American Journal of Animal and Veterinary Sciences

Original Research Paper

Antimicrobial Susceptibility of Bovine Subclinical Mastitis and During Milk Separation Isolates and the Accompanying Hygienic Practices

[1]Sultan Farag Nagati and [2]Safaa Khamis Hassan

[1]Department of Bacteriology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt
[2]Public Health and Community Medicine Department, Faculty of Medicine, Fayoum University, Egypt

Article history
Received: 22-01-2021
Revised: 08-03-2021
Accepted: 13-03-2021

Corresponding Author:
Sultan Farag Nagati
Department of Bacteriology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt
Email: sultan_farag99@yahoo.com

Abstract: Milk is considered one of the most perfect foods for humans and milk contains many nutrients. Subclinical mastitis has been counted as a great problem for dairy manufacture. The disease causes severe economic losses due to lower milk production, treatment costs and increased labor and milk retained for human consumption after treatment and premature culling. A cross-sectional study was conducted in 3 villages of Tamia district, Fayoum governorate, Bacteriological examination, Antibiotic susceptibility test, to study the presence of sub-clinical mastitis and milk contamination during milk separation and milking process and to detect the most resistance isolate to antibacterial agents and find the suitable antibacterial and disinfectants. 25.7% of the milk samples were positive for the California test. Skim milk and cream showed the isolation of *Staphylococcus aureus* (*S. aureus*), *Streptococcus aglacteae* (*S. aglacteae*), *Coliform bacteria*, *Arcanobacterium pyogenes* (*A. pyogenes*) and *Escherichia coli* (*E. coli*). The microbiological quality of the two disinfectants tested was considered satisfactory, as no significant number of microbial contaminants were recovered from them following the sterility test. Only 26.7% of milkers use water and soap for washing their hands. The participants had a lack of information about subclinical mastitis and improper hygienic practices during the milking and milk-handling process.

Keywords: Subclinical Mastitis, Milk Processing Machines, Bacteriology, Disinfectants

Introduction

Milk is considered one of the most perfect foods for humans, milk contains many nutrients needed for growth and development and prevention of many nutritional disorders (Dhanashekar *et al*., 2012). Raw milk is still used by a large number of farm families, workers and growing sectors of the population because they believe this raw milk is not only safe, but pasteurization destroys the health benefits of milk (LeJeune and Rajala-Schultz., 2009) milk is rich in proteins, lipids and sugars, milk is an example of ideal culture medium for various pathogens, including bacteria, viruses, fungi and parasites, which can be important sources of foodborne pathogens. These pathogens could be found in milk by direct contact with the contaminated animal environment and secretion from the udder of infected animals including mastitis and subclinical mastitis (Dhanashekar *et al*., 2012; Zeinhom and Abdel-Latef, 2014).

Mastitis is the most common inflammatory disease of the mammary gland in dairy animals, which causes several changes in milk such as, physical, chemical and microbiological changes (Eshratkhah *et al*., 2012). Mastitis is categorized into two forms, clinical which is diagnosed clinically and subclinical which is mainly diagnosed by estimation of somatic cell count and bacterial examination (Blowey and Edmondson, 2010).

Over 100 diverse micro-organisms have been listed as a cause of intramammary infection in dairy animals, the main causative micro-organisms were classified into infectious pathogens including (*Staphylococcus aureus* and *Streptococcus agalactiae*) and environmental organisms most encountered are species of streptococci other than *Streptococcus*

agalactiae and Gram-negative bacteria such as *E. coli* and Klebsiella (Dhanashekar *et al*., 2012; Zeinhom and Abdel-Latef, 2014; Fox and Gay, 1993).

Subclinical mastitis has been counted as a great problem for dairy manufacture; the infected quarter appears seemingly natural and could act as a hidden source of infection among dairy cattle (Sorensen *et al*., 2015). The disease causes severe economic losses due to lower milk production, treatment costs and increased labor and milk retained for human consumption after treatment and premature culling, therefore early detection of mastitis at the subclinical stage is important for most dairy farmers to take proper measures toward treatment and prevention of transmission of infection to another healthy one in the animal and thus reducing production losses. The aim of the study is pointed to study the presence of subclinical mastitis and milk contamination during milk separation and the milking process and to detect the most resistant isolate to antibacterial agents and find suitable antibacterial and disinfectants.

Materials and Methods

Study Design and Setting

A cross-sectional study was conducted in 3 villages of Tamia district, Fayoum governorate. Fayoum governorate is located in the southwest of Cairo in Egypt. The information on animals being studied was collected from the owners. The owners of the animals are individual farmers every farmer had 1 to 3 cows or buffaloes. The sampled milking animals were; Egyptian buffaloes and local or crossbred cows. The farmers are mainly keeping their animals in the back yard of their house. The backyard units are connected directly to the owner's home. In Fayoum, the diet of the animals (home-prepared concentrates) is not devised according to the physiological needs, neither in terms of quality nor quantity. The animals are taken to the field early morning after milking every day for feeding by green ration and return home just before sunset. At home, the concentrates (1-3 kg/animal) are provided once daily, in addition to dry wheat hay. The average daily milk yields from buffalo ranged from 15 to 18 L/d and for local cow 10 to 13L/d crossbreed cow from 19 to 22 L/d. Most of the sampled animals were in the mid-lactation stage (from 3 to <6 months) based on the age of the calves. The target group was the milk separator machine owners and people using the milk separator machine for their animals (6 machine owners and all the women who milk animals and come to separate the milk with the separation machine which is operated manually). The data were collected and analyzed over 6 months from June to September 2020 Table 1.

The study was designed as follow:

a. Cows and buffalo milk under manufacturing was tested for subclinical mastitis using the California test
b. Milk samples were collected from cows and buffaloes milk before and after separation (skim milk)
c. Hand swabs from workers and milking persons were collected before using the separator machine directly. Dorsal and palmar surfaces and fingertips were swabbed using a moistened cotton sterile swab (using sterile ringer's solution) which was rubbed gently against surfaces
d. Swabs were collected from the separator machine before and after the operation
e. Samples were collected from cream
f. All samples were submitted for isolation of bacteria, followed by a susceptibility test for different antimicrobial agents
g. Study the effect of the used two disinfectants on bacterial isolates
h. An interview structured questionnaire was designed to assess the knowledge of farmers on clinical and subclinical mastitis and to identify their hygienic practices during the milking process. The data of the study were collected by the authors

California Mastitis Test (*CMT*): (*Leach et al., 2008*)

A small sample of milk (approximately 2 mL) per liter was collected in a plastic paddle of four shallow cups marked A, B, C and D with an equal amount of CMT reagent added to the milk. To mix the content, the paddle was rotated and after a few seconds (about 20 seconds) the result was read. The test was performed daily to support the data obtained with an accurate somatic cell count Table 2.

