Animal Health Research Journal Vol. 7, No. 2, June 2019 pp. 163-180

ISSN: 2356-7767

Clinicopathological studies on bacterial pathogens of ewes' mastitis with molecular detection of some virulence genes of *Pseudomonas aeruginosa*

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Received in 6/5/2019
Accepted in 11/6/2019

Abstract

The objective of the study was to investigate the outbreak of ewes' mastitis due to *Pseudomonas aeruginosa* infection with PCR detection of some of virulence and antibiotic resistance genes as well as studying the effect of bacterial infection on blood constituent, serum biochemical analysis oxidative stress and antioxidant enzymes activity in clinical mastitis (CM) and subclinical mastitis (SCM) ewes. Eighty five of milk mastitis cases (45 CM and 40 apparently healthy), 50 blood samples and 20 environmental samples (drinking water, milk containers and animal bedding) were collected from five sheep farms at Ismailia and Sharkia Governorates. All apparently healthy animals were individually tested for SCM by the California (rapid) mastitis test (CMT). The milk samples of grade 2, 3 or 4 reactions were considered CMT positive. All collected milk and environmental samples were examined bacteriologically for detection of *Pseudomonas aeruginosa* and other bacterial causes for ewes' mastitis. Blood samples were subjected to hematological and biochemical analysis as well as evaluating the level of oxidative stress/antioxidant enzyme activity. The bacteriological investigation showed that *P. aeruginosa* was mainly isolated (27.4%) either alone (8.4%) or in mixed cultures (18.9%). Gram positive bacteria like: *S. aureus* (21.1%), CNS (10.5%) and *Streptococcus agalactia* (1.1%) and Gram negative organisms like: *E. coli* (22.1%), *Proteus vulgaris* (3.2%) and *Klebsiella pneumoniae* (3.2%) were also isolated from all mastitis milk and animal environmental samples. The antibiotic resistance profile of *P. aeruginosa* isolates showed wide range of resistance (85%-100%) against most tested antibiotics however, gentamycin and ciprofloxacin recorded the highest sensitivity rates (65.4% and 53.8%), respectively. Some virulence genes of *P. aeruginosa* isolates (*las I, tox A,* and *opr L*) were detected with PCR in (71.43%, each) of the isolates, mean- while the *tet A* and *sul1* antibiotic resistance genes were found in all *P. aeruginosa* isolates (100%). The hematological examination revealed a significant decrease in RBCs count, hemoglobin concentration, and packed cell volume (PCV) as well as leucocytosis, neutrophilia, lymphopenia and eosinophilia were observed. The biochemical analysis showed a significant increase in AST, ALT, ALP, urea, creatinine and calcium. There were significant increased levels of total globulin, alpha and beta globulins in clinical mastitic ewes. However, the levels of total globulin, alpha and gamma globulins were increased in the subclinical cases. Total protein, albumin, and Catalase test (CAT) were significantly decreased but antioxidant enzymes (GSH and SOD) and oxidative stress (MDA and NO) were significantly increased in all examined ewes. In conclusion, *P. aeruginosa* was incriminated as the main cause for this outbreak of ewe mastitis in this study consequently; it was adversely affected on blood constituent, serum biochemical analysis and level of oxidative/ antioxidant enzymatic activity.

***Keywords**: Mastitis, P. aeruginosa, blood analysis and oxidative stress/ antioxidant enzymes activity.*

Introduction

Mastitis is a major health problem in the dairy sheep flocks worldwide. It is defined as the inflammation of the mammary gland or udder regardless of its origin, severity or evolution **(Bergonier and Berthelot, 2003)**. The major sheep dairy losses were attributed to reduced milk production, bad quality milk, low weaning weight, drugs cost and veterinary services in addition to the increased culling rate and reduced reproductive efficiency. California mastitis test (CMT) is used for diagnosis of both forms of mastitis (clinical and subclinical forms) as a special field screening test **(Al-Khazay and Kshash, 2014)**. Ewes with inflamed mammary glands with no clinical signs, giving apparently normal milk, but bacteriologically positive and with a somatic cell count (SCC) of $\geq 500 \times 10^3$ cells/ml are considered to have subclinical mastitis **(Kiossis *et al*., 2007)**.

Pseudomonas aeruginosa is occasionally involved in enzootic or epizootic outbreaks of mastitis in small ruminants **(Sela *et al*., 2007)**. It is an environmental pathogen that can cause severe clinical mastitis with systemic signs as well as subclinical chronic mastitis **(Kelly and Wilson, 2016).**

Virulence of *P. aeruginosa* is multifactorial process. They have many cell associated factors such as lipopolysaccharide (LPS), flagellum, and pilus and non-pilus adhesins, in addition to other exo-enzymes and secretory virulence factors as: protease, elastase, phospholipase, pyocyanin, exotoxin A, exoenzyme S, hemolysins, rhamnolipids and siderophores **(Hentzer *et al*., 2003 and Green, *et al.,* 2008)**. Exotoxin A encoded by the *toxA* gene has the ability to inhibit protein biosynthesis just like diphtheria toxin **(Hamood, *et al.,* 2004)**. PCR detection of some virulence genes (*oprL, lasI, toxA, exoU, Exos, rhlR, rhlI* and *ecfx*) is recommended for the molecular identification of *P. aeruginosa* organisms which could reflect different levels of intrinsic virulence and pathogenicity of these organisms **(Nikbin *et al*., 2012)**.

