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Comparison of incidence of three major pathogens causing bovine subclinical mastitis in relation to SCC and enzymatic activities in large and small dairy herds in Egypt

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Abstract

The study was designed to investigate the current incidence of Staphylococcus aureus, Streptococcus agalactiae and Escherichia coli in bovine subclinical mastitis (SCM) in two groups of herds, large dairy herd: group 1 (≥100 head/ farm) and small and medium dairy herds: group 2 (≤50 head / farm + small owner's herds) in Egypt. *S. aureus* and *S. agalactiae* are contagious major pathogens of mastitis, while *E. coli* is an environmental major pathogen.

PCR amplification and gene detection were carried out on the extracted DNA of the pathogens' isolates which were identified by conventional methods. Three pairs of specific primers, one for each of the pathogens were used in PCR amplification. Detection of the amplified three products of sizes 231 bp, 280 bp and 108 bp resulted in confirmation of 100%, 72% and 40% of isolates of the *E. coli*, *S. agalactiae* and *S. aureus* respectively.

Additionally, the study referred to the associated variable values of somatic cell count, enzymatic activities of lactate hydrogenase (LDH), glutamic oxaloacetic transaminase (GOT) and alkaline phosphatase (ALP). The study included examination of randomly selected 476 of apparently normal milk samples belonged to 137 apparently healthy Holstein Friesian cows. Incidence of SCM was 42.6% of the examined quarters and 62% of cows represented by 203 samples and 85 cows with the result of recovered 242 isolates. In relative to total number of examined quarters, SCM quarters and total number of isolates, *S. agalactiae* constituted 2.31 %, 5.41% and 4.54 % while *S. aureus* was 3.6%, 8.37% and 7.2% whereas *E. coli* represented 7.4%, 17.2% and 14.5% respectively. SCC Geometric mean of SCM samples in the

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overall study was 483000 cell/ mL. In the present study, the higher SCC mean was in SCM samples with *S. aureus* (611000) followed by *E. coli* (545000) then *S. agalactiae* (394000). Geometric mean of LDH, GOT and ALP were 378, 147 and 469 IU/L in the overall study SCM samples. The higher mean of LDH was recorded in case of *S. aureus* (402) followed by *S. agalactiae* (398) then *E. coli* (369). The higher GOT value was in case of *S. agalactiae* (150) followed by *E. coli* (149) then *S. aureus* (147). The higher ALP value was in *E. coli* (614) followed by *S. aureus* (560) then *S. agalactiae* (553).

INTRODUCTION

Although, mastitis which is an inflammatory reaction within mammary tissues may be sometimes due to non-infectious agents that may be of physical, chemical or mechanical cause, the predominant cause is still the bacterial pathogens (Zadoks *et al.*, 2002). 135 different pathogens are recorded as causative agents of mastitis in bovine (Hawari and Al-Dabbas, 2008). The most prevalent pathogens are members of *Streptococcus sp*., *Staphylococcus sp*. and coagulase negative *Staphylococcus sp*. (CNS) (Piepers *et al.*, 2007). Mastitis may be clinical (CM) or subclinical (SCM). In the subclinical form, the resulted inflammatory reaction doesn't represent any apparent changes on milk, udder nor the cow. Additionally, pathogens in sometimes may be not shed in milk (Sordillo *et al.*, 1997). Additionally, SCM pathogens are classified into contagious and environmental pathogens. Infections due to contagious pathogens spread through transmission from cow with infected mammary tissues to another non-infected one. Great financial losses resulted from SCM and mostly due to the steady deteriorating of milk production (Abdel Hameed and Sender, 2006). *S. aureus*, S. agalactiae, *E. coli*, *S. dysgalactiae* and *S. uberis* are the most impacting pathogens of mastitis (Hegde *et al.*, 2013). The most important contagious pathogens are *S. agalactiae* and *S. aureus* (Goli *et al.*, 2012). While Escherichia coli is regarded as one of the forefront major environmental pathogens of SCM (Hinthong *et al.*, 2017). A substantial decrease in the cases of *S. agalactiae* as well as *S. aureus* is recorded and explained by increasing application of control hygienic measures (Pitkälä *et al.*, 2004).

E. coli is considered the preliminary Gram-negative pathogens causing mastitis (Burvenich *et al.*, 2003). In study in-between 56 pathogens isolated in metagenomic study of bovine milk samples with subclinical mastitis; *E. coli* was the most detected pathogen (Bhatt *et al.* 2012).

Most dairy losses due to SCM, originate from the double significance of the SCC. The first purpose of the SCC is related to the milk quality when introducing milk to dairy plants while the second purpose is related to udder health. While dairy producers give their attention towards first, they ignore the second significance. Whereas the first is concerned with losses in current revenue that still not received and includes avoidance of the penalties and milk withdrawn when dealing with dairy plants, while treating with SCC through the second, needs extra expenses (Seegers *et al.*, 2003). SCM Cases may persist undetected and may change into a chronic state if still standing for two months (Erskine, 2020). Chronic SCM is mostly recorded in cases of *S. aureus* when compared with *S. agalactiae* SCM (Goli *et al.*, 2012). Because of the apparent healthy state of cow, udder and milk of SCM cases, laboratory examination is considered the only tool to detect SCM cases (Lakic, 2007)

Culturing of milk samples is considered the typical technique for mastitis pathogens identification (Mattila *et al.*, 1985). Nevertheless, identification of mastitis needs more than one bacteriological investigation. In spite that most cases of mastitis are due to pathogens colonization, many of the chronic SCM examinations may be false negative when are conducted once (Babaei *et al.*, 2007).

According to the International Dairy Federation (IDF) both somatic cell count (SCC) and microbiological examination are needed for diagnosis of mastitis (Pyörälä, 2003). The need for the combination of bacteriological identification and isolation together with SCC is triggered by presence of many other factors that may affect and alter the SCC within many non-infection conditions (Katsoulos *et al.*, 2010). Other factors in addition to mastitis, may alter values of the SCC as cow age, parity, seasonal effectors, breed, lactation stage and oestrus (Paura *et al.*, 2002). California Mastitis Test (CMT) and many other indirect methods are used in evaluation of SCC, but the typical standard method is the direct microscopic SCC (Bremel et. al. ,1977 and Kamal *et al.* 2014). Suggested values for SCC in both healthy or in altered milk are greatly variable in between authors, locations, regions, dates and countries. An average between 250000 and 300000 cells/mL was proposed (Dohoo and Meek, 1982). Mastitis causing pathogens are either major or minor pathogens. It was recorded that major pathogens as *S. agalactiae* and *S. aureus*, are related to high figures of SCC while minor pathogens as CNS, are inducing low SCC (Oliver and Calvinho, 1995).