Table 1: Types and number of samples collected

		Milk byproduct		Swabs*			
Animal	Milk samples	Cream	Skim milk	Before use machine	After use	Human hand	Total sample
Time	-	-	-	6	6	12	24
Buffalo	24	3	3	-	-	-	30
Cow	11	3	3	-	-	-	17
Total	35	6	6	6	6	12	71

Table 2: Interpretation of California test

Category	Score	Description of reaction
Negative	0	A mixture of milk and California test fluid stays unchanged and can easily be shaken
Weak positive/trace	1	The mixture is slightly mucous but can still be shaken
Positive	2	With a movement of the mixture, an unmistakable mucous formation can be seen. A small portion of the mixture can still be taken out.
Strong positive	3	Jelly-like, mucous consistency is formed and is difficult to shake the mixture. The excess fluid can no longer be removed.

Bacteriological Examination

Samples were submitted for isolation and identification of different bacteria causing mastitis (the milk sample was collected from the four-quarter and considered as one sample) (Quinn *et al*., 2002; Abera *et al*., 2010), by plating on the following culture media plates (Oxoid): Sheep blood agar, MacConkey agar mannitol salt agar, Staph-Strept, media, Aloa agar, XLD, CN Pseudomonas-specific media Morphological characterization of the colonies, the effectiveness of hemolysis on sheep blood agar, microscopic morphology evaluation on Gram Stained samples and biochemical characterization by oxidase test, catalase test and Staphtect (Oxoid) or by commercial API (Biomèurieux).

Milk, swabs and cream samples were cultured on blood agar plates, incubated at 37°C for 16-24 h. The growth on the plate was confirmed by additional laboratory tests according to routine in vitro procedures. *Staphylococcus aureus* was recognized by the typical colony morphology, alpha and beta hemolysis, or by coagulant reaction (positive coagulase) when typical hemolysis regions were not present. Coagulase-negative staphylococcus was recognized by typical colony morphology and coagulation reaction. *Streptococcus agalactia* was determined by colony morphology and CAMP-reaction and biochemical reactions were used for typing to the species level. Enterococci were confirmed by Gram staining and the growth of model colonies on SlaBa plates (Oxoid Ltd. Basingstoke, England). Gram-negative bacteria of typical colony shape, p-nitrophenyl-b-D-Glucupyranosiduronic Acid (PGUA) and indole were considered *E. coli*. For other Gram-negative bacteria, the oxidase reaction and API 20 E biochemical test profile (BioMérieux) was used.

Antibiotic Susceptibility Test

The disc diffusion method (according to NCCLS, 2002) was placed using differing kinds of antibacterial discs with changing concentrations to detect the susceptibility of isolates. These discs were gotten from (Oxoid). Tetracycline (TE 30 μg), Ampicillin (AM 10 μg), Neomycin (N 30 μg), Erythromycin (E 10 μg), Sulfa/trimethoprim (SXT 25 μg), Cephalothin (KF 30 μg), Amikacin (KA 30 μg), Clindamycin (DA 2 μg), Colistin sulfate (CT 2 μg), Gentamicin (CN 10 μg), Lincomycin (L 2 μg) and Enrofloxacin (Er 10 μg).

Assessment of the microbiological quality of two disinfectants (A and B) used on surface and farm equipment (Samson *et al*., 2017).

Types of Disinfectants Used and Their Neutralizers:

1. Type B disinfectant (Combination of four Quaternary Ammonium (QAC), glutaraldehyde and two ter) pene derivatives it neutralizer is lecithin
2. Type A disinfectant contains peracetic acid, its neutralizer is glycine

Sterility Check of Test Disinfectants

0.1 mL sample of each disinfectant was added to 0.9 mL sterile diluents, 0.02 mL volume of the diluted disinfectant was placed on each Nutrient Agar (NA) and Sabour Dextrose Agar (SDA) plates prepared. The NA plate was incubated at 37°C for 3 days; the SDA plate was incubated at 25°C (room temperature) for 7 days. Five or more colonies on either plate indicate contamination of the test disinfectant. Fungal isolates were identified on the basis of microscopic (using Lactophenol cotton blue staining) and macroscopic characteristics.

Evaluation of Disinfectant Activity on Each Test Isolate

Standardization of Isolates

A single isolated colony of each isolate was obtained from Tryptic Soy Agar (TSA) plates and inoculated separately in 10 mL of Tryptic Soy Broth (TSB) and incubated for 24 h at 37°C. After incubation, the 24 h broth culture was filtered with a saline pre-wet filter paper to remove slime and centrifuged at 2000 rpm for 20 min. The cell pellets were washed with 10 mL TSB. Then the population density of the bacterial suspensions in the TSB (about 10^7 CFU/mL) was adjusted by matching 0.5 McFarland Standard (10^5 CFU/mL) then diluting 1:100 in sterile TSB.

Quantitative Suspension Test (*QST*)

0.1 mL of the standard bacterial suspension will be added to 0.9 mL of the disinfectant solutions and softly mingle at room temperature for contact times of 0, 1, 3, 5, 10 and 15 min. The timer was started when the bacterial test suspension was combined with the

disinfectant. Then, at time 15 min, the specified contact time, 0.1 mL of the disinfectant-organism mixture was removed and transferred to a tube containing 0.9 mL neutralizer (tube A) and mix thoroughly. Within 5 min of transfer to the neutralizer tube, three additional ten-fold dilutions in saline blanks shall be made to achieve 10^{-1}, 10^{-2} and 10^{-3} dilutions (Tube B, Tube C and Tube D, respectively). 0.1 mL of each dilution was plated onto TSA plates in duplicate by the spread-plate technique and incubated at 37°C for 24 h. After incubation, TSA plates were noted for no visible growth. The surviving microbial colonies were enumerated, multiplied with factor hundred (100) and expressed as Colony-Forming Units per milliliter (CFU/mL). Controls were set for all organisms tested to indicate the neutralizer activity. For control, 0.1 mL each of 0.5 McFarland broth from the test object was mixed with 0.9 mL of neutral in separate tubes and so transferred to TSB, as a procedure described with disinfectants. Later, all the controls were striped onto TSA plates. The presence of growth indicates that the neutralizer is not inhibiting the microbes tested. Similarly, 0.1 mL of every disinfectant was mixed with 0.9 mL neutralizer, then 0.1 mL suspension of the test organism (0.5 McFarland standard) was added to every tube, later, they are directly transferred and incubated in TSB and streaked onto TSA plates. Growth on TSA plates shows effective neutralization of the disinfectant activity (USEPA, 2014).