Mastitis disease could lower the average values of RBCs, Hb and PCV **(Zaki *et al*., 2010)**. Also, leucocytosis, neutrophilia and lymphopenia were reported by **Zaki *et al*., (2008) and Sarvesha *et al*., (2017).** Biochemical analysis in mastitis cases revealed significantly ($P< 0.05$) higher average values of Ca, P, Na, Cl, and K compared with healthy animals however, no significant ($P> 0.05$) changes were observed in Mg and ALT levels. AST and TP values were significantly increased in subclinical mastitis compared to healthy animals how- ever, no significant change were observed in clinical mastitis infected animals **(Sarvesha *et al*., 2017)**. Additionally, Mg, P, Fe, Zn and Cu parameters and GOT and ALP enzymes were significantly increased in both cattle and buffaloes **(Awale *et al.*, 2012).** However, homeostasis disturbance occurs, oxidative processes result in oxidative stress that causes mastitis in high yielding dairy cows **(Ibrahim *et al*., 2016)**. During mastitis, an increase in lipid peroxidation level was recorded leading to a decrease in levels of some antioxidant molecules making consequently an oxidative stress **(Weiss *et al*., 2004).**

Therefore, this study aimed to investigate the prevalence, virulence and antibiotic resistance gene of *P. aeruginosa* in an outbreak of ewe mastitis via bacteriological isolation, biochemical and PCR identification methods and also, the detection of bacterial effects on blood constituent, serum biochemical analysis and to evaluate the role of oxidative stress and antioxidant enzymes activity in mastitic ewes.

Materials and Methods

I - Samples

a) Animal samples: The current study was carried out in five sheep private farms at Ismailia and Sharkia Governorate, Egypt. The age ranged from 3-5 years. Eighty five of milk samples (45 from clinical mastitis and 40 from apparently healthy). The clinical signs in acute mastic ewes were: history of stopping of eating food, dullness, agonizing swelling in the left mammary gland for two days, rejected its lamb for suckling and lambed sixty days back. By examination of drain from the left quarter watched yellow shading with flakes and from

right quarter no obvious any abnormalities in clinical mastitic ewes. On the other hand, the clinical examination showed pyrexia, watery nasal discharge, cough, cessation the motility of rumen, hard painful swelling of left quarter of udder and injury noticed on the left teat.

b) Environmental samples: Twenty samples from each sheep farm (including: drinking water, milk containers and animal bedding) were collected in this study under sterile conditions in separate sterile plastic bags and/or containers then they were transported as soon as possible to the bacteriological laboratory for bacterial examination

c) Blood samples: Fifty blood samples were collected by jugular vein puncture from diseased (clinical mastitis and subclinical mastitis) and healthy ewes. The first blood sample was collected into a clean centrifuge glass tube (Digisystem Laboratory Instrument, His Chih city, Taiwan) containing 5mg of sodium ethylene diaminetetraacetic acid (EMD Chemicals Inc.) as an anticoaguant for determining of blood parameters and to separate plasma for evaluation of the activity of some oxidative stress and antioxidant enzymes. Meanwhile, the second blood sample was collected without anticoagulant into a clean centrifuge glass tube to separate serum for evaluation of some biochemical tests. The separated serum and plasma were kept frozen at −80°C.

II- Detection of California Mastitis Test (CMT): It is used for field diagnosis of SCM cases as a special field screening test. The degree of viscosity after homogenization of each sample was illustrated in (Table1) according to **(Al-Khazay and Kshash, 2014)**.

Estimation of Somatic Cells Count (SCC): It was performed automatically using SOMACOUNT, 150 from Bentley (USA). Milk samples were classified into 2 categories, normal (values below 500,000 cell /ml^{-1}) and subclinical mastitis (values above the limit of 500,000 cells /ml^{-1}) according to the **National Mastitis Council, (1999) (Table, 1).**

Table (1). The viscosity degree and somatic cell count values of milk samples.

Score	Milk viscosity	Somatic cell count X 10^3
0 (-)	The consistency of the mixture is homogeneous, liquid, without visible change	<200
1 (-)	Forms slight flakes, which by turning the plate disappear	200-500
2 (+)	Clot is formed, the mixture viscosity is increased	500-1500
3 (++)	Viscous mixture, by turning the plate clot is visible, localized on one plate.	1500-5000
4 (+++)	Forms ropey, viscous mixture, significantly visible clot, pouring mixture falls of the plate	>5000
0: (-) Negative reaction, 1(-): trace, 2: (+) slightly positive, 3: (++) moderately positive, 4 (+++) strongly positive.		

IV. Preparation of milk samples for bacteriological examination: Udder halves were cleaned and disinfected prior to sampling with 70% alcohol and dried with sterile cotton. Milk samples were collected from each ewe before morning milking in a sterile McCartney tube for bacteriological examinations. The samples were stored in ice box and transported as soon as possible to the laboratory for further examination.

Bacterial isolation and identification: Ten ml of each milk sample in a sterile tube was incubated at 37ºC for 24h then the sample was centrifuged for 20 minutes at 3000 rpm. The cream and supernatant fluid were discarded to get the sediment. Milk from CM (clinical mastitis) and positive SCM (subclinical mastitis) cases, drinking water, milk containers and animal bedding samples were streaked onto (nutrient agar enriched with 7% sheep blood, macConkey's agar and Pseudomonas agar base with 0.1% cetramide plates) and incubated at 37°C for 24-48 h. The suspected colonies were identified according to colonial morphology, hemolytic activity and biochemical characters **(Quinn *et al.*, 2002)**. Films from the pure suspected colonies were stained by Gram's stain and examined microscopically. The pure isolates of *P. aeruginosa* were transferred to 1% nutrient agar slant and stored at 4°C. In addition, the Integral system stafilococchi kit (Liofilchem) was used for specific confirmation and biochemical identification of coagulase negative *Staphylococci* (CNS) isolates.

Antibiotic susceptibility testing of *Pseudomonas aeruginosa* isolates: The identified *P. aeruginosa* isolates were tested against a panel of eleven commercial antimicrobial agents: gentamycine (120 µg), ciprofloxacin (5 µg), norfloxacin (10µg), enrofloxacin (5 µg), ceftriaxone(5 µg), amoxicillin+clavulanic acid (10 µg), erythromycin (15 µg), sulphamethoxazole (25µg), streptomycin (10 µg), trimethoprim (30 µg) and tetracycline (10 µg) with the standard Kirby–Bauer disc diffusion method **(Bauer *et al*., 1996)**. The results were interpreted according to the criteria recommended by **(CLSI, 2011).** The susceptibility of identified isolates resistant to three or more antibiotics was classified as multidrug drug resistance (MDR) strains.