Appropriateness, duration, sensitivity and specificity are the required criteria for the method of detection, isolation and identification of the etiologic agents of the SCM. Conventional bacteriological techniques are currently the used golden method, nevertheless, they are time consuming and in some cases are unsuitable for some pathogens to be distinguished from other closely related. Development of PCR techniques was used for diagnosis of the major pathogens S. agalactiae, *S. aureus*, *E. coli*, *S. uberis* and *S. dysgalactiae* and were suggested (Riffon *et al.* 2001).

Mastitis which is an inflammatory changes of mammary tissues. Consequently, both positive bacteriological examination as well as inflammatory alteration should be accomplished for diagnosis of mastitis. Regarding to

complicated issues relating to the precise bacteriological identification techniques, the second, that is the inflammatory alteration, is used as screening for SCM and consequently, depending to its results, the crucial bacteriological identification is performed. SCC has been always used for that purpose (Mattila *et al.*, 1985). Nevertheless, there has been always, a need for new screening biomarkers that are characterized by more specificity, sensitivity, fast and ease to detect SCM (Åkerstedt *et al.*, 2008). Acute phase proteins and different enzymes in milk fulfill these criteria as screening detectors of SCM (Hirvonen *et al.*, 1999). High cost inappropriateness of the use of acute phase proteins was recorded (Åkerstedt *et al.*, 2008). Evaluation of activities of milk enzymes were considered more appropriate technique for SCM screening method (Kitchen, B. 1981). Released Enzymes that are shed in milk from destructed mammary epithelial as well as inflammatory leucocytes during SCM may be used as indicators of SCM (Oliszewski *et al.*, 2002).

Many of mastitis pathogens present in milk are responsible for production of part of the ALP in addition to the bovine ALP (Rankin *et al.*, 2010). Some studies suggested ALP to be the most milk enzymes that could be used as indicator in bovine SCM (Babaei *et al.*, 2007). LDH was suggested to be a diagnostic biomarker of SCM (Chagunda *et al.*, 2006) (Harmon, 1994).

The aim of this study is Investigation of the S. agalactiae, *S. aureus* and *E. coli* prevalence's in SCM cases in relation to SCC, LDH, GOT and ALP in different types of dairy herds including large, medium and small farms, as well as smallholder dairy herds.

MATERIALS AND METHOD

1- Animals and samples:

From data of Table (1), the study involved randomly selected 528 bovine quarters' milk samples classified into two groups of herds. They all are from ten dairy farms' animals. The first included 338 quarter milk samples recovered from 85 cows belonged to four large farms (≥100 dairy heads). The second included 190 quarter milk samples from 52 cows belonged to herds of three small farms (≤ 50 heads), One medium farm herds (70 dairy heads) and two groups of small owners' animals that were submitted to the Animal Reproduction Research Institute (A.R.R.I.).

Table 1. List of study samples, animals, farms, and groups of samples

Milk samples sources		Examined animals	All Examined milk samples	Apparently abnormal Milk samples	Apparently normal samples
Group 1					
1- Nubaria Region farm	large Farm	25	98	5	93
2- Al Tal Al Kabeer city farm	large Farm	25	100	10	90
3- Ismailia Governorate farm	large Farm	10	40	14	26
4- Monofia Governorate farm	large Farm	25	100	5	95
		85	338	34	304
Group 2					
1- El Fayom Governorate medium farm	medium Farm	18	71	1	70
2- Al Sharkia Governorate small farm	Small farm	9	31	15	16
3- Al Giza small farm no.1 submitted to A.R.R. I.	Small farm	8	29	2	27
4- Al Giza small farm no.2 submitted to A.R.R. I.	Small farm	9	31	0	31
5- Small owners' herd group 1 submitted to A.R.R. I	Small Owners	4	13	0	13
6- Small owners' herd group 2 submitted to A.R.R. I	Small Owners	4	15	0	15
		52	190	18	172

Large farms ≥100 dairy heads, medium farm (50-100 dairy heads), small farms (≤ 50 heads)

Apparent normal 304 and 172 milk samples were recovered from group1 and group 2 respectively. They were obtained from 137 cows that are free from any apparent abnormalities in the milk, as flacks, clots or blood, nor in the mammary gland and cows like swelling, hotness, induration, redness nor discolorations.

2- Milk sampling

Udders and teats were washed, dried and first four strips of milk were thrown. The teats were disinfected with ethyl alcohol 70%. Two samples of 25 and 10 mL aseptic milk samples were gathered from each quarter. The first was used for SCC and enzymatic analysis while the second was used for conventional bacteriological examination as well as further PCR. Bacteriological examination of samples was performed within 24 hours after sampling.

3- Screening for subclinical mastitis

On the basis of SCC 250000 cell/mL as threshold for SCM in apparently healthy samples recovered from healthy udder and animals SCC, bacteriological examination and PCR **(Schultz, 1977).** Accordingly, samples with SCC ≥ 250000 cell/mL were considered as SCM cases. They were bacteriologically examined for screening of ***S. agalactiae, S. aureus*** and ***Escherichia coli*** and values were calculated as percentages to all apparently normal 476 milk samples. Cow was assigned as SCM case, when at least has one SCM quarter.

4- Direct microscopic SCC

Somatic cells in milk samples were counted by the direct microscopic method as the reference procedure as recommended by Commission of European Communities, (1991).

4.1. Preparation and staining of the milk film (Nierman, 2004):

Levowitz-Weber / Newman-Lampert xylene stain (LW-N L-X stain) was used to stain milk sample film. Briefly, on

well-defined area of 1 cm^2 on glass slide, 0.01 mL of smoothly agitated milk sample is spread. The slide is left to be dried, rinsed with worm water then left to dry. The slide is then examined microscopically and the somatic cells in a strip at the most middle zone of the slide, are counted as shown in the Figure (1) which represents fields of four samples of milk with different counts of somatic cell. Other objects in the field as pathogens cells were not counted Figure (2).

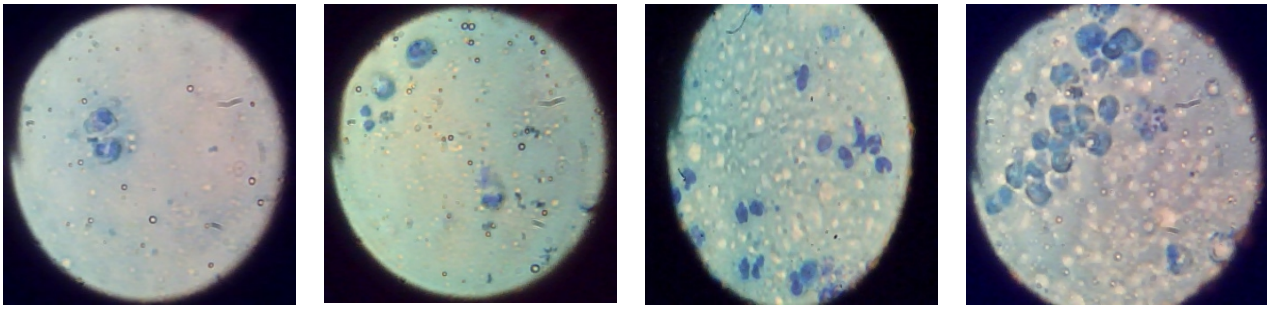

Figure 1. Fields of four milk samples with different SCC.