Determination of Bactericidal Effect of the Disinfectants:

$$RF = \log NC - \log ND$$

RF: The bactericidal effect (Logarithm reduction factor) of the disinfectants
NC: Number of colonies from control plates (no disinfectant)
ND: Number of colonies from test plates (after contact with disinfectant)

Data Analysis

Data were collected, coded and analyzed using Statistical Package for Social Science (SPSS version 16. Chicago IL, USA). Simple descriptive analysis in the form of frequencies and percentages were performed.

Results

Detection of Subclinical Mastitic Milk (*California Test*)

Machines one and six used milk with subclinical mastitis of both types of animals (mixed), Machines two and four used milk of the same animal with no subclinical mastitis (cattle milk pure), Machine three used milk of the same animal with subclinical mastitis (cattle milk infected) and Machine 5 used milk both animals (mixed) with no subclinical mastitis (cattle milk pure). 74.3% of milk samples used in this study were negative to the California test, which clarified that most of the milk samples were non-mastitic milk, while 25.7% were positive for the California test Table 3.

The isolation of different pathogens in cow and buffalo`s milk (machines 1, 3 and 6) corroborate the results of the California test, which discovered the presence of a subclinical mastitis Tables 4 and 6.

Tables 5 and 6 illustrated the milk of both cows and buffalos were free from pathogens, in machines (2, 4 and 5) which also showed negative results with the California test. Table 5 showed the isolation of *S. aureus, S. agalecteae and* other coliforms bacteria from machine swabs collected before used and *A. pyogenes*, other coliforms bacteria and *E. coli* were isolated from one hand of the personal handle with the machine (1). In the case of a machine (4), *S. aureus* was isolated from the machine before used; the Hand swab showed the isolation of other coliforms, *A. pyogenes* and *E. coli* (50% for each). Skim milk and cream showed the isolation of *S. aureus, S. aglacteae, other coliforms bacteria, A. pyogenes and E. coli* Table 5.

In Table 6, coliform was isolated from both machine and hand swabs of a personal handle with the machine (5). Also, *S. aureus* and *E. coli* were isolated from the hand swab during the handle with a machine (5). The ratio of different isolates among different samples, where all isolates isolated from machine swabs, hand swabs and subclinical mastitis milk were isolated from skim milk and cream Table 7.

In this study sensitivity test was applied to most of the isolated strains. *A. pyogenes* showed complete resistance to all antimicrobial agents used, followed by *S. aureus* which showed only 10% sensitivity to enrofloxacin, 40% of *S. agalacteae* were susceptible to enofloxacin, 30% to Erythromycin and Sulfa/trimethoprim and 20% to tetracycline. *E. coli* were susceptible to enrofloxacin (80%), Amikacin (70%) colistin sulfate and neomycin (40%), tetracycline (30%) and Sulfa/trimethoprim (20%) Table, 8.

The microbiological quality of the two disinfectants tested was considered satisfactory, as no significant number of microbial contaminants were recovered from them following the sterility test. The bactericidal effect of B disinfectant (0.5%) and A disinfectant (2%) solution on isolates per contact time is presented in (Table 9, Figs. 1 and 2) respectively. On one hand, all isolates were grown when exposed for 3 min to both disinfectants except coliform no growth observed, on the other hand, *A. pyogenes* were grown when exposed to both disinfectants for 10 min. A period of ten minutes of

exposure or contact was sufficient time for killing most isolates. Only A disinfectant has its biocides effect on *A. pyogenes* when exposed for 15 min.

Analysis of Data Collected from the Owners of the Milk Separator Machine and Milkers

All the owners of the milk processing machine use the machine for personal and business use. The machine is used for more than one type of animal by 100%. The milk used each time is a mixture of milk from both cows and buffaloes by 100%. No one of the owners knows subclinical mastitis. All the owners wash the machine. The machine is washed once a time at the end of the day by 100% of them. 50% of the owners use water only for cleaning and 50% use water and soap (Table 10).

Table 3: The occurrence of subclinical mastitis in milk samples

Animals	Milk samples	No. of +ve samples	%*	No. of -ve samples	%
Buffalo	24	5	20.8	19	79.2
Cow	11	4	36.4	7	63.6
Total	35	9	25.7	26	74.3

*: Percentage calculated according to the total No. of animals

Table 4: The pervasiveness of different bacteria isolated from different samples (machines 1&6)

				Type of isolated m.o							
				S. aureus		*S. agalcteae*		*Coli form*		*E. coli*	
Machine	Type of. animals/No.	Type of samples	No. of samples	No.	%	No.	%	No.	%	No.	%
Machine 1	Cow/5	Milk	5	3	60.0	1	20.0	5	100.0	2	40.0
	Buffalo/3	Milk	3	1	33.3	0	0.0	2	66.6	0	0.0
		Machine swab before	1	1	100.0	1	100.0	1	100.0	0	
		Machine swab after	1	1	100.0	1	100.0	1	100.0	1	
		Hand swab	2	0	0.0	0	0.0	2	100.0	0	0.0
		cream	1	1	100.0	1	100.0	1	100.0	1	100.0
		Skimmed milk	1	1	100.0	1	100.0	1	100.0	1	
Total			14	8	57.1	4	28.6	11	78.6	4	28.6
Machine 6	Cow/3	Milk	3	3	100.0	0	0.0	3	100.0	1	33.3
	Buffalo/2	Milk	2	2	100.0	2	100.0	2	100.0	0	0.0
		Machine swab before	1	1	100.0	1	100.0	1	100.0	0	
		Machine swab after	1	1	100.0	1	100.0	1	100.0	1	
		Hand swab	2	0	0.0	0	0.0	1	50.0	1	50.0
		Cream	1	1	100.0	1	100.0	1	100.0	1	100.0
		Skimmed milk	1	1	100.0	1	100.0	1	100.0	1	
Total			11	9	81.8	6	54.5	11	100.0	5	45.5