Molecular identification of some virulent genes of *P. aeruginosa* isolates:

PCR amplification: As shown in **table (2),** the primers for the selected virulence genes of *P. aeruginosa* isolates (elastase, or "*LasI*", exotoxin A gene or "*Tox A*", and outer membrane protein gene or "*opr L*") and tetracycline and sulphamethoxazole antibiotics resistant genes (*Tet A* and *Sul 1*) were utilized in a 25µl reaction containing 12.5µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µL of each primer of 20 pmol concentrations, 4.5µl of water, and 6µl of DNA template. The reactions were performed in an applied biosystem 2720 thermal cycler. The cycle for each gene was tabulated in **table (3).**

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20µl of the products was loaded in each gel slot. Gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) and gene ruler 100bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (2). Oligonucleotide primer sequences of virulence and antibiotic resistance genes of *P. aeruginosa*

Target gene	Primer sequence (5'-3')	References
LasI	F: ATGATCGTACAAATTGGTCGGC	Bratu *et al*., (2006)
	R: GTCATGAAACCGCCAGTCG	
OprL	F: ATG GAA ATG CTG AAA TTC GGC	Xu *et al.*, (2004)
	R: CTT CTT CAG CTC GAC GCG ACG	
ToxA	F:GACAACGCCCTCAGCATCACCAGC	Matar *et al.*, (2002)
	R: CGCTGGCCCATTCGCTCCAGCGCT	
Tet A	F: GGTTCACTCGAACGACGTCA	Randall *et al*., (2004)
	R: CTGTCCGACAAGTTGCATGA	
Sul1	F: CGGCGTGGGCTACCTGAACG	Ibekwe *et al*., (2011)
	R: GCCGATCGCGTGAAGTTCCG	

Table (3). Cycling conditions and predicted sizes of PCR products for virulence and antibiotic resistance genes

Target gene	Initial denaturation °C/ min	Actual cycles (35 cycles) °C/sec			Final extention °C/ min	Amplified product Size (bp)
		Denaturation	Annealing	Extension		
ToxA	94˚C/ 5	94˚C/ 30	55˚C/ 40	72˚C/ 45	72˚C/10	396
LasI	94˚C/ 5	94˚C/ 30	56˚C/ 40	72˚C/ 45	72˚C/ 10	606
oprL	94˚C/ 5	94˚C/ 30	55˚C/ 40	72˚C/ 45	72˚C/ 10	504
Sul1	94˚C/ 5	94˚C/ 30	60˚C/ 40	72˚C/ 45	72˚C/10	433
Tet A	94˚C/ 5	94˚C/ 30	55˚C/ 40	72˚C/ 45	72˚C/10	576

Clinicopathological examination: All blood analysis was performed on –ve CMT (n=10) as healthy ewes and mastitic ewes (clinical and subclinical cases).

Hematological Procedures: Determination of total erythrocytic count, hemoglobin concentration, packed cell volume, and total leucocytic count. Also, differential leukocyte count was carried out in blood films prepared and stained with Giemsa and counting up to 100 cells then take the per- cent of eosinophils, lymphocytes, basophils, heterophils and monocytes were determined for healthy ewes, subclinical and clinical mastitic ewes **(Jain, 2000).**

Biochemical serum analysis: The collected sera were assayed for serum biochemistry. The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) **(Reitman and Frankel, 1957),** Inorganic phosphorus **(El-Merzabani *et al*., 1977)**, alkaline phosphatase (ALP) **(Tietz *et al*., 1983)**, creatinine **(Henry, 1979)** and urea (**Patton and Crouch, 1977)** were spectrophotometrically assayed using semi-automated spectrophotometer **(Erba-Chem7, Germany)** and commercial kits **(Spectrum, Cairo, Egypt)**.

Protein electrophoresis: It was done using SDS-Polyacrylamide gel electrophoresis according to **Laemmli, (1970)** in animal health research institute Biochemistry department**.**

Oxidative stress and antioxidant enzymes activity: Plasma samples were measured spectrophotometerically by using (Robonik biochemistry automatic analyzer, Robonik India PVT LTD, India) following standard methods using commercially available test kits (Biodiagnostic, Cairo, Egypt), for the following parameters: MDA **(Ohkawa *et al*., 1979)**, GSH-Px **(Paglia and Valentine, 1967),** SOD activity **(Goldstein and Czapski, 1996)** and CAT according to **(Aebi, 1983)**. NO level was measured using ELISA reader according to **(Rajaraman *et al*., 1998)**.

Statistical Analysis: The obtained data were statistically analyzed by an ANOVA (one way) variance method considering $P < 0.05$ using MiniTab17© software. The significant differences were taken to Fisher multiple range tests to compare the means **(Ryan and Joiner, 2005).**

Results

The prevalence ratio of subclinical mastitis (SCM) in ewes' farms: In the present study, subclinical mastitis (SCM) cases were detected in 30/40 (75%) with CMT test (grades 2(+), 3 (++) and 4 (+++) reactions).

Evaluation of somatic cell count in milk (SCC): The level of SCC in ewes which were suffered from subclinical mastitis (SCM) is highly significant than those of the control healthy animals. The mean levels are found 267.2×10^3 and 700×10^3 in healthy and subclinical mastitic animals, respectively as shown in **table (4)**.

Table (4). The values of somatic cell count/ ml. of the examined milk samples

Animal group	No.	Somatic cell count		
		Min.	Max.	Mean
Healthy animals somatic cell count ≤500.000	10/30	200×10^3	450×10^3	$267.2\times10^3\pm12.3\times10^3$
Infected animals somatic cell count ≥500.000	20/30	500×10^3	900×10^3	$700\times10^3\pm20.5\times10^3$

Bacteriological isolation and identification results: The bacteriological examination of total samples (45 from CM, 30 from SCM and 20 environmental samples) showed that *P. aeruginosa* was recorded as in total percent- age of 27.4% which was distributed as a pure isolates (8.4%) of the samples and mixed infections with others like: *S. aureus, E. coli, Coagulase negative staphylococcus* (CNS) and *Proteus vulgaris* with percentage of (18.9%) as shown in **(Table 5).**