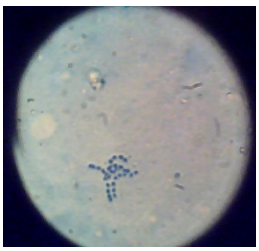

Figure 2. Milk sample with chains of cocci seen during SCC and were not counted.

4.2. Calculation of the somatic cell count per one milliliter milk sample (Commission Decision 1991):

SCC in one milliliter milk sample was calculated according to the following equation: SCC in 1 mL milk sample = counted cells in the strip × $\frac{100 \times 1}{d \times r}$

d = diameter of the microscopic field by the use of stage micrometer

r = diameter of the middle-located strip which is counted

5- Bacteriological Examination:

Milk sample films were subjected to defattening and centrifuged at 3000 r.p.m. for 2 minutes. Milk samples were stained by Gram's stain and subjected to direct microscopic examination (Quinn *et al.,* 2002) Figure (2).

5.1. Cultivation on ordinary and selective media:

All milk samples were incubated for 18-24 hours at 37°C (Seadawy, 2004). During the incubation period, direct microscopic SCC were conducted and the samples with somatic cell count ≥ 250000 cell/mL were assigned as SCM cases. SCM samples were screened for the presence of *S. agalactiae, S. aureus* and *E. coli.* Selected pathogens are three major pathogens of subclinical mastitis. *S. agalactiae* and *S. aureus* are contagious major pathogens of mastitis while *E. coli* is an environmental major pathogen. 0.01–0.03 ml of SCM milk samples were streaked onto nutrient agar medium and blood agar medium then positive cultures were conveyed to be cultured onto Edward's, Mannitol, MacConkey's and Eosine methylene blue agar (EMB) media as selective media for *S. agalactiae* and *E. coli*.

Plates were incubated at 37 °C, and examined for culture after 24 and 48 hours. Plates without growth were examined for growth after 48 hours and considered negative if no growth up to 72 hr.

Resulted colonies were examined for color, size, and changes on the media as hemolysis, color. Pure identical colony was picked and subcultured on nutrient slant for being examined microscopically and biochemically identified. While another colony was picked and cultivated on semisolid agar media to be used for PCR amplification.

5.2. Biochemical identification of Gram positive cocci:

5.2.1. Biochemical identification of *S. agalactiae* (Quinn *et al.*, 2002), (Koneman *et al.*, 2004) and (Collee *et al.*, 1996):

S. agalactiae were identified by their characteristic's morphology and catalase negative reaction. Then they were subjected for identification by their hemolysis pattern on blood agar, CAMP test and fermentation on sugars fermentation test as shown in Table (2):

Table 2. Biochemical identification of ***S. agalactiae***

Features and tests	*S. agalactiae*
Aggregations pathogen cells	Cocci in chains
Catalase tests	–
Coagulase test (tube test)	–
Oxidase tests	–
Hemolysis	β - hemolysis
CAMP test	+
Voges Prosk test	+
Lactose fermentation test	+
Mannitol salt agar	–
Bacitracin sensitivity	R
R over 90% of strains are resistant + over 90% of strains are positive. – over 90% of strains are negative	

5.2.2. Biochemical identification of Gram-positive *S. aureus*: (Quinn *et al.*, 2002), (Koneman *et al.*, 2004) and (Collee *et al.*, 1996)

Biochemical identification of pure cultures of the isolates were performed by Catalase tests, coagulase tests using tube method, hemolysis pattern, pigmentation, mannitol and maltose fermentation test, salt tolerance using mannitol salt agar, novobiocin sensitivity tests were conducted and interpreted as in table (3):

Table 3. Biochemical identification of ***S. aureus***

Tests	*S. aureus*
aggregations pathogen cells	Cocci in clusters

Catalase tests	+
Coagulase test (tube test)	+
Hemolysis	+
Colony pigmentation	+W
Maltose fermentation test	+
Mannitol salt agar	W
Novobiocin sensitivity	S
S sensitive W weak reaction + W positive to weak reaction + over 90% of strains are positive. – over 90% of strains are negative	

5.3. Biochemical identification of Gram-negative Escherichia coli:

As described by Quinn *et al.* (2002), Gram-ve negative bacilli (*E. coli)* were identified on basis of the colony growth characteristics on both MacConkey's bile salt and Eosine methylene blue agar (EMB) media, lactose agar medium, Gram's stain, oxidase test, sugar fermentation test, and the IMViC test, which included indole test, methyl red test, Voges Proskauer test and citrate utilization test. *E. coli* isolates were identified as shown in Table (4)

Table 4. Biochemical identification of Escherichia coli

Features and tests	*Escherichia coli*
Shape	Gram negative bacilli
Spore formation	Non spore former
Colony size,	Medium sized (3-6 mm in diameter),
Colony shape	opaque, sticky
Colony pigmentation	greyish white colonies.
Haemolysis on blood agar	Some strains show haemolysis
Catalase test	+
Oxidase test	–
Indole test	+
Methyl red test	+
Voges Proskauer test	–
Citrate utilization test	–
Lactose fermentation test	+
Mannitol fermentation test	+
+ over 90% of strains are positive. – over 90% of strains are negative	

6- Molecular detection of *S. agalactiae, S. aureus* and *E. coli* by PCR technique

6.1. DNA extraction and isolation

Of each of the pathogen isolates, an identical colony was picked and stabbed on semi solid nutrient agar for PCR examination. Isolates' DNA were prepared for amplification by use of Dneasy kits of Qiagen. DNA extraction was conducted to be used in detection and amplification of the selected gens for each pathogen according to the manufacturer's recommendations. Isolates were re-cultured in 20 mL TSB (Difco Laboratories, Detroit, Mich.) incubated for 18 hours at 37ºC before the extraction. 1.5 mL of the cultured TSB was centrifuged for 5000 3g for 10 minutes. The resulted pellet is resuspended in 200 µL of enzyme incubation buffer (brought in the kit) and incubated for 30 min at 37°C. 25 ml of proteinase K (brought in the kit) was supplemented and incubated at 70°C for 30 min. 20 µg of RNase H /ml (involved in the kit) was added with 200 µl of AL buffer. The mixture was incubated for at 70°C for10 min. the mixture is the transferred into column and eluted using deionized sterilized water.