Table 5: Pervasiveness of different bacteria isolated from different samples (machines 2&4)

				Type of isolated m.o									
				S. aureus		*S. agalcteae*		*S. coliform*		*A. pyogenes*		*E. coli*	
Machine	Type of animals/No.	Type of samples	No. of samples	No.	%	No.	%	No.	%	No.	%	No.	%
Machine 2	Cow /2	Milk	2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
		Machine swab before	1	1	100.0	1	100.0	1	100.0	0	0.0	0	0.0
		Machine swab after	1	1	100.0	1	100.0	1	100.0	1	100.0	0	0.0
		Hand swab	2	0	0.0	0	0.0	0	0.0	1	50.0	0	0.0
		cream	1	1	100.0	1	100.0	1	100.0	1	100.0	0	0.0
		Skimmed milk	1	1	100.0	1	100.0	1	100.0	1	100.0	0	0.0
Total			8	4	50.0	4	50.0	4	50.0	4	50.0	0	0.0
	Buffalo /5	Milk	5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Machine 4		Machine swab before	1	1	100.0	0	0.0	0	0.0	0	0.0	0	0.0
		Machine swab after	1	1	100.0	0	0.0	0	0.0	1	100.0	1	100.0
		Hand swab	2	0	0.0	0	0.0	1	50.0	1	50.0	1	50.0
		Cream	1	1	100.0	0	0.0	1	100.0	1	100.0	1	100.0
		Skimmed milk	1	1	100.0	0	0.0	1	100.0	1	100.0	1	100.0
Total			11	4	36.4	2	18.0	4	36.4	3	27.3	3	27.3

Table 6: The pervasiveness of different bacteria isolated from different samples (machines 3&5)

				Type of isolated m.o							
				S. aureus		*S.agalcteae*		*Coliform*		*E. coli*	
Machine 3	Type of animals/No.	Type of samples	No. of samples	No.	%	No.	%	No.	%	No.	%
	Buffalo /7	Milk	7	2	28.6	2	28.6	3	42.9	0	0.0
		Machine swab before	1	0	0.0	0	100.0	0	0.0	1	100.0
		Machine swab after	1	1	100.0	1	100.0	1	100.0	1	100.0
		Hand swab	2	0	0.0	0	0.0	2	100.0	0	0.0
		cream	1	1	100.0	1	100.0	1	100.0	1	100.0
		Skimmed milk	1	1	100.0	1	100.0	1	100.0	1	100.0
Total			13	5	38.5	3	23.1	5	38.5	4	30.8
	Cow /1	Milk	1	0	0.0	0	0.0	0	0.0	0	0.0
	Buffalo 7	Milk	7	0	0.0	0	0.0	0	0.0	0	0.0
		Machine swab before	1	1	100.0	1	100.0	1	100.0	0	0.0
Machine 5		Machine swab after	1	1	100.0	1	100.0	1	100.0	1	100.0
		Hand swab	2	1	50.0	0	0.0	1	50.0	1	50.0
		Cream	1	1	100.0	1	100.0	1	100.0	1	100.0
		Skimmed milk	1	1	100.0	1	100.0	1	100.0	1	100.0
Total			14	5	35.7	4	28.6	5	35.7	3	21.4

Table 7: Total ratio of pathogens isolated from different samples

	Cow`s milk (11)		Buffalos milk (24)		Machine swabs (12) (before and after using)		Hand swabs (12)		Skimmed milk (6)		Cream (6)	
Type of sample												
Type of isolates	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
S. aureus	6	54.5	5	20.8	7	58.3	1	8.3	6	100.0	6	100.0
S. agalacteae	1	9.1	2	8.3	7	58.3	0	0.0	5	83.3	5	83.3
Coliform	8	72.7	5	20.8	6	50.0	6	50.0	6	100.0	6	100.0
E. coli	3	27.2	0	0.0	2	16.7	3	25.0	6	100.0	6	100.0
A, puogenes	0	0.0	0	0.0	0	0.0	2	16.7	2	33.3	2	33.3
Total (71)	18	25.4	12	16.9	22	31.0	12	16.9	25	35.2	25	35.2

Table 8: Sensitivity test results of different isolates against different antibacterial agents (10 isolates of each strain)

Antibacterial disc	*S. aureus* (*No./%*)		*S. agalacteae* () (*No./%*)		*E. coli* (*No./%*)		*A. pyogenes* (*No./%*)	
	S	R	S.	R.	S.	R.	S.	R.
Tetracycline (TE 30 µg),	0	10/100	2/20	8/80	3/30	7/70	0	7/100
Ampicillin (AM 10 µg),	0	10/100	0	10/100	0	10/100	0	7/100
Neomycin (N 30 µg),	NA	NA	NA	NA	4/40	6/60	0	7/100
Erythromycin (E 10 µg),.	0	10/100	3/30	7/70	NA	NA	0	7/100
Sulfa/trimethoprim (SXT 25 µg)	0	10/100	3/30	7/70	2/20	8/80	0	7/100
Cephalothin (KF 30 µg),	0	10/100	0	10/100	0	10/100	0	7/100
Amikacin (KA 30 µg),	0	10/100	0	10/100	7/70	3/30	0	7/100
Clindamycin (DA 2 µg),	0	10/100	0	10/100	0	10/100	0	7/100
Colistin sulfate (CT 2 µg),	NA	NA	NA	NA	4/40	6/60	0	7/100
Gentamicin (CN 10 µg),	0	10/100	0	10/100	0	10/100	0	7/100
Lincomycin (L 2 µg)	0	10/100	0	10/100	3/30	7/70	0	7/100
Ernofloxacin (Er 10 µg)	1/10	9//90	4/40	6/60	8/80	2/20	0	7/100

Table 9: Bactericidal effect of A and B disinfectant on isolates per contact time

		Contact time						
Disinfectant	Isolates	0 min	1 min.	3 min.	5 min	7 min	10 min	15 min
Type A	*S. aureus*	G	G	G	NG	NG	NG	NG
	S. agalacteae	G	G	G	NG	NG	NG	NG
	E. coli	G	G	G	NG	NG	NG	NG
	Coliform	G	G	NG	NG	NG	NG	NG
	A. pyogenes	G	G	G	G	G	G	NG
Type B	*S. aureus*	G	G	G	G	G	NG	NG
	S. agalacteae	G	G	G	NG	NG	NG	NG
	E. coli	G	G	NG	NG	NG	NG	NG
	Coliform	G	G	NG	NG	NG	NG	NG
	A. pyogenes	G	G	G	G	G	G	G

