (25%) D3 O 1, 9, 12: g,m H: 1,7. *S. infantis* (12.5%) C1 O 6, 7: H r: 1, 5. *S. kentucky* 12.5% C3 O8, 20: H i: Z6. *S. montevideo* (6.25%) C1 O 6, 7: g,m,s 1,2,7. *S. shangani* (12.5%) E1 O 3, 1 0 1, 4, 12: H i e, n, z 15. *S. tsevie* (12.5%) B D: 1, 5. *S. typhimurium* (18.75%) O1, 4, 5, 12: H i: 1, 2.

Table 1. Incidence of Enteropathogenic *E. coli* detected in raw milk samples.

E. coli Strains	No.	%*	Strain characteristics
O44: H18	2	18.20%	EPEC
O127: H6	5	45.50%	ETEC
O159	2	18.20%	EIEC
O15: H2	1	9.10%	EPEC
O91: H21	1	9.10%	EHEC
Total No. (%)	11	100%	

EPEC: Enteropathogenic *E. coli*; ETEC: Enterotoxigenic *E. coli*; EIEC: Enteroinvasive *E. coli*; EHEC: Enterohaemorrhagic *E. coli*; %*: in relation to Enteropathogenic *E. coli* (11).

The identified *S. aureus* strains was 50% of tested strains. The entero-toxigenic strains of *S. aureus* were 31.25% of the identified strains; A enterotoxin was 60%, A&C enterotoxin was 20% and D enterotoxin was (20%).

The isolated *E. coli* strains in Table 3 demonstrated resistance to Ampicillin, amoxicillin / clavulanic acid, cefpodoxime, cefovecin, ceftiofur, and trimethoprim / sulfamethoxazole. Intermediate sensitive to cefalothin while they were sensitive to cefalexin, imipenem, amikacin, gentamicin, neomycin, enrofloxacin, marbofloxacin, pradofloxacin, doxycycline, tetracycline, nitrofurantoin and chloramphenicol.

The isolated *S. aureus* in Table 4 demonstrated intermediate resistance to rifampicin as well as resistance to tetracycline,

Table 2. Incidence of *Salmonella* strains isolated from the examined samples of raw milk.

*Salmonella*e strains	No. 16	Percent (%)	Group	Antigenic structure	
				O	H
S. enteritidis	4	25	D3	1,9,12	g,m : 1,7
S. infantis	2	12.5	C1	6,7	r : 1,5
S. kentucky	2	12.5	C3	8,20	i : Z6
S. montevideo	1	6.25	C1	6,7	g,m,s: 1,2,7
S. shangani	2	12.5	E1	3,10	D:1,5
S. tsevie	2	12.5	B	1,4,12	i: e,n,z15
S. typhimurium	3	18.75	B	1,4,5,12	i:1,2

Table 3. Performance of AST-GP73 card for *E. coli*.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	NEG	-	Gentamicin	<=1	S
Ampicillin	>=32	R	Neomycin	<=2	S
Amoxicillin/Clavulanic acid	4	R	Enrofloxacin	<=0.12	S
Cefalexin	<=4	S	Marbofloxacin	<=0.5	S
Cefalothin	16	I	pradofloxacin	<=0.12	S
Cefpodoxime	>=8	R	Chloramphenicol	<=0.5	S
Cefovecin	>=8	R	Tetracycline	<=1	S
Ceftiofur	>=8	R	doxycycline	<=16	S
Imipenem	<=0.25	S	Nitrofurantoin	<=2	S
Amikacin	<=2	S	Trimethoprim/Sulfamethoxazole	80	R

R: Resistance; S: Sensitive; I: Intermediate

Table 4. Performance of AST-GP71 card for *S. aureus*.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin screen	POS	+	Erythromycin	>=8	R
Benzylpenicillin	>=0.5	R	Clindamycin	>=8	R
Oxacillin	>=4	R	Quinupristin/Dalfopristin	0.5	S
Gentamicin	>=16	R	Linezolid	2	S
Ciprofloxacin	>=8	R	Vancomycin	1	S*
Levofloxacin	>=8	R	Tetracycline	>=16	R
Moxifloxacin	4	R	Tigecycline	0.25	S
Inducible Clindamycin Resistance	NEG	-	Nitrofurantoin	<=16	S
Rifampicin	2	I	Trimethoprim/Sulfamethoxazole	<=8	S

*Some strains are Vancomycin resistance. R: Resistance; S: Sensitive; I: Intermediate

benzylpenicillin, oxacillin, gentamycin, ciprofloxacin, levofloxacin, and moxifloxacin. The identified *S. aureus* strains were also susceptible to linezolid, nitrofurantoin, vancomycin, tigecycline, and trimethoprim / sulfamethoxazole.