6.2. Selected primers of molecular detection of *S. agalactiae, S. aureus and E. coli* by PCR technique:

Selected PCR primers of each of *S. agalactiae, S. aureus and E. coli* are highly divergent and applied in previous studies. Their sequences were screened by BLAST procedure through the universal gene bank using the BLAST procedure of the NCBI website (www.ncbi.nlm.-nih.gov). The primers listed in Table (5).

Table 5. List of selected *S. agalactiae* and *E. coli* primers

Organism	Primer	Sequence (5¢–3¢)	Annealing temp	Product size
S. agalactiae	*STRA*- AgI *STRA*- AgII	5'-GGAAACCTGCCATTGCG -3' 5'-TAACTTAACCTTATTAACCTAG-3'	*43 oC*	280
S. aureus	SSa442-1 Sa442-2	5'-AATCTTTGTCGGTACACGATATTCTTCACG-3 5'-CGTAATGAGATTTCAGTAGATAATACAACA-3	*40 oC*	118
E. coli	*ECO*455F *ECO*223R	5'ATCAACCGAGATTCCCCCAGT 3' 5' TCACTATCGGTCAGTCAGGAG 3'	*62 oC*	231

6.3. PCR Amplification for DNA of S. agalactiae, *S. aureus* and *E. coli* Gene:

The PCR method was used to investigate the presence of the *S. agalactiae* gene, *S. aureus* gene and *E. coli* gene defining *S. agalactiae, S. aureus* and *E. coli* respectively, among the isolates. The quality of purified DNA was investigated using electrophoresis. 5 µL of each DNA was amplified in 25 µL of mixture reaction consisting of a 2X Taq polymerase master mix, 10 ρmol of primers. PCR Amplification and Gene Detection on the extracted DNA were carried out as follow:

6.3.1. PCR Amplification for *S. agalactiae* Gene Detection

PCR was performed by Conventional PCR (Applied biosystems Veriti 96 TC) with an initial denaturation step of 5 minutes at 95°C; 35 cycles of 15 second at 94°C; 30 second at 40°C annealing temperature for the *S. agalactiae* genes; and 40 seconds at 72°C followed by a 5 minutes' final extension at 72°C. For determining the presence of the desired amplicon, electrophoresis was done on 1.5% gel agarose stained by ethidium bromide, and then the products were visualized by UV transilluminator. The sizes of the PCR product of amplifying Str. Agalactiae gene are 280 bp.

6.3.2. DNA Isolation and PCR Amplification for *S. aureus* Gene Detection

PCR was performed by Conventional PCR (Applied biosystems Veriti 96 TC) with an initial denaturation step of 5 minutes at 95°C; 35 cycles of 15 second at 94°C; 30 second at 43°C annealing temperature for the *S. aureus (Sa442)* gene and 40 seconds at 72°C followed by a 5 minutes' final extension at 72°C. For determining the presence of the desired amplicon, electrophoresis was

done on 1.5% gel agarose stained by ethidium bromide, and then the products were visualized by UV transilluminator. The size of the PCR product of amplifying *S. aureus* (*Sa442*) gene is 108 bp.

6.3.3. DNA Isolation and PCR Amplification for *E. coli* Gene Detection

PCR was performed by Conventional PCR (Applied biosystems Veriti 96 TC) with an initial denaturation step of 5 minutes at 95°C; 35 cycles of 15 second at 94°C; 30 second at 62°C annealing temperature for the *E. coli* genes; and 40 seconds at 72°C followed by a 5 minutes' final extension at 72°C. For determining the presence of the desired amplicon, electrophoresis was done on 1.5% gel agarose stained by ethidium bromide, and then the products were visualized by UV transilluminator. The sizes of the PCR product of amplifying ***E. coli*** gene are 231 bp.

7- Biochemical analysis of milk enzymatic Activities:

Enzymatic activities of the three enzymes were performed by spectrophotometric analysis technique using kits of "Spectrum Diagnostics", following instruction of the manufacture. Preparation of milk samples before analysis involved defattening by cooling centrifugation for 30 minutes at 15000 g at temperature 4 °C. Samples with values beyond the values of the used kits range was diluted with 0.9% saline solution at 1/10 dilution, then the obtained value was multiplied by ten as dilution factor (Kalantari *et at.* 2013).

RESULTS AND DISCUSSION

The present study was designed to investigate the bacterial etiology of subclinical mastitis in bovine and to highlight the correlation between variation of SCC and recovered *S. agalactiae, S. aureus* and *E. coli*.

Table 6. SCM in the two herds' groups

Dairy herds' Group	Apparently normal Milk samples	No. of SCM quarters	Percentage of SCM quarters	Examined animals	No. of SCM animals	Percentage of SCM animals
Large Farms group (1)	304	118	38.8%	85	49	57.6%
Small / medium group (2)	172	85	49.4%	52	36	69.2%
Overall study figures	476	203	42.6 %	137	85	62%

Large farms ≥100 dairy heads, medium farm (50-100 dairy heads), small farms (≤ 50 heads)

As shown in Table (6), In the current study, 203 milk samples were found of SCC ≥ 250000 cell / mL and were belonged to 137 cows. SCM in the overall study, in relative to apparently normal quarters, was 42.6 % while in between cows, it was 62 %. On the quarter level, similar result of SCM in milk quarters was recorded by Suleiman *et al.* (2017), which was 42.9%, while it was higher than results of Hussein *et al.* (2018) and Olivares-Pérez *et al.* (2015) which were 32.73% and 20.5% respectively. On the animal level, present study result was lower than results Suleiman which was 70.9% (Suleiman *et al.*, 2017).

In between the two studied groups, higher incidence of SCM was recorded in small /medium herds group (2) in relative to large farm herds group (1). On the quarter level, incidence of SCM in small / medium herds group was higher (49.4%) than the large herd group (38.8%). While on the animal level, SCM in small farms was higher (69.2%) than large farms (57.6%).

Although it was confirmed that SCC couldn't be used as indicators for each specific mastitis causative organisms (Dohoo and Meek, 1982), great variation in between SCC in each SCM causative pathogen (Ward and Schultz 1972). It was pointed out that higher SCC values are recorded in the major mastitis pathogens like *S. aureus* while moderate elevations are recorded in case of minor ones as coagulase negative staphylococci (Oliver *and* Calvinho, 1995).