Table 10: The behavior of the owners of the cream separator milk machine

Question on …?	Number	%
the machine use for		
Personal	0	0
Business	0	0
Both	6	100
The machine is used for		
One type of animal	0	0
More than one type	6	100
The type of milk used in each time		
Cows	0	0
Buffalo	0	0
Mixed milk	6	100
Have you ever heard of subclinical mastitis		
Yes	0	0
No	6	100
Washing and disinfecting the machine before use		
Washing only	6	100
Disinfection only	0	0
The number of washing times per day		
After each use	0	0
Once-daily before starting use and after finishing work	0	0
Once at the end of the day	6	6
The material used for cleaning		
Water and soap	3	50
Water only	3	50

Table 11: The characteristics and behavior of animal milkers

Question on …?	Number	%
Sex (females)	105	1000
Education		
Illiterate	68	64.8
Primary	29	27.6
Secondary	8(7.6)	
Do you ensure that the machine is cleaned?		
Yes	0	0.0
No	105	100.0
The number of animals that you milk each time		
One	9	8.60
More than one	96	91.40
Presence of animals with mastitis		
Yes	13	92.00
No	92	87.60
What to do if a quarter is infected (swelling of one quarter without a change in color)		
Use the non-infected ones and Discard the infected one	11(	10.5
Mix milk from both udder (the infected by non-infected)	94	89.5
What to do if a quarter infected (swelling of one quarter with a change in color		
Using milk	12	11.4
Not using milk	93	88.5
Have you heard of subclinical mastitis before		
Yes	0	0.0
No	105	100.0
Have you ever had an animal with a low milk production?		
Yes	105	100.0
No	0	0.0
Action taken when decreased milk yield occurs		
ask vet	61	58.1
Lab analysis	0	0.0
No action	12	41.9
When a decrease in the usual amount of milk occurs		

Table 11: Continue

Use the milk without inquiring about the reasons	105	100.0
Not using the milk	0	0.0
cleaning hands before milking		
Yes	88	83.8
Sometimes	17	16.2
cleaning hands after milking		
Yes	35	33.3
No	70	66.7
The material used for cleaning		
Water only	77	73.3
Water and soap	28	26.7
Use an antiseptic for hands and mammary		
Yes	0	0.0
No	100	100.0
Make karish cheese at home		
Yes	100	100.0
No	0	0.0

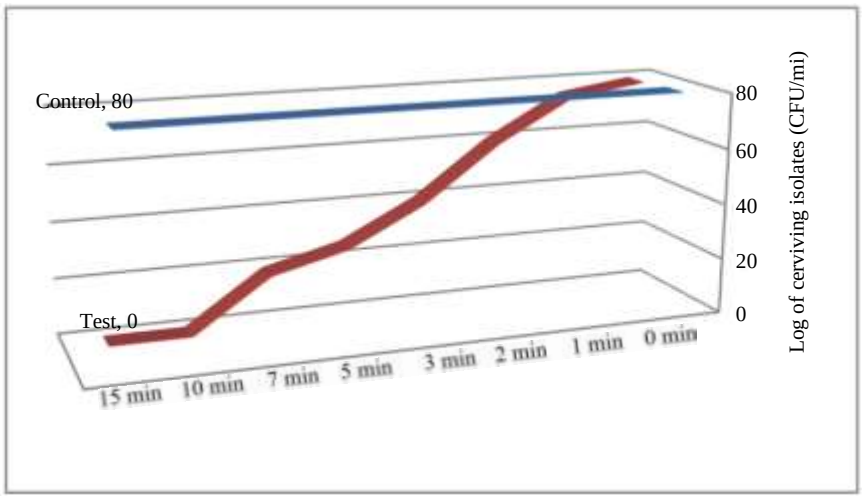

Fig. 1: Line charts showing the killing rate of *S. aureus* isolates when exposed to 0.5% (B disinfectant) for 15 min

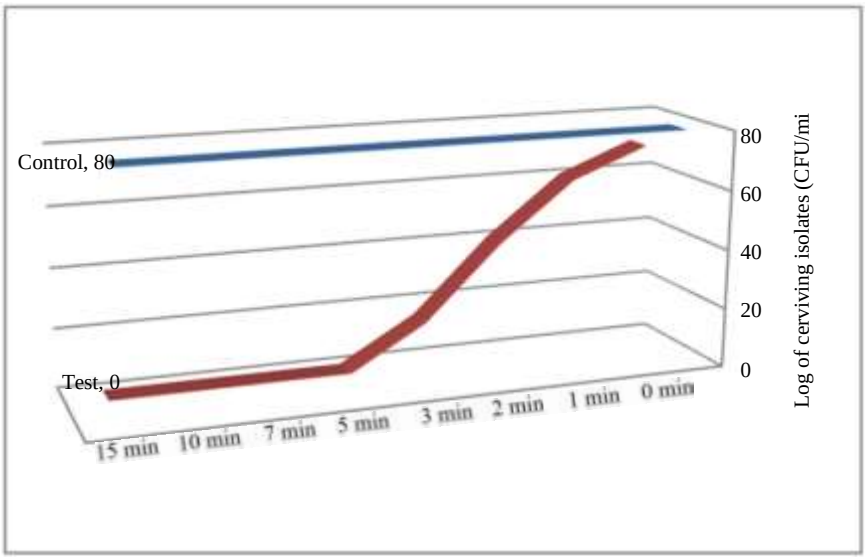

Fig. 2: Line charts showing the killing rate of *S. aureus* isolates when exposed to 2% of (A disinfectant) for 15 min

The data were collected from 105 females aged from 18 to 55 years with a mean of 31.5±10.5 years. The majority (64.8%) were illiterate. Most of the participants (91.4%) milking more than one type of animal each time. Most of them (87.6%) had one or more animals with a history of the previous mastitis. 89.5% of milkers mix the milk from both quarters of the udder if there was no change of the milk color. Only, 11.4% of milkers using milk from infected udder with color milk change. No one of the milkers knows about subclinical mastitis. 100% of them have ever experienced a decrease in the production of animal milk. 68.1% of them asked veterinarians about low animal milk yield. All participants (100%) used milk in the presence of decreased animal milk production. The majority (83.8%) wash their hands before milking the animals. Only 26.7% of them use water and soap for hand-washing (Table 11).