Table (5). The prevalence of *P. aeruginosa* isolates as pure or mixed among examined milk and environmental samples

Isolates		Clinical mastitis (45)	Subclinical mastitis (30)	Environmental samples (20)	Total (95)
		No. (%)	No. (%)	No. (%)	No. (%)
Pure *P. aeruginosa*		4 (8.9%)	3 (10%)	1 (5%)	8 (8.4%)
Mixed *P. aeruginosa* With	*S. aureus* + *E. coli*	5 (11.1%)	2 (6.7%)	1 (5%)	8 (8.4%)
	E. coli+ CNS	3 (6,7%)	1(3.33%)	1 (5%)	5 (5.3%)
	CNS	2 (4.4%)	2 (6.7%)	--	4 (4.2%)
	Streptococcus agalactia	1 (2.2%)	--	--	1 (1.1%)
	Total mixed isolates	11 (24.4%)	5 (16.67%)	2 (10%)	18 (18.9%)
Total *P. aeruginosa* isolates		15 (33.4%)	8 (26.67%)	3 (15%)	26 (27.4%)

CNS= Coagulase Negative Staphyloccoci

Biochemical identification tests revealed that other Gram +ve bacteria like *S. aureus,* CNS (Coagulase Negative Staphelococci) and *Streptococcus agalactia* were isolated in percentages of: 21.1%, 10.5% and 1.1%, respectively from all samples as shown in **(Table 6)**. However, Gram -ve bacteria like *E. coli*, *Klebsiella pneumoniae* and *Proteus vulgaris* were also recovered in 22.1%, 3.2% and 3.2%, respectively from all samples. Biochemical identification of coagulase negative *staphylococci* (CNS) with integral system stafilococchi kit (ISSK) revealed the detection of 6 different species of coagulase negative staphylococci: *S. hominis, S. sim- ulans, S. hyicus, S. saprophyticus, S. cohni* and *S. lentus* from all samples

Table (6). Prevalence of other Gram positive and Gram negative bacteria in all samples.

Mastitis cases		Clinical mastitis (45)	Subclinical mastitis (30)	Environmental samples (20)	Total (95)
		No. (%)	No. (%)	No. (%)	No. (%)
Gram +Ve bacteria	*S. aureus*	10 (22.2%)	5 (16.7%)	5 (25%)	20 (21.1%)
	CNS	6 (13.3%)	3 (10%)	1 (5%)	10 (10.5%)
	Streptococcus agalactia	1 (2.2%)	--	-	1 (1.1%)
Gram -Ve bacteria	*E. coli*	8 (17.8 %)	4 (13.3%)	9 (45%)	21 (22.1%)
	Klebsiellapneumonia	2 (4.4 %)	1 (3.3%)	-	3 (3.2%)
	Proteus vulgaris	1 (2.2 %)	1 (3.3%)	1 (5%)	3 (3.2%)
Total bacterial isolates		28 (62.2%)	14 (46.7%)	16 (80%)	58 (61.1%)

On the other hand, bacteriological examination of twenty environmental samples from five examined sheep farms (drinking water, milk containers and animal bedding) showed that *P. aeruginos*a was isolated either pure or mixed cultures with other organisms in 3 out of twenty of the environmental samples (15%) however, *E. coli*, *S. aureus*, CNS and *Proteus vulgaris* were isolated separately in: 9/20, 5/20, 1/20 and 1/20, respectively of all environmental samples in this study as shown in table (7).

Table (7). Different isolated bacteria from environmental samples (drinking water, milk containers and animal bedding)

Mastitis cases	Animal drinking water	Milk containers	Animal litter	Total No. (20)
E. coli	6	3	-	9 (45%)
S. aureus	2	2	1	5 (25%)
*P. aeruginos*a	1	1	1	3 (15%)
CNS	-	-	1	1 (5%)
Proteus vulgaris	1	-	-	1 (5%)

Antibiotic sensitivity patterns of *P. aeruginosa* isolates: The antibiotic susceptibility testing of twenty six recovered *P. aeruginosa* isolates exhibited multidrug resistance patterern (MDR) against most used antibiotics. The isolates showed 100% resistance to: tetracyclin, sulphamethoxazole, amoxicillin + clavulanic acid, erythromycin, streptomycin and trimethoprim. However, only gentamycine and ciprofloxacin were the most effective drugs with percentages of 65.4% and 53.8%, respectively **(Table, 8).**

Table (8). Antibiotic sensitivity testing of the recovered *P. aeruginosa* isolates of most sensitive antibiotic.

Antibiotic (potency/µg)	No. of *P. aeruginosa* isolates showing susceptibility pattern (26)	
	Sensitive	Resistant
Gentamicin (120 µg)	17 (65.4%)	9 (34.6%)
Ciprofloxacin (5 µg)	14 (53.8%)	12(46.2%)
Norfloxacin (10 µg)	4 (15.4%)	22 (84.6%)
Enrofloxacin (5 µg)	3 (11.5%)	23 (88.5%)
Ceftriaxone	2 (7.7%)	24 (92.3 %)

Detection of some virulence and resistant genes of *P. aeruginosa* isolates: PCR identification on seven *P. Aeruginosa* isolates (from pooled CM, SCM and environmental samples) showed the presence of (*Las I, Tox A,* and *oprL*) virulence genes in five out of seven (71.43% of each) of the examined isolates (**fig.1; A, B & C**). *oprL* virulence gene of *P. aeruginosa* acts as reliable factor for rapid identification of the species of *P. aeruginosa.* Meanwhile, the tetracycline and sulphamethoxazole resistant genes (*TetA* and *SUL1*) were found in all tested isolates (100% of each), where clear bands were shown at 576 bp and 433 bp, respectively, as shown in (**fig. 1; D&E**).