Table 7. Number of SCM milk samples according to the recovered number of isolates per sample

No of SCM milk samples	Samples with one isolate	Samples with two isolates	Samples with three isolates	No of samples with isolates	No of samples with no isolates
203	70	59	18	147	56
% to examined samples	34.48%	29.06%	8.86%	72.4%	27.6%

Table 8. Number and percentage of isolates for each used media in relative to the total isolates

Edward's media Isolates	Mannitol media 56Isolates	MacConkey's media Isolates	No of isolates
101	94	47	242
41.74 %	38.84 %	19.42 %	100 %

Bacteriological examination of milk samples with SCC ≥ 250 000 were performed. As seen in Table (7), 56 milk samples were negative for culture while 147 milk samples were positive and represented 72.4% of the examined samples. 34.48%, 29.06% and 8.86 of the samples contain one isolate, two isolates and three isolates respectively. As shown in Table (8), 101, 94 and 47 isolates representing 41.74%, 38.84% and 19.42% were recovered from Edward's media, mannitol media and MacConkey's media respectively.

Table 9. Number of isolates of each media and pathogens

		S. agalactiae	*S. aureus*	*E. coli*	% of isolates to /
Number of isolates		11	17	35	
Total isolates	242	4.56 %	7.02 %	14.46 %	Total isolates
SCM quarters	203	5.41%	8.37 %	17.42%	SCM quarters
All examined quarters	476	2.31%	3.6 %	7.35%	Total examined quarters

From data of Table (9), *S. agalactiae* were recovered in 11 quarter milk samples with SCC ≥ 250000 cell /mL that represent 5.41% of all quarter samples. Study results are higher than results reported by Abdel Hameed and Sender (2006) and Abd El-Razik *et al.* (2010) which were 1.4% and 3.12%. While Keef (1997), Östensson *et al.* (2013) and Erskine *et al.* (1987) reported higher figures 11%, 21% and 26% respectively. In relative to the total recovered isolates in the study, *S. agalactiae* isolates constituted 4.56 % of the total recovered isolates in the study. This result was higher than the result which was reported by Gangwal and Kashyap (2017) who recorded 2%. Current result is much lower than that obtained by Abdelrady *et al.* (2009) and Östensson *et al.* (2013) who recorded 31.5% and 35.7 % respectively.

From data of Table (9), 17 *S. aureus* isolates were recovered in 17 quarter milk samples of SCC ≥ 250000 cell /mL that represent 8.37% of all quarter samples. Close figure was recorded which was 7.6 % (Erskine *et al.*, 1987). While reported higher figures were 10.7%, 15.62%, 16.6% and 59.9% by Pumipuntu *et al.* (2019), Abd El-Razik *et al.* (2010), Abdel Hameed and Sender (2006) and Shitandi and Kihumbu, 2004) respectively. lower values were recorded by Thorberg *et al.* (2009) and Östensson *et al.* (2013). In relative to the total recovered isolates in the study, recovered *S. aureus* isolates constituted 7.02% of the total isolates. This result was higher than the figures that were reported by Östensson *et al.* (2013), Mamache *et al.* (2014) and Thorberg *et al.* (2009) which were 4.2%, 4.5%, and 6.3% respectively. while higher figures were recorded Gangwal and Kashyap (2017), Seddek *et al.* (1999), Saidi *et al.* (2013), Abdelrady *et al.* (2009) and Shitandi and Kihumbu, (2004) and were 24%, 29.1%, 40%, 52.5%, 58% and 59.9% respectively.

E. coli is one of the major mastitis pathogens. In the current study, 35 *E. coli* isolates were recovered in 35 quarter milk samples of SCC ≥ 250000 cell /mL that represent 17.2% of all samples. These results are approximately agreed with results obtained by Abdelrady *et al.* (2009), Sayed (2014) and Mamache *et al.* (2014) which were 16.25%, 17.8 and 17.97% respectively. Meanwhile the study results were higher than those achieved by Thorberg *et al.* (2009) and Mahmoud *et al.* (2015) and Shitandi and Kihumbu (2004) which were 1.8%, 3.7% and 5.9% respectively. In relative to the total recovered isolates in the study, *E. coli* isolates constituted 14.5% of total isolates. This result was much higher than those obtained by Thorberg *et al.* (2009) and Hameed and Sender (2006) which were 0.3% and 2.9% respectively. the results were lower than results of Harini and Sumathi (2011) and Abd El-Razik *et al.* (2010) which were 28%. And 23.75% respectively.

Table 10. Lower and higher SCC values and Number and types of isolates of each group

Group / Farm	Lower – higher SCC values / farm	*S. agalactiae*	*S. aureus*	*E. coli*	Examined animals	SCM quarter	Apparently normal sample	No of all isolates
Large farm Group (1)								
1- Nubaria Region farm	265300 - 856000	0	0	4	25	42	93	28
2- Al Tal Al Kabeer city farm	265000 - 862000	0	5	8	25	33	90	51
3- Ismailia Governorate farm	280000 - 848000	0	0	0	10	9	26	3
4- Monofia Governorate farm	251000 - 854500	5	11	8	25	34	95	73
		5	16	20	85	118	304	155
Small /medium farm Group (2)								
5- El Fayom Governorate medium farm	260000 - 854000	6	0	11	18	27	70	53
6- Al Sharkia Governorate small farm	528000 - 852200	0	0	0	9	2	16	2
7- Al Giza small farm no.1 submitted to A.R.R. I.	350000 - 817000	0	0	1	4	13	13	8
8- Al Giza small farm no.2 submitted to A.R.R. I.	261000 - 854000	0	0	0	8	13	27	4
9- Small owners' herd group 1 submitted to A.R.R. I	329000 - 856000	0	1	3	9	18	31	16
10- Small owners' herd group 2 submitted to A.R.R. I	277900 - 639000	0	0	0	4	12	15	4
		6	1	15	52	85	172	87

Large farms ≥100 dairy heads, medium farm (50-100 dairy heads), small farms (≤ 50 heads)

From data of Table (10), five *S. agalactiae* isolates were recovered from group (1) while six isolates were obtained from group (2). On the farm level, *S. agalactiae* constituted 6.85% of total isolates in the first farm while it was 11.32% of the second. It is worth notifying that the higher *S. agalactiae* incidence was in the farm of the higher incidence of recovered pathogens in the study. No *S. agalactiae* isolates were recovered from the other farms.

In case of *S. aureus,* 16 isolates were obtained from samples of large farm group (1) while only one isolates were from small / medium farm group (2). No *S. aureus* isolates were recovered from the other farms. On the farm level, *S. aureus* constituted 15.1% of total isolates in the first farm while it was 9.8% of the second and 6.25% in the third one. It is worth notifying that the two higher *S. aureus* incidences were belonged to the two farms of the higher and the third higher incidences of total pathogen isolates in the study.