DISCUSSION

Since milk is a vital source of nutrients essential to human health, it must be produced in sterile conditions to avoid any biological risks. Direct or indirect interaction between healthy dairy animals and/or their milk and different sources of contamination is the main pathway for the presence of foodborne bacteria in milk (Owusu-Kwarteng *et al*., 2020).

VITEK is characterized by accuracy and high speed for detecting Gram positive and Gram negative pathogens (Kacmaz *et al*., 2017). Similar results obtained by Eltokhy *et al*. (2021) who isolated *E. coli* from 76.7% of the higher incidence of *E. coli* was detected by Metwally, and Ali (2015) and Elbastawesy *et al*. (2016), but they were less than the results of Saad *et al*. (2012) and Bonyadian *et al*. (2014). The level of *E. coli* found in dairy products is used as a gauge for the cleanliness of the workplace, the water quality used to handle and process milk products, and the personal hygiene of food handlers (Metz *et al*., 2020).

Also, Bousbia *et al*. (2018) failed to isolate *Salmonella* species from raw milk. Due to their consistent pathogenicity, *Salmonella*e are not tolerated in a food such as milk (Rechidi-Sidhoum *et al*., 2021). The investigations of (Singh *et al*., 2018) revealed the presence of *Salmonella* species, that are resistant to many antibiotics with a higher prevalence in raw milk (11.9%).

Higher results recorded by Bogdanovičová *et al*. (2014) and Eltokhy *et al*. (2021) who detected *S. aureus* in 73.3% of raw milk samples and lower results reported by Al-Gamal *et al*. (2019).

Furthermore, Eltokhy *et al*. (2021) found *B. cereus* in 13.3% of raw milk samples. Lower results recorded by Yobouet *et al*. (2014); while was not found in other examined samples. *Bacillus cereus* is widely distributed in the environment and may thrive in a variety of media, including the earth and plants, as well as in the digestive tracts of insects, animals, and foods like raw milk (Arnesen *et al*. 2008). Additionally, several *B. cereus* strains are well known for producing toxins that cause food poisoning (Tewari and Abdullah, 2015).

Entero-pathogenic *E. coli* is a food–borne pathogen that causes severe fatal diarrhea for children, have been reported in developing nations (Alonso *et al*., 2011). Additionally, the presence of *S. aureus* in raw milk, its byproducts, and mastitic udder, which is thought to be a source of toxic strains in raw milk, as well as the high storage temperature of raw milk prior to separation, promote *S. aureus* growth and can promote the generation of toxins (Fagundes *et al*., 2010).

Many opportunistic bacteria that cause disease and food deterioration can grow well in milk and dairy products. *S. aureus*, *Salmonella* species., *Listeria monocytogenes*, and *E. coli* are the pathogens most frequently found in connection with milk and dairy products in industrialized nations. Additionally, these organisms mimic the primary microbiological dangers associated with raw milk, tainted cheese that has been processed after, or incorrectly handled milk (Cancino-Padilla *et al*., 2017). These bacteria are significant because they are now included in the microbiological evaluation programs of both industry and governing bodies (Al-Gamal *et al*. 2019) as they are regarded as indicators of the quality or safety of dairy products (Price and Wildeboer, 2017).

Enteropathogenic *E. coli* is the most significant bacterial pathogen that causes fatal illnesses, notably in humans globally, including hemorrhagic colitis (HC), stomach discomfort, bloody diarrhoea, hemolytic uremic syndrome, and kidney failure (Pal *et al*. 2016). Due to inadequate handling procedures, milk is primarily contaminated with *E. coli* during direct contact with faeces and causes intestinal or extra intestinal illness (Bélanger *et al*., 2011).

The *E. coli* strains known as Shiga toxin-producing *E. coli* (STEC), also known as verotoxin-producing *E. coli*, are those that produce at least one Shiga toxin, a type of powerful cytotoxin (Gyles, 2007). A subclass of the Vero (Shiga) toxin-producing *E. coli* (VTEC or STEC), enterohemorrhagic *E. coli* (EHEC) strains produce either Vtx or Stx (Bai *et al*., 2016).

One of the leading infections causing mastitis in dairy cattle all over the world is *S. aureus*. The enterotoxin it produces is a substantial source of food poisoning, and it is a serious opportunistic pathogen of raw milk. For the purpose of determining the *S. aureus* risk, it is useful to monitor the antibiotic resistance of *S. aureus* in raw milk (Kou *et al*. 2021). Because of its widespread distribution, high rate of contamination, and rapid transmission, *S. aureus* has a strong pathogenicity.