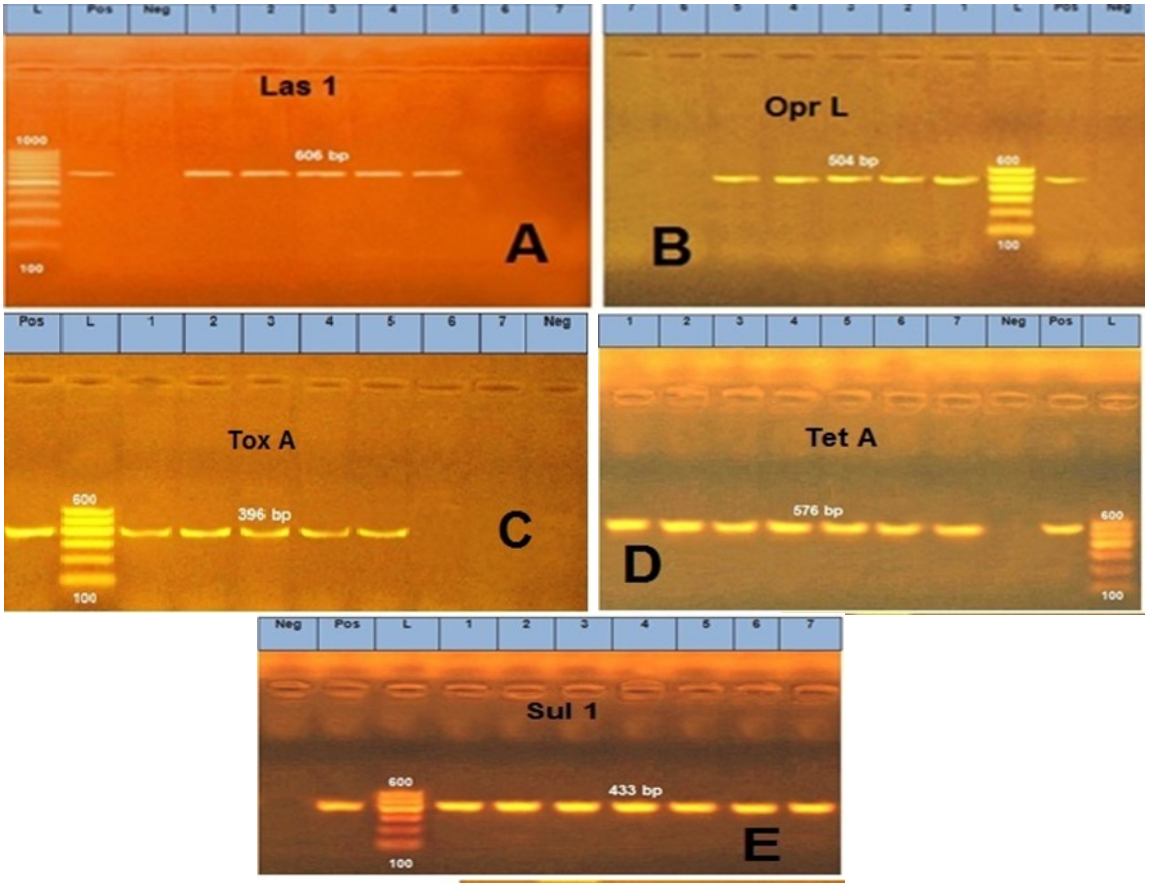

Fig. (1): Agarose gel electrophoresis of PCR ampli- fied products of virulent genes**:** *Las I* **(A)***, Opr L* **(B)** and *ToxA* and resistant genes: *Tet*A **(D)** and *Sul 1* **(E). Lane L**: DNA molecular size marker (100 bp), **lanes 1-7**: *P. aeruginosa* strains**, lane (Pos):** positive control and **lane (Neg):** negative control. The size in base pairs (bp) of each PCR product is indicated on the bands.

Hematological findings in table (9) revealed a significant decrease in RBCs count, Hb conc., and PCV value in mastitic ewes as compared with apparently healthy ewes.

Also, there were leucocytosis, neutrophilia, eosinophilia, monocytosis and lymphopenia in mastitic ewes milk a as compared with healthy ewes.

Table (9). Hematological studies on in healthy and mastitic ewes' milk

Parameters \ Groups	Healthy ewe's milk (N=10)	Subclinical Mastit- ic ewes (N=20)	Clinical Mastitic ewes (N=20)
RBCs (10^6 /µl)	5.2 ± 0.11[a]	3.1 ± 0.05[b]	2.8± 0.07[b]
HB (gm %)	10.5 ±0.17[a]	8.3 ± 0.07[b]	8.0± 0.12[b]
PCV (%)	33.4 ± 0.3 [a]	20.1 ± 0.36[b]	21.5± 0.23[b]
WBCs (10^3/ µl)	9.06±0.11 [b]	12.25 ± 0.1 [a]	11.53±0.3 [a]
Neutrophil (10^3/ µl)	5.00±0.22 [b]	7.6 ±0.5[a]	7.0± 0.3[a]
Lymphocyte (10^3/ µl)	4.50 ± 0.2 [a]	3.1 ± 0.13 [b]	3.00± 0.2 [b]
Monocyte (10^3/ µl)	0.46 ±0.14 [b]	1.1 ± 0.03 [a]	1.0±0.06[a]
Eosinophil (10^3/ µl)	0.10 ± 0.11 [b]	0.45± 0.04 [a]	0.53±0.15 [a]

Means within the same row with different superscripts are significantly different (P<0.05).

The biochemical analysis of blood showed a significant increase in AST and ALT, ALP, urea, creatinine and calcium. However, Phosphorus was significantly decreased in clinical and subclinical mastitic ewes (**table 10**).

Table (10). Serum biochemical analysis results in healthy and mastitic ewes' milk

Parameters \ Groups	Healthy N=10	Subclinical Mastitic ewes (N=20)	clinical Mastitic ewes (N=20)
ALT (u/l)	11.5 ± 0.03 [b]	16.7 ± 0.11[a]	18.5 ± 0.05 [a]
AST (u/l)	59.50±2.4 [b]	72.5 ± 3.1 [a]	80.8± 2.6 [a]
ALP (u/l)	305.8±5.3 [b]	510.7± 22.3 [a]	530.3±22.61 [a]
Urea (mg/dl)	20.25±2.71 [b]	24.26± 1.58 [a]	23.4 ±2.3 [a]
Creatinine (mg/dl)	1.1 ± 0.02 [b]	1.7 ± 0.01 [a]	1.5 ± 0.02 [a]
Ca (mg/dl)	2.1 ± 0.33 [b]	3.2 ±0.5 [a]	3.4± 0.07 [a]
P (mg/dl)	7.66±0.2 [a]	5.34 ±0.3[b]	5.00 ±0.27 [b]

Means within the same row with different superscripts are significantly different (P<0.05).