20 isolates of *E. coli* were recovered from large farm group (1) while 15 isolates were obtained from samples of small /medium farm group (2). *E. coli* isolates were

recovered from 28.8% from examined cows in the group (1) which constitute 16.9% at the SCM quarter level and 6.6% of the all apparently normal samples and 12.9% of the isolates while it is recovered from 23.5% of the animals in group (2) constituting 17.6%, 8.7% and 17.2% of SCM quarter level, all apparently normal samples and the total isolates respectively. It is worth notifying that *E. coli* was isolated in six herds involved in the study in-between 10 herds.

Table 11. Relation between ***Streptococcus agalactiae*** and somatic cell count

	S. agalactiae	***S. aureus***	***E. coli***
Group1			
Lower – Higher SCC	291 000- 649 000	298 000 - 855000	298 000- 857000
Geometric mean SCC	370000	599000	615000
Group 2			
Lower – Higher SCC	260 000- 853900	855000	260 000- 853 000
Geometric mean SCC	370 000	855000	464000
Allover study pathogen SCC geometric mean	394 000	611 000	545 000

From data in Table (11), the higher SCC geometric mean was of *S. aureus* followed by *E. coli* then *S. agalactiae* and were represented by 611000, 545000 and 394000 respectively. SCC geometric mean of both *E. coli* and *S. agalactiae* in large farm group (1) were higher than it in the group (2). While *S. aureus* was higher in the group (2) than the group (1). In allover study, (260000 – 854000), (298000– 855000) and (260000- 857000) were recorded as minimum and maximum SCC values of samples of *S. agalactiae*, *S. aureus* and *E. coli* respectively (shown in Tables 13,14 and 15).

Table 12. Number of isolates per sample for each pathogen.

	One isolate	**Two isolates**	**Three isolates**
S. agalactiae	0	9	2
S. aureus	1	7	9
E. coli	5	20	10

As shown in Table (12), *S. agalactiae* isolates were recovered in milk samples with another two recovered pathogens in 2 samples while recovered in the rest milk samples were with another one pathogen isolate. While *S. aureus* isolates were recovered in milk samples with 2 other recovered pathogens in 9 samples while recovered in 7 samples with another one pathogen isolate and obtained alone in one sample. *E. coli* was recovered alone in 5 samples, with another pathogen in 20 samples and with two pathogens in 10 samples.

Table 13. *Streptococcus agalactiae* isolates with the other pathogen isolates recovered in each recovered milk sample

S.N.	**SCC**	***St. epidermidis***	***St. saprophticus***	***St. aureus***	***B. cereus***	***M. luteus***	***S. Dysagalactiae***	***S. agalactiae***	***S. uberis***	***E. coli***	***Proteus vulgaris***	***P. aeruginosa***	***no of isolates***
1.	496440	+						+					***2***
2.	649000		+					+		+			***3***
3.	365000		+					+					***2***
4.	411000	+						+					***2***
5.	291000	+						+			+		***3***
6.	360000		+					+					***2***
7.	380000		+					+					***2***
8.	300000						+	+					***2***
9.	854000	+						+					***2***
10.	280000						+	+					***2***
11.	260000						+	+					***2***

Data seen in Table (13) represent that *S. epidermidis* and *S. saprophyticus* were the most prevalent pathogens found in common sample with *S. agalactiae* (4 isolates in 4 samples for each), followed by *S. dysgalactiae* isolates (3 isolates in 3 samples). Then one isolate of *S. dysgalactiae, E. coli and Proteus vulgaris* each in one sample. Three patterns were recorded each of two pathogens, *S. agalactiae* + *S. epidermidis, S. agalactiae* + *S. saprophyticus and S. agalactiae* + *S. dysgalactiae* Each pattern was distinguished in three samples.

Table 14. *S. aureus* isolates with the other pathogen isolates recovered in each recovered milk sample

S.N.	SCC	*S. epidermidis*	*S. saprophyticus*	*S. aureus*	*B. cereus*	*M. luteus*	*S. Dysgalactiae*	*S. agalactiae*	*S. uberis*	*E. coli*	*Proteus vulgaris*	*P. aeruginosa*	*No of isolates*
1.	641000	+		+									2
2.	542000	+		+						+			3
3.	489000	+		+						+			3
4.	679000			+									1
5.	298000			+			+			+			3
6.	819000	+		+			+						3
7.	440000		+	+			+						3
8.	854500		+	+									2
9.	762000			+			+						2
10.	851000			+			+			+			3
11.	812000			+			+						2
12.	393000			+			+			+			3
13.	606000		+	+			+						3
14.	655000			+			+			+			3
15.	715000		+	+									2
16.	855000			+					+				2
17.	440000		+	+									2

Data seen in Table (14) highlight that *S. dysgalactiae* was the most prevalent pathogen found in common sample with *S. aureus* (9 isolates in 9 samples) followed by *E. coli* isolates (6 isolates in 6 samples) then *S. saprophyticus* with 5 isolates in 5 milk samples. The most recorded three pathogen-pattern was the *S. aureus + S. dysgalactiae + E coli and* found in 4 samples. The pattern of *S. aureus + S. epidermidis + E. coli,* was distinguished in 2 samples.

As presented in Table (15), *S. dysgalactiae* was the most prevalent pathogen found in common sample with *E. coli* (12 isolates in 12 samples) followed by *S. saprophyticus* isolates (10 isolates in 10 samples) then *S. aureus* with 6 isolates in 6 milk samples. In between examined samples with 3 recovered isolates, the most rated patterns were *E. coli + S. dysgalactiae + S. aureus* which is demonstrated in 4 samples as well as the *E. coli + S. epidermidis+ S. saprophyticus* that was identified in 2 samples, On the samples with two recovered isolates, the most pattern was *E. coli + S. dysgalactiae* pattern and was distinguished in 7 samples.

Examination of the enzymatic activities

As shown in table (16), examination of the enzymatic activities included in addition to analysis of samples with SCC ≥ 250000, examination of the enzymatic activities of selectively selected 15 milk samples with SCC ≤ 250000 cell/ mL to compare values with that of the normal milk samples. In normal samples, mean value of LDH, GOT and ALP were 154, 132 and 287 µL/ml respectively.

Mean value of LDH in the study in SCM milk samples was 378 µL /ml which was lower than results recorded by Kalantari *et at.* (2013), Batavani *et al.* (2007) and Babaei *et al.* (2007) which were 839, 1524 and 1098 µL/ml respectively while it was lower than results of Hiss *et al.* (2007) who recorded 104 µL/ml. In our study, the higher mean was recorded in samples with *S. aureus* which was 402 µL/ml followed by *S. agalactiae* (398 µL/ml) then *E. coli* (369 µL/ml) that result was lower the results of Hiss *et al.* (2007) which were 167-284 µL/ml respectively.