Discussion

Subclinical mastitis is an annoying problem in dairy farms, it is responsible for virtually accounted for 70% of economic losses of mastitis cases and its frequency is about 15 to 40 times the frequency of clinical mastitis. (Losinger, 2005; El-Awady and Oudah, 2011). In the present study, a total of 71 samples were collected from six milk manual separating machine used for bacteriological isolation, 35 (24 cows and 11 buffaloes' milk before used), 12 milk byproduct (6 cream and 6 skimmed milk) and 24 swabs (6 swabs before using the machines, 6 swabs after using and 12 hand swabs from human) to determine the presence of the bacterial contamination of the milk byproduct. The hygienic measures taken by the handlers before, during and after milking have a greater effect on the safety and hygiene of milk and its products. Infected personnel involved in milk handling, containers used to put milk during milking, storage and delivery may be possible sources of contamination. Under bad sanitary conditions, milk can be easily contaminated by diverse microorganisms (Chatterjee *et al*., 2006). Studies exhibited that bovine clinical mastitis is currently one of the most serious problems found on dairy farms, where the disease is with the highest economic impact on milk production. The impact on public health should be taken into account as dairy animals produce milk and milk byproducts as a cream for consumption (Riekerink *et al*., 2008).

In this study, regarding the hygienic process of the owners of milk processing machine, all the owners used the machine for both types of animals (cows and buffalo) and all of them wash the machine once a day at the end of the milk operation process, no hygienic practices were followed between each time of work (100%) and none of them use disinfectants to clean the machine. These poor hygienic practices may be sources of contamination to milk and its products besides no one of the female milkers (100%) asked if the machine was cleaned or not. The majority (83.8%) wash hands before animal milking and 33.3% wash their hands after milking. 41% of them clean the udder before milking and only 26.7% of them use water and soap for washing the hands. These findings were similar to a study conducted in Ethiopia. All the milkers were also females, 76.5% of them practiced udder washing and drying before milking. However, 64.7% did not practice post milking udder wash. On the other hand, 58.8% practiced washing milking equipment with detergents before milking (Regasa *et al*., 2019), others reported that more than 60% of the milkers did not clean their hand nor cleaning the udder of the animal before milking, indicated that the microorganisms on hand could lead to contamination of the milk (Shija, 2013) Use of unsterilized containers and other practices such as milking with unsterilized bare hands and allowing calves to feed without cleaning udder nipples, exposing the milk to microbial contamination (Owusu-Kwarteng *et al*., 2020) and avoid cross-contamination proper cleaning and disinfectant of the containers is important (kivaria *et al*., 2006).

Mastitis constitutes one of the most important and expensive diseases of the dairy industry. It is difficult to discover Subclinical Mastitis (SCM) due to the absence of visible designation, in the mammary gland and milk. The alteration in milk yield and composition depends on the severity and duration of mammary gland infection (Oliver and Calvinho, 1995). In our findings, although no one of the milkers stated that they knew or heard of subclinical mastitis all of them (100%) reported that they had a reduction in animal milk production once and 58.1% asking vet advice on animal milk reduction and no one of them has done laboratory tests for their animals and this may reflect the high illiteracy rates and poverty in rural areas in Fayoum governorate (HDR, 2008). In India, (Sinha *et al*., 2014) revealed that economic losses due to mastitis were due to the reduced milk yield by 48.53% followed by veterinary expenses, which account for 36.57% of the total loss.

Our findings revealed that 25.7% of the milk samples were positive to the California test which revealed that their mastitic milk appeared before separating operation, this result may be explained by the fact that this study was conducted under high humid conditions during the summer months in Egypt, which increase the rate of mammary infections, this was similar to what reported by (Dang *et al*., 2013). We used the California Mastitic test in our study according to (Leach *et al*., 2008), the CMT was supported by (Galfi *et al*., 2017) who revealed that CMT represents a valuable diagnostic method in detecting subclinical mastitis in dairy cows and high sensitivity and specificity.

The isolation of different pathogens in cow and buffalos milk (machines 1, 3 and 6) corroborates the results of the California test which discovered the presence of subclinical mastitis. These results proved a possible explanation for this finding could be that most farmers in the study area do not run-through proper farming management and screen for mastitis at an earlier stage (Mpatswenumugabo *et al*., 2017; Bekuma and Ulfina, 2018).

In machines 2 and 4, the isolation of different microorganisms from hand swabs and machine swab (machine 2) revealed that bad cleaning of personal handling with machines and bad cleaning and no disinfecting of the machine (2) before use, which lead to the presence of all isolates present in cream and skimmed milk.

All isolates were isolated from machine swabs, hand swabs and subclinical mastitis milk from skim milk and cream. The dairy industry is known for its health policies, performance and high health standards. This industry is especially essential for good sanitation practices to ensure the safety and well-preservation of dairy products. (Marriott, 1997; Schlegelova *et al*., 2010).

Clean and sterile equipment and buildings are essential for the production, processing and distribution of healthy dairy products and it is important to use appropriate cleaning vehicles and equipment so that workers can clean the facility in a shorter period and with less work. (Marriott, 1997).

Enrofloxacin was the best antimicrobial agent for most isolates except for *A. pyogenes* and these results agree (Sripad *et al*., 2016). Most of the isolates displayed MDR to different classes of antimicrobial agents. The evidence for the effectiveness of antimicrobial treatments for mastitis is limited. Antibiotic resistance has turned into a considerable worry for human and animal health. The detection of MDR-from *S. aureus* and *A. pyogenes* in various samples is of concern and represents a major public health concern. These results agree with (Salauddin *et al*., 2020). Antiseptics used on equipment and surfaces should be tested periodically for effectiveness. Since some disinfectants lose their effectiveness upon standing in addition to organic matter, their effectiveness must be tested. While some methods help in choosing the right dilution of sanitizer for others to use, test the effectiveness of the sanitizer used. The present study evaluated the microbiological quality and efficacy of two common disinfectants (A and B) used to disinfect the environment, surfaces and equipment. The microbiological quality of the tested disinfectants. No bacterial or fungal growth of any of these isolates occurred after appropriate days of incubation on Nutrient Agar (NA) and Sabour Dextrose Agar (SDA). All isolates were grown upon exposure for 3 min to both antiseptics except coliform no growth was observed.