Table (11) showed a significant decrease in total protein (TP), albumin level and a significant increase in total globulin in clinical and subclinical mastitis. The level of alpha and beta globulin increased in clinical mastitis. On the other hand, alpha and gamma globulins were increased in the subclinical mastitic cases as compared to apparently healthy ones.

Table (11). Proteinogram parameters in healthy and mastitic ewes' milk.

Parameters \ Groups	Healthy ewe's milk N=10	subclinical Mastitic ewes (N=20)	clinical Mastitic ewes (N=20)
T. protein (gm/dl)	7.47±0.07[a]	6.90 ± 0.05 [b]	7.10± 0.06 [b]
Albumin (gm/dl)	3.50±0.20 [a]	1.30 ± 0.01 [b]	2.00± 0.25 [b]
Globulin (gm/dl)	3.97±0.55 [b]	5.60 ±1.8 [a]	5.10± 0.9 [a]
α– globulin (gm /dl)	1.32 ± 0.58 [b]	1.89± 0.3 [a]	1.81± 0.45 [a]
β– globulin(gm /dl)	0.85 ± 1.04 [b]	1.81± 0.5 [a]	0.80± 1.00 [b]
l– Gamma globulin (gm /dl)	1.80 ± 0.95 [b]	1.90 ± 0.6[b]	2.50± 0.85 [a]

Means within the same row with different superscripts are significantly different (P<0.05).

In **table (12),** the ewes with clinical mastitis and subclinical mastitis revealed a significant decrease in the CAT. However, there was a significant increase in the activity of GSH, MDA, NO, and SOD

Table (12). Oxidative stress / antioxidant enzymes activity in healthy and mastitic ewes' milk.

Parameters \ Groups	Healthy ewe's milk N=10	Subclinical Mastitic ewes (n=20)	Clinical Mastitic ewes (n=20)
MDA (nmol/ml)	5.66±1.5[b]	20.23±1.8[a]	23.37 + 2.4 [a]
NO (µmol/l)	40.40± 2.4 [b]	70.30±3.02 [a]	68.94 + 3.1 [a]
GSH (mg/dl)	0.65± 0.04[c]	0.95±0.3 [a]	0.82 + 0.05 [b]
CAT (u/l)	188.0±0.19 [a]	100.0±0.18[b]	125.3 + 0.20 [b]
SOD (u/ml)	115.3± 12.2 [b]	136.5±27 [a]	147.0± 25.2 [a]

Means within the same row with different superscripts are significantly different (P<0.05).

Discussion

Mastitis is considered as one of the most important diseases in dairy ewe industry **(Tormod *et al*., 2007)**. The variation in the incidence ratio of subclinical mastitis was attributed to the change of the management condition **(Radostitis *et al*., 2000)**. In the current study, the subclinical mastitis cases were recorded in 30/40 of apparently healthy ewes with a percentage of (75%) in different ewes' farms at Ismailia and Sharkia Governorate using CMT. Similar studies recorded the percentage of subclinical mastitis cases in ewes' farms according to CMT as 69.3% and 64.9%, respectively **(Al-Khazay and Kshash, 2014)**. **Radostits *et al*., (2000) and Khanal and Pandit, (2013)** attributed the high prevalence of subclinical mastitis (SCM) cases to late lactation period; they found that SCM was higher in high milk yielding animals as compared to low milk yielding animals.

SCC of milking ewes could be used to define subclinical mastitis and a threshold of 200,000 to 400,000 cell /ml could accurately identify most infected ewes **(Ruegg, 2011**). In ewe milk samples with SCC > 1,000,000 cells/ml, clear differences were found in leukocyte production and their counts between infected glands and healthy glands. More recent data reported subclinical mastitis cases in small ruminants after diagnosis with the CMT and SCC (**Marogn *et al*., 2012)**. The milk somatic cell count of animals suffered from subclinical mastitis is in highly significant levels than those of the healthy animals as the mean levels were $267.2\times10^3$and 700×10^3 in healthy and subclinical mastitic animals, respectively. **Jones, (2006**) reported that the higher level of the SCC was associated with the greater risk of raw milk contamination with pathogens.

P. aeruginosa is usually present in soil, water

and feces of animals. It acts as opportunistic pathogen and predisposing factors for mastitis especially in case of wound or lacerations in the teat depending on the immunological status of animal **(Carter *et al.*, 1994).** Regarding the bacteriological examination of ewe milk samples in this study, it was clear that some pathogenic Gram positive and Gram negative bacteria contributing for ewe mastitis. *S. aureus, streptococcus spp.,* coagulase negative staphylococci (CNS)*, P. aeruginosa, E. coli*, *Klebsiella pneumoniea.,* and *Proteus vulgaris* were highly pathogenic isolated bacterial species from all mastitis milk samples (CM and SCM) and environmental samples (drinking water, milk containers and animal bedding).

Specifically, as shown in table (5) that main bacterial species isolated from all examined ewe samples in this study, was *P. Aeruginosa* organisms (27.4%). They were isolated from clinical mastitis cases (33.4%) and subclinical mastitic cases (26.67%). Similar results of isolation of *P. aeruginosa* in ewes were recorded with **(Al-Khazay and Kshash, 2014)** in which 33.3% were from clinical mastitis and 27.5% from subclinical mastitis milk samples. Meanwhile, lower isolation rate of *P. aeruginosa* (9.09%) from clinical mastitis cases was recorded **(Geetha *et al.,* 2016)**. This variation in percentages of clinical and subclinical mastitis due to *P. aeruginosa* infections between present and other studies could be attributed to many factors such as the seasonal variation, age, nutritional status, type of animal housing, animal breed, as well as milking hygiene, different degree of sanitary measurement that applied in the dairy ewes **(Al-Khazay and Kshash, 2014)**.