It results in a range of clinical presentations, including minor localized skin lesions, major invasive infections, and even life-threatening conditions (Turner *et al*., 2019). Infections brought on by *S. aureus* have symptoms and severity that are correlated with certain virulence factors (Xing *et al*., 2016). Staphylococcal enterotoxins (SEs), one of them, are the main cause of foodborne illness. *S. aureus* food poisoning develops and manifests itself quite quickly, posing serious risks to human health (Messaoudi *et al*., 2013). These enterotoxins retain activity in the digestive tract after consumption due to their high stability (Fisher *et al*., 2018). 95% of staphylococcal food poisoning cases, according to reports, are caused by the traditional enterotoxins (Papadopoulos *et al*., 2019).

Due to their affordability and convenience of use, commercial automated systems for bacterial identification and susceptibility testing are employed in the majority of clinical microbiology laboratories in the United States. Antimicrobial susceptibility testing and identification are frequently performed using the VITEK 2 small system. Recent studies describing the effectiveness of VITEK 2 against Staphylococci concentrated on a single antimicrobial drug, like vancomycin (Swenson *et al*., 2009), cefoxitin (Junkins *et al*., 2009), clindamycin (ICR test) (Gardiner *et al*., 2013), or linezolid (Tenover *et al*., 2007). *S. aureus* with clindamycin (ICR test), which had a stated sensitivity of 91 to 95%, showed notable

differences (Gardiner *et al*., 2013), and with vancomycin, which had a reported sensitivity of 91% (Swenson *et al*., 2009).

Over 8 years have passed since a thorough analysis of VITEK 2's Staphylococci performance was published in peer-reviewed literature (Eigner *et al*., 2005). *E. coli* is described as one of the most frequently isolated causative agents associated with bovine intramammary infection (Keane *et al*., 2013). Due to the fact that *E. coli* is pervasive in the environment, there are numerous opportunities for it to enter cow udders through the teat canal (Burvenich *et al*., 2003). Antimicrobial therapy is currently the main strategy for preventing and treating dairy cow mastitis. Many antimicrobials have been approved in the United States, including -lactams, sulphonamides, quinolones, macrolides, and tetracyclines (Bengtsson *et al*. 2009).

Concern over the spread of antibiotic resistance is widespread and affects both human and animal health. Animals that produce food and people can contract resistant germs from each other (Roth *et al*., 2019). Antimicrobials can combat bacteria, protozoa, viruses, fungi, and other organisms, but the antibacterial class is the one that is most important for public health (Page and Gautier, 2012). However, due to prolonged antibiotic misuse, bacterial drug resistance is becoming more significant and treatment failure is frequent, especially for pathogenic bacteria that are multidrug resistant (Sweeney *et al*., 2018). Similar studies found that between 20 and 33% of *E. coli* isolates from raw milk samples from mastitis cases were resistant to at least one antimicrobial agent, and about 20 percent of the isolates were resistant to more than two classes of antimicrobial drugs (Fairbrother *et al*., 2015).

Since *S. aureus* is one of the main organisms responsible for dairy cow mastitis, tracking the usage of antibiotics will be helpful in determining the risk of *S. aureus* in raw milk. Antimicrobial therapy is an important tool in mastitis control programs, but *S. aureus* responds poorly to therapy with antimicrobial agents (Gomes and Henriques, 2016).

S. aureus is a pathogen with a remarkable ability to withstand antimicrobial agents and evade the human immune system. By recognizing the resistance mechanisms of *S. aureus*, effective measures in mastitis control programs can be established. Intrinsic resistance and acquired resistance have been shown to contribute to the ability of *S. aureus* to survive specific antimicrobial stress (Baym *et al*., 2016). It possesses numerous intrinsic factors that limit the effectiveness of specific antimicrobial agents (Rajagopal *et al*., 2016) and can develop acquired resistance to many other antimicrobial agents by carrying various traits on plasmids or transposons (Chajęcka-Wierzchowska *et al*., 2016). Acquired antimicrobial resistance has a transmission potential to humans (Ruegg *et al*., 2015). Therefore, monitoring antimicrobial resistance in *S. aureus* from raw milk is very important in order to predict the rate and type of antimicrobial resistance development and to make decisions regarding antibiotic treatments of animals from a food safety standpoint (Liu *et al*., 2021).

CONCLUSION

In view of the results obtained from microbiological and serological criteria, it appears that the quality of the milks studied does not comply with the standards as several pathogens that have public health importance were isolated. Also, the antibiotic resistance of *E. coli* and *S. aureus* was evaluated.

ACKNOWLEDGMENTS

We would like to thank Animal Health Research Institute members for their support.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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