The period of ten minutes of exposure or contact was sufficient time for killing all isolates except *A. pyogenes*. Biocides (BC) resistance among bacterial pathogens is not frequent, but resistance may develop to user concentrations after exposure to sublethal concentrations of BC. (Sidhu *et al*., 2004). *A. pyogenes* were not grown when exposed to a disinfectant A for 15min. Hydrogen peroxide was the only disinfectant found to be effective versus all of the tested microorganisms. The biocidal effect of hydrogen peroxide is attributable to the-OH radical, formed by the decomposition of peroxide in the presence of catalysts, such as iron and copper ions, commonly found in microorganisms. The radical acts via an oxidative mechanism against the membrane, DNA and other cellular constituents of the microorganism (Montagna *et al*., 2019).

Selecting a disinfectant registered for a particular pathogen is crucial. It is also important to recognize that observed effects in laboratory studies of small increases in intolerance to some disinfectants and association with antibiotic resistance have to be balanced versus the general health benefits of using them (Gerba, 2015).

Conclusion

The results showed that the owners of the milk separation machine and small farmers, who account for the cattle milk production in Egypt, the participants had a lack of information about subclinical mastitis and improper hygienic practices during the milking and milk-handling process. The use of on-farm written protocols to treat mastitis encourages the rational use of antimicrobials and reduces antimicrobial use. The reason for the strains to remain after Cleaning and Disinfectant (C&D) is not resistant to the disinfectant, but could be due to insufficient C&D in critical locations and/or residual organic matter and/or other factors affecting the efficacy of disinfectants (such as diluting disinfectant with residual rinse water and environmental temperature. If this behavior continues and is consistent, this may lead to the presence of germs that are resistant to C&D in this area.

Acknowledgment

We would like to thank prof. Somyia El Shafaii, Agriculture research center, for helping and supporting us.

Author's Contributions

Sultan Farag Nagati: Select the research topic, shared in questionnaire design, editing and revising the manuscript.

Safaa Khamis Hassan: Planned the research methodology, shared in questionnaire design, data analysis and shared in drafting of the manuscript.

Ethical Approval and Consent to Participate

The study protocol was approved by the ethics committee of the animal health research institute at 27 Aught 2020 that complies with guidelines of the World Medical Association Declaration of Helsinki. Informed consent was obtained from all participants.

References

Abera, M., Demie, B., Aragaw, K., Regassa, F., & Regassa, A. (2010). Isolation and identification of Staphylococcus aureus from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. Journal of Veterinary Medicine and Animal Health, 2(3), 29-34.

Bekuma, A., & Galmessa, U. (2018). Review on Hygienic Milk Products Practice and Occurrence of Mastitis in Cow's Milk. Agricultural Research & Technology: Open Access Journal, 18(2), 1-11. https://doi.org/10.19080/ARTOAJ.2018.18.556053

Blowey, R. W., & Edmondson, P. (2010). Mastitis control in dairy herds.: CABI. International, UK. https://www.lactoscan.com/editor/ufo/manuals/SCC/Mastitis_Control_in_Dairy_Herds_2nd(veterinary-student.blogfa.com).pdf

Chatterjee, S., Bhattacharjee, I., Chatterjee, S. K., & Chandra, G. (2006). Microbiological examination of milk in Tarakeswar, India with special reference to coliforms. African Journal of Biotechnology, 5(15). https://www.ajol.info/index.php/ajb/article/view/43120

Dang, A. K., Prasad, S., De, K., Pal, S., Mukherjee, J., Sandeep, I. V. R.,... & Prakash, B. S. (2013). Effect of supplementation of vitamin E, copper and zinc on the in vitro phagocytic activity and lymphocyte proliferation index of peripartum Sahiwal (Bos indicus) cows. Journal of Animal Physiology and Animal Nutrition, 97(2), 315-321. https://doi.org/10.1111/j.1439-0396.2011.01272.x

Dhanashekar, R., Akkinepalli, S., & Nellutla, A. (2012). Milk-borne infections. An analysis of their potential effect on the milk industry. Germs, 2(3), 101. https://doi.org/10.11599/germs.2012.1020

El-Awady, H. G., & Oudah, E. Z. M. (2011). Genetic and economic analysis for the relationship between udder health and milk production traits in Friesian cows. Asian-Australasian Journal of Animal Sciences, 24(11), 1514-1524. https://doi.org/10.5713/ajas.2011.10328

Eshratkhah, B., Beheshti, R., & Shayegh, J. (2012). Variation of some minerals values in subclinical mastitic milk of Buffalo during different ages and lactation stages. Global Vet, 8, 333-337.

Fox, L. K., & Gay, J. M. (1993). Contagious mastitis. Veterinary Clinics of North America: Food Animal Practice, 9(3), 475-487. https://doi.org/10.1016/S0749-0720(15)30615-0

Galfi, A. L., Radinović, M. Ž., Davidov, I. N., Erdeljan, M. M., & Kovačević, Z. R. (2017). Detection of subclinical mastitis in dairy cows using California and Draminski mastitis test. Biotechnology in Animal Husbandry, 33(4), 465-473. http://scindeks.ceon.rs/article.aspx?artid=1450-91561704465G

Gerba, C. P. (2015). Quaternary ammonium biocides: efficacy in application. Applied and Environmental Microbiology, 81(2), 464-469. https://doi.org/10.1128/AEM.02633-14

HDR. (2008). Egypt Human Development Report 2008. Human Development Report. https://hdr.undp.org/sites/default/files/2008_egypt_nhdr_en.pdf.