Mean value of GOT in the study in between SCM milk samples was 147 µL /ml that was higher than result of Babaei *et al.* (2007) which was 140.6. The higher mean was recorded in samples with *S. agalactiae* which was 150 µL/ml followed by *E. coli* (149 µL/ml) then *S. aureus* (147 µL/ml).

Mean value of ALP in the study in between SCM milk samples was 469 µL /ml which was higher than result of Babaei *et al.* (2007) which was 136 but lower than results of Kalantari *et at.* (2013) which was 918 µL/ml). The higher mean was recorded in samples with *E. coli* which was 614 µL/ml followed by *S. aureus* (560 µL/ml) then *S. agalactiae* (553 µL/ml).

Table 15. *E. coli* isolates with the other pathogen isolates recovered in each milk sample

S.N.	*SCC*	*S. epidermidis*	*S. saprophticus*	*S. aureus*	*B. cereus*	*M. luteus*	*S. dysagalactiae*	*S. agalactiae*	*S. uberis*	*E. coli*	*Proteus vulgaris*	*P. aeruginosa*	No of isolates
1.	542000	+		+						+			*3*
2.	489000	+		+						+			*3*
3.	617000		+							+			*2*

4.	649000		+					+		+			3
5.	436000		+				+			+			3
6.	652000		+						+	+			3
7.	298000			+			+			+			3
8.	851000			+			+			+			3
9.	393000			+			+			+			3
10.	567000					+				+			2
11.	400000						+			+			2
12.	857000	+								+			2
13.	852500									+			1
14.	655000			+			+			+			3
15.	855700								+	+			2
16.	852000	+					+			+			3
17.	280000		+							+			2
18.	420000								+	+			2
19.	430000								+	+			2
20.	260000		+							+			2
21.	270000						+			+			2
22.	290000						+			+			2
23.	853200						+			+			2
24.	485000						+			+			2
25.	350000	+								+			2
26.	700000		+							+			2
27.	370000						+			+			2
28.	852600									+			1
29.	615600		+							+			2
30.	856000								+	+			2
31.	570600		+							+			2
32.	696700									+			1
33.	662900									+			1
34.	852200									+			1
35.	651200		+							+			2

Table 16. Relation between geometric mean of SCC-LDH-GOT-ALP in allover study and each Pathogen

	Total geometric mean			
	SCC	**LDH**	**GOT**	**ALP**
Normal milk	99000	154	132	286
Total SCM study	483000	378	147	469
S. aureus	611000	402	147	560
S. agalactiae	394000	398	150	553
E. coli	545000	369	149	614

From data in Table (17), meanwhile the minimum and maximum values of LDH in the study SCM milk samples were 102 and 798 respectively, the higher LDH values were in *S. aureus* and *S. agalactiae* and were 731 and 751 µL/ml respectively. The lower figure was in *E. coli* (102 µL/ml).

Table 17. milk LDH values in overall study

	Milk Enzyme	**Higher value**	**Lower value**	**Geometric mean**
1	Normal milk	301	103	154
2-	Milk LDH	798	102	378
3	***S. aureus***	731	247	402
4	***S. agalactiae***	751	240	398
5	***E. coli***	564	102	369

According to results in Table (18), the higher GOT in the total allover study samples was 159 µL/ml while the lower was 114 µL/ml, the lower and the higher values in the examined pathogens were recorded in case of samples of ***E. coli*** (131, 159 µL/ml).

Table 18. GOT in overall study

	Milk Enzyme	**Higher value**	**Lower value**	**Geometric mean**
1	Normal milk	139	119	132
2	SCM Milk GOT	159	114	147
3	*S. aureus*	158	140	147
4	*S. agalactiae*	154	144	150
5	*E. coli*	159	131	149

As shown in Table (19), ALP represent wide average in between the minimum and maximum figures in the total SCM study samples (205 and 930 µL/ml) both the three pathogen express higher close figures which were 930,913 and 852 µL/ml in *E. COLI, S. aureus* and *S. agalactiae.* The same was noticed in the lower values.

Table 19. Alp in overall study

	Milk Enzyme	Higher value	Lower value	Geometric mean
1	Normal milk	470	199	286
1-	Milk Alp	930	205	469
2	*S. aureus*	913	234	560
3	*S. agalactiae*	852	239	553
4	*E. COLI*	930	207	614

Staphylococcus aureus isolates PCR amplification

All isolates of Staphylococcus aureus gave the characteristic growth on both Blood agar medium and Mannitol salt Medium. Biochemical characterization of the isolates with Catalase tests, coagulase tests using tube method, hemolysis pattern, pigmentation, mannitol and maltose fermentation test, salt tolerance using mannitol salt agar, novobiocin sensitivity tests were conducted and interpreted. All the 17 isolates are identified as Staphylococcus aureus. Only, seven of the isolates were confirmed by the PCR amplification which represented 40% of the isolates while the rest 10 were negative for *S. aureus* (Sa442) gene amplification as seen in Figure (3).

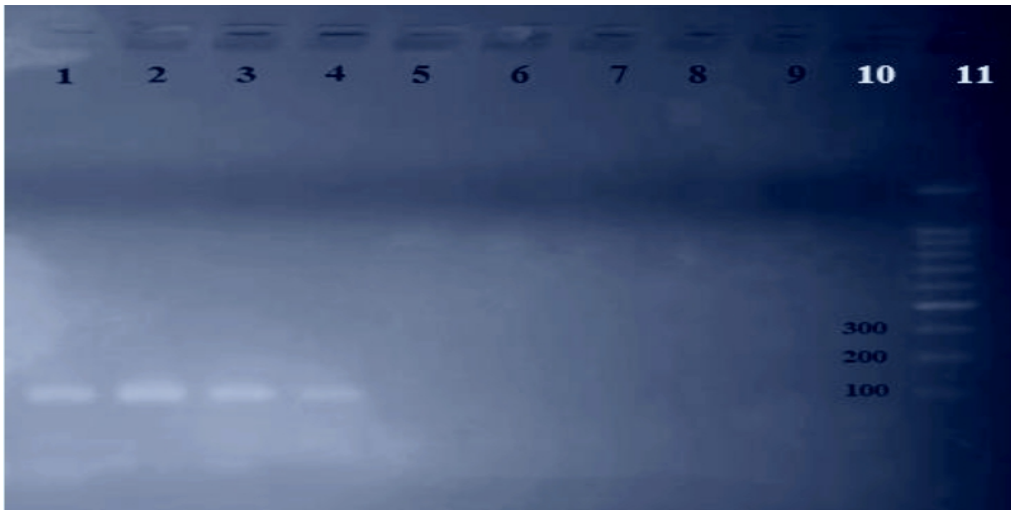

Figure 3. Lane 1,2, 3, 4 are positive for *S. aureus* (*Sa442*) gene: 108 bp
Lane 5, 6,7,8, 9, 10 are negative for *S. aureus* (*Sa442)* gene
Lane 11: molecular ladder

Streptococcus agalactiae isolates PCR amplification

All isolates of Streptococcus agalactiae gave the characteristic growth on Blood agar medium and Edwards' Medium. Biochemical characterization and identification of the isolates was conducted by their hemolysis pattern on blood agar, CAMP test, Esculin hydrolysis, Inulin test and fermentation on sugar fermentation. 8 isolates of were confirmed by the PCR amplification as seen in Figure (4) representing 72.7% while three isolates were negative for Streptococcus agalactiae gene amplification.