Staphyloccal infections of mammary glands in sheep farms could be occurred during milking operation leading to the transmission of the infection to most ewes in the flock. The transmission of *S. aureus* infection among ewes could be due to herdsman during manual contact of udder, contaminated bedding material from infected ewes and lambs sucking ewes other than their dam **(Contreras, 1995)**. In the recent study, *S. aureus*, CNS and *St. agalactia* were harbored from CM cases in percentages of: 22.2%, 13.3% and 2.2%, respectively. Also, *S. aureus*, CNS were isolated from SCM cases in percentage of: 12.7% and 10%, respectively.

Many studies worldwide investigated the prevalence of CNS among sheep mastitis cases **(Burriel, 1997)**. *S. hominis, S. simulans, S. hyicus, S. saprophyticus, S. cohni* and *S. lentus* were the most identified species of CNS from mastitic cases in the present study. These results were in accordance with **(Thorberg *et al.*, 2009 and Piessens *et al.*, 2011)**. As in table (6), CNS was isolated in 13.3% and 10% of CM and SCM ewes. Similarly, **Onnasch *et al.*, (2002)** recorded the prevalence of CNS in Norway in 16%, meanwhile, in Germany, it was recorded in 9% in the milk samples of 80 dairy herds.

Environmental mastitis is caused by many organisms such as *E. coli* which do not normally live on the skin or in the udder but they enter the teat canal when the animal comes in contact with a contaminated environment. The pathogens normally found in feces bedding materials, and feed **(Heeschen, 2012).** In this study, as shown in table (7)*, P. aeruginosa* was isolated in 3/20 (15%) of the environmental samples which include drinking water, milking containers and animal bedding. Also, other bacteria like: *E. coli*, *S. aureus*, CNS and *Proteus vulgaris* were isolated in different ratio (9/20, 5/20, 1/20 and 1/20), respectively from all environmental samples.

Antimicrobial therapy is an important tool in the scheme of mastitis control and misuse or intensive use of antimicrobials could lead to the development of antibiotic resistance among different bacterial strains **(Libera *et al.*, 2010)**. *P. aeruginosa* is a clinically significant pathogen characterized by intrinsic resistance to many antimicrobials. Consequently, its resistance to antimicrobial agents is encountered with increasing frequency and cross resistance to chemically unrelated antibiotics **(Mura- matsu *et al.,* 2005)**.

Screening of antibiotic sensitivity exhibited MDR resistance of the examined *P. aerugino-*

sa isolates against tetracycline, sulphamethoxazole, norfloxacin, enrofloxacin, ceftriaxone, amoxicillin+clavulanic acid, erythromycin, streptomycin, trimethoprim meanwhile, gentamycine (65.4%) and ciprofloxacin (53.8%) recorded the highest sensitivity rates against *P. aeruginosa* isolates from both clinical and subclinical cases (Table 8). These results were compatible with previous studies **(Amany *et al.*, 2006)** in which *P. aeruginosa* isolates were sensitive to gentamycine and ciprofloxacin. Also, **Nahla *et al.*, (2013)** indicated that ciprofloxacin was the most effective drug (60.7%) against *P. aeruginosa* isolates from milk samples however; gentamycin showed a very low sensitivity (21.4%), this might be due to the variation in the usage of antibiotics. The same results were recorded by **(Nashwa *et al.*, 2016)** who stated that the most sensitive antibiotics against *P. aeruginosa* from sheep milk were gentamicin however, they were highly resistant to cefepime, cefotaxime, tetracycline and erythromycin with a percentage ranged from 78.6% - 92.9%.

Virulence of *P. aeruginosa* is multifactorial which may contribute to its pathogenicity **(Hentzer *et al.*, 2003)**. It also has a large number of virulence factors such as exotoxin A and Las genes **(Van Delden and Iglewski, 1998)**. The outer membrane proteins of *P. aeruginosa* (*OprI* and *OprL)* play important roles in the interaction of the bacterium with the environ- ment as well as the inherent resistance of *P. aeruginosa* to antibiotics where the consequence of the presence of these specific outer membrane proteins that have been implicated in efflux transport systems that affect cell per- meability **(Nikaido, 1994)**. As these proteins are found only in this organism, they could be a reliable factor for rapid identification of *P. aeruginosa* in clinical samples (**De Vos *et al.*, 1997**). *Las I* virulence gene is required for tran- scription of three proteases genes: elastase (*lasB*) and protease (*LasA)* and alkaline prote- ase gene (apr) which are associated with the virulence. *OprL* virulence gene is a lipoprotein in outer membrane which is used for the detection species of *P. aeruginosa* and *ToxA* is a gene expressing for an exotoxin A (*ETA*). Several of these virulence factors, acting alone or synergistically with each other, are believed to cause cell death, severe tissue damage, and necrosis in the human host **(König *et al.*, 1996)**.

PCR assays were developed with specific primers for the detection of some virulence genes (*lasI*, *oprL* and *toxA*) of *P. aeruginosa* isolates. In this study, these genes were found in 71.43% of seven examined isolates from examined samples. Similarly, *lasI* gene was detected in 4/6 strains only while *oprL* and *toxA* genes were detected in six tested strains of *P. aeruginosa* **(Gihan *et al.*, 2015)**. The differences in the distributions of virulence factor genes confirmed that some *P. aeruginosa* strains were better adapted to the specific conditions found in specific infectious sites **(Lanotte *et al.*, 2004)** that may returned to the different environmental and geographical sources.

P. aeruginosa expresses a multi resistance to antibiotics and this resistance could be acquired either on (plasmids or transposons) or natural. The resistance of *P. aeruginosa* is most often related to chromosomal mechanisms or to hydrolyzing enzymes production **(Sheetal and Srivastava, 2016)**. PCR evaluation of the examined isolates in this study confirmed the presence of tetracycline and sulfonamide resistant genes of (Tet A and Sul1) in 100% of examined isolates as shown in **fig 1 (D, E)** where clear bands were shown at 576 bp and 433 bp, respectively.