Kivaria, F. M., Noordhuizen, J. P. T. M., & Kapaga, A. M. (2006). Evaluation of the hygienic quality and associated public health hazards of raw milk marketed by smallholder dairy producers in the Dar es Salaam region, Tanzania. Tropical Animal Health and Production, 38(3), 185-194. https://doi.org/10.1007/s11250-006-4339-y

Leach, K. A., Green, M. J., Breen, J. E., Huxley, J. N., Macaulay, R., Newton, H. T., & Bradley, A. J. (2008). Use of domestic detergents in the California mastitis test for high somatic cell counts in milk. Veterinary Record, 163(19), 566-570. https://doi.org/10.1136/vr.163.19.566

LeJeune, J. T., & Rajala-Schultz, P. J. (2009). Food safety: unpasteurized milk: A continued public health threat. Clinical Infectious Diseases, 48(1), 93-100. https://europepmc.org/article/med/19053805

Losinger, W. C. (2005). Economic impacts of reduced milk production associated with an increase in bulk-tank somatic cell count on US dairies. Journal of the American Veterinary Medical Association, 226(10), 1652-1658. https://doi.org/10.2460/javma.2005.226.1652

Marriott, N. (1997). Esentials of Food Sanitation (Edited by: Gill Robertson) Chapman and Hall. https://doi.org/10.1007/978-1-4615-6045-6

Montagna, M. T., Triggiano, F., Barbuti, G., Bartolomeo, N., De Giglio, O., Diella, G.,... & Caggiano, G. (2019). Study on the in vitro activity of five disinfectants against Nosocomial bacteria. International Journal of Environmental Research and Public Health, 16(11), 1895. https://doi.org/10.3390/ijerph16111895

Mpatswenumugabo, J. P., Bebora, L. C., Gitao, G. C., Mobegi, V. A., Iraguha, B., Kamana, O., & Shumbusho, B. (2017). Prevalence of subclinical mastitis and distribution of pathogens in dairy farms of Rubavu and Nyabihu districts, Rwanda. Journal of Veterinary Medicine, 2017. https://doi.org/10.1155/2017/8456713

NCCLS. (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2, 2nd ed. National Committee for Clinical Laboratory Standards, Wayne, Pa. National Committee For Clinical Laboratory Standards.

Oliver, S. P., & Calvinho, L. F. (1995). Influence of inflammation on mammary gland metabolism and milk composition. Journal of Animal Science, 73(suppl_2), 18-33. https://doi.org/10.2527/1995.73suppl_218x

Owusu-Kwarteng, J., Akabanda, F., Agyei, D., & Jespersen, L. (2020). Microbial safety of milk production and fermented dairy products in Africa. Microorganisms, 8(5), 752. https://doi.org/10.3390/microorganisms8050752

Quinn, P. J., Markey, B. K., Carter, M. E., Donnelly, W. J. C., & Leonard, F. C. (2002). Veterinary microbiology and microbial disease. Blackwell science. https://www.cabdirect.org/cabdirect/abstract/20013163051

Regasa, S., Mengistu, S., & Abraha, A. (2019). Milk safety assessment, isolation and antimicrobial susceptibility profile of *Staphylococcus aureus* in selected dairy farms of mukaturi and sululta town, oromia region, Ethiopia. Veterinary Medicine International, 2019. https://doi.org/10.1155/2019/3063185

Riekerink, R. O., Barkema, H. W., Kelton, D. F., & Scholl, D. T. (2008). Incidence rate of clinical mastitis on Canadian dairy farms. Journal of Dairy Science, 91(4), 1366-1377. https://doi.org/10.3168/jds.2007-0757

Salauddin, M., Akter, M. R., Hossain, M., Nazir, K. H. M., Noreddin, A., & El Zowalaty, M. E. (2020). Molecular Detection of Multidrug Resistant *Staphylococcus aureus* Isolated from Bovine Mastitis Milk in Bangladesh. Veterinary Sciences, 7(2), 36. https://doi.org/10.3390/vetsci7020036

Samson, E. S., Okeleke, O. J., Awharentomah, D. K., Richard, A.Y., Josephine, N., & Paul, A. C. (2017). Assessment of the Microbiological Quality and Efficacy of Two Common Disinfectants Used in Hospital Laboratory. Advances in Biomedical Sciences, 2(6) 31-43. http://eprints.abuad.edu.ng/459/

Schlegelova, J., Babak, V., Holasova, M., Konstantinova, L., Necidova, L., Šišák, F.,... & Jaglic, Z. (2010). Microbial contamination after sanitation of food contact surfaces in dairy and meat processing plants. Czech Journal of Food Sciences, 28(5), 450-461. https://doi.org/10.17221/65/2009-CJFS

Shija, F. (2013). Assessment of milk handling practices and bacterial contaminations along the dairy value chain in Lushoto and Handeni districts, Tanzania (Doctoral dissertation, Sokoine University of Agriculture). http://www.suaire.sua.ac.tz/handle/123456789/522

Sidhu, J. S., Volberda, H. W., & Commandeur, H. R. (2004). Exploring exploration orientation and its determinants: Some empirical evidence. Journal of Management Studies, 41(6), 913-932. https://doi.org/10.1111/j.1467-6486.2004.00460.x

Sinha, M. K., Thombare, N. N., & Mondal, B. (2014). Subclinical mastitis in dairy animals: incidence, economics and predisposing factors. The Scientific World Journal, 2014. https://doi.org/10.1155/2014/523984

Sorensen, L. P., Engberg, R. M., Løvendahl, P., & Larsen, T. (2015). Effects of Bos taurus autosome 9-located quantitative trait loci haplotypes on enzymatic mastitis indicators of milk from dairy cows experimentally inoculated with Escherichia coli. Journal of Dairy Science, 98(8), 5440-5447. https://doi.org/10.3168/jds.2014-9071

Sripad, K., Upendra, H. A., & Isloor, S. (2016). Efficacy of Enrofloxacin and Enrofloxacin with Ampicillin sulbactam Combination in the Treatment of Subclinical Mastitis in Dairy Cow. Frontier J. Vet. Anim. Sci,5(2): 111-113. https://www.kvafsu.edu.in/fjvas/FJVAS_Vol%205_Issue%202_Jul-Dec%202016_Articles.pdf

USEPA. (2014). Standard Operating Procedure for Quantitative Suspension Test Method for Determining Tuberculocidal Efficacy of Disinfectants against Mycobacterium Bovis (BCG). US Environmental Protection Agency, Office of Pesticide Programs, Microbiology Laboratory, Environmental Science Center, SOP Number: MB-16-02: pp. 1-12. https://www.epa.gov/sites/production/files/2014-11/documents/mb-16-02.pdf

Zeinhom, M. M., & Abdel-Latef, G. K. (2014). Public health risk of some milk borne pathogens. Beni-Suef University Journal of Basic and Applied Sciences, 3(3), 209-215. https://doi.org/10.1016/j.bjbas.2014.10.006