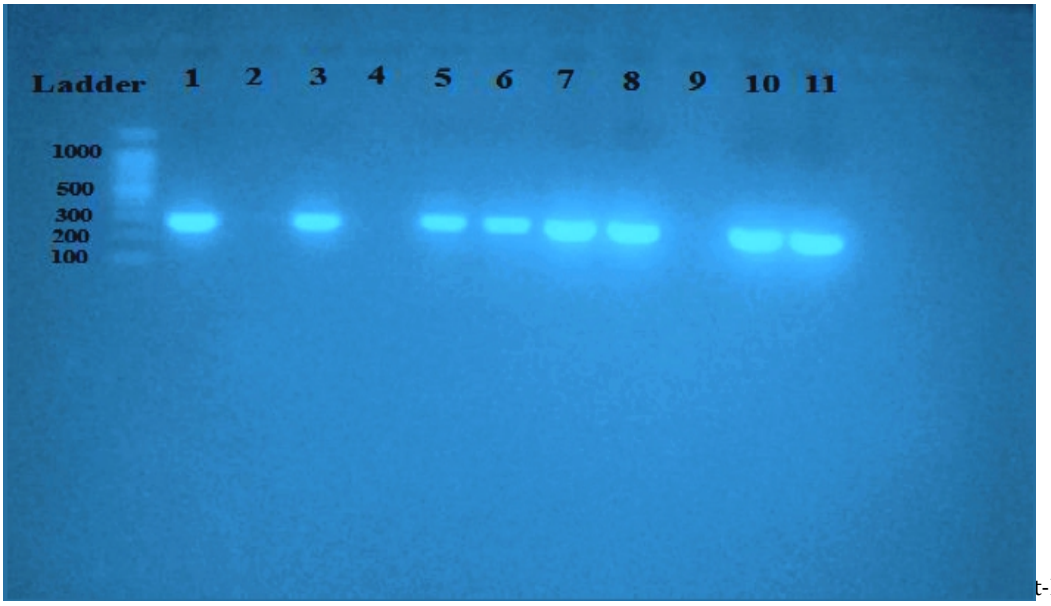

Figure 4. Molecular ladder
Lane 1, 3, 5, 6, 7, 8, 10, 11 are positive for ***Streptococcus agalactiae*** gene: 280 bp
Lane 2, 4, 9 are negative for ***S. agalactiae*** gene

***E. coli* isolates PCR amplification**

All isolates of *E. coli* gave the characteristic growth on both Eosine methylene blue agar (EMB), MacConkey's. Biochemical characterization of the isolates with IMViC pattern, growth on TSI (Y/Y/-) and fermented different sugars. All of the 35 isolates were confirmed by the PCR amplification as seen in Figure (5).

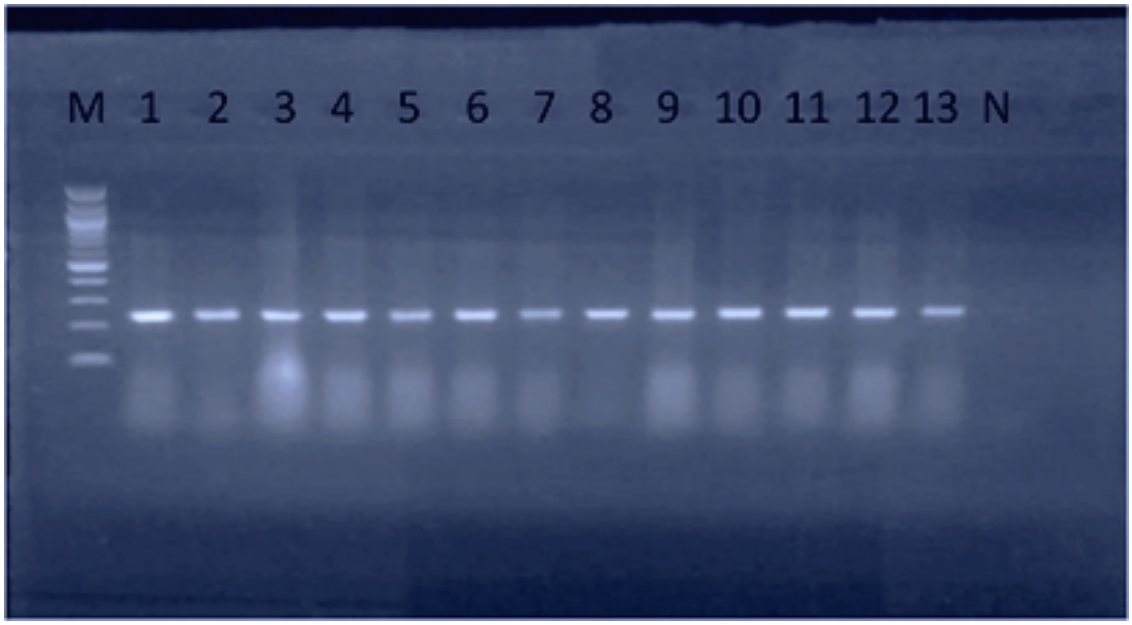

Figure 5. Agarose gel electrophoresis of PCR production from *Escherichia coli*
M: 100 bp ladder DNA marker
Lane 1: positive control
Lanes 2-13: positive *E. coli* isolates
N: negative control

CONCLUSION

In conclusion, the study referred to the relative low prevalence of SCM due to *S. aureus* and *S. agalactiae* constituted isolates 3.6% and 2.31 % respectively in relative to *E. coli* (7.4%) of apparently normal animals. Meanwhile, it highlighted the increased overall figures of SCC in farms involved in the experiment and consequently, increase of the prevalence of SCM. This may be due the double use of the SCC significance as milk quality indicator and udder health indicator. Most dairy producers are interested in the first purpose which is related to the current financial losses due to penalties and milk withdrawn due to the elevated SCC when dealing with dairy product plants. While they are ignoring SCC as a significance for the udder health condition. I recommended a regime of periodical and authorized somatic cell counting for the earlier evaluation and treatment of the SCM cases.

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