Hematological findings of apparent healthy and mastitic ewes' milk were reported in Table (9), a significant decrease in RBCs count, Hb conc., and PCV value in mastitic ewes that indicated the anemia was correlated with the severity of bacterial infection. These results were in accordance with **(Jain, 2000 and Azza and Ebtisam, 2013).** Also, **Zaki *et al.*, (2010)** who recorded significant reduction in RBC, Hb and PCV values leading to anemia in animals affected with mastitis. **Sarvesha *et al.*, (2017)** observed significantly ($P< 0.05$) lower average values of TEC, Hb and PCV were observed in subclinical mastitis of infected animals, In contrast with our findings, no significant trends in PCV and Hb in clinical mastitic cases were observed by **Sarvesha *et al.*,**

(2017). Leucocytosis, neutrophilia, eosinophilia, monocytosis and lymphopenia were recorded in the present study in clinical and subclinical mastitis. **Zaki *et al*., (2008) and Sarvesha *et al*., (2017)** reported an increase in leucocyte in affected animals along with monocytosis, neutrophilia and eosinophilia. In contrast, **Jain, (2000)** stated that the neutropenia and lymphocytosis was seen in ruminant with peracute and acute sever inflammatory disease.

Table (10) revealed a significant increase in levels of AST, ALT and ALP. These changes might be attributed to hepatic degenerative and necrotic changes due to bacterial infection and toxin **(Sarvesha *et al*., 2017)**.

Significant elevation of ALP in SCM milk might be due to both mammary epithelial dam- age and a breach in the blood-milk barrier selectively damaged by bacterial toxins **(Katsoulos *et al*., 2010).** They reported also that the origin of elevated ALP activity was from leukocyte and mammary epithelial and interstitial cells damaged during inflammation, particularly from disintegrated leukocytes. Similarly, **Azza and Ebtisam, (2013)** reported highly significant increases in AST values in mastitic cow. Similar highly significant increases in AST values of SCM were recorded by **(Chandrasekaran *et al*., 2015)**.

Our investigation on the biochemical analysis of kidney function recorded significant increase in the level of urea and creatinine in mastitic ewes. This was attributed to excessive protein catabolism and present azotaemia. Hypoproteinemia and hypoalbuminaemia in mastitic ewes might possibly be attributed to renal dysfunction **(Coles, 1986**). Hypoalbuminemia was an important feature of the liver disease **(Kaneko, 1980**). These results were agreed with **Azza and Ebtisam, (2013)**. A significant increased level of alpha and beta globulins in clinical mastitic ewes however, in subclinical cases, the increased level of alpha and gamma globulins were recorded. Increased alpha and beta or gamma globulins in the blood of mastitic ewes indicated an activation of immune response following the infection of the mammary gland. These proteins were mainly immunoglobulins which were implicated in udder defense mechanisms **(Tsenkova *et al*., 2001 and Azza and Ebtisam, 2013)**.

Serum calcium level of the mastitic ewes was significantly ($P< 0.05$) higher than the healthy animals which were attributed to the reduced milk production in the affected animals decreasing the excretion of Ca in milk **(Wegner and Stull, 1978).** Similar reports by **(Sarvesha *et al*., 2017)** in which higher plasma levels of Ca in mastitis affected buffaloes and cows were reported but in contrast to the present study (**Zaki *et al*., 2008 and 2010)** observed a reduction in the Ca values in the infected animals. There is significant decreased in phosphorous in mastitic ewes that may be resulted from the injury in the udder wall of ewes lead to high secretion of phosphorus in milk, resulting in decreasing phosphorus in blood which is in accordance with **(Krishnappa *et al*., 2016 and Sarvesha *et al*., 2017)**.

During inflammatory disease like mastitis, lipid peroxidation was increased which caused a decrease in levels of some antioxidant molecules leading consequently to oxidative stress **(Weiss *et al*., 2004 and Ibrahim *et al*., 2016).**

In this study, the plasma GSH concentration in dairy ewes with acute clinical mastitis and sub - clinical mastitis was significantly increased ($p<0.01$) compared to control group. The increased GSH concentration could be explained by the enhancement of activities of both types of enzymes glutathione peroxidase (GPx) and GR, leading to intense regeneration of GSH from the oxidized form (GSSH) obtained after reduction of peroxides into alcohols **(Kizil *et al*., 2007 and Ibrahim *et al*., 2016).** In contrast, some studies recorded a decrease in GSH in dairy cows with acute clinical mastitis **(Jhambh *et al*., 2013).** This variation could be attributed to different GPx activity associated with different bacterial pathogens **(Matei *et al*., 2011).** There was a significant increase in the level of GSH and SOD in diseased ewe compared to healthy ones which was probably a response to the higher superoxide radicals ($\bullet O_2-$) generation as a result of inflammatory

reactions in the mammary gland tissue. SOD catalyzes the dismutation of $\bullet O_2-$ into oxygen and H_2O_2, and it is an important antioxidant defense mechanism in aerobic organisms, although too much SOD may sometimes be deleterious. These findings were in accordance with the findings of **(Halliwell and Chirico, 1993).** The protective effect of CAT had been demonstrated in bovine neutrophil induced model of mammary cell damage **(Boulanger *et al*., 2002).** In the present study, there was a significant decrease in CAT activity in dairy ewes with acute clinical mastitis. Such decrease might be attributed to its increased consumption to counteract reactive oxygen species produced from inflamed mammary glands, suggesting a compromise in antioxidant defense of the body **(Jhambh *et al*., 2013).**

Nitric oxide (NO) production was considered as a primer defense system as it has antimicrobial properties due to peroxynitrite, a reactive nitrogen metabolite, derived from oxidation of NO, however, peroxynitrite could cause alteration in antioxidant balance in microorganism when produced in excess **(Chaiyotwittayakun *et al*., 2002)**. Nitric oxide is one of the most important reactive nitrogen radicals; which operate in a variety of tissues such as epithelial cells and macrophage of mammary gland, producing a significant amount of NO that mediates inflammation during mastitis **(Bouchard *et al*., 1999).** The clinical mastitis and SCM ewes in our study recorded a significant increase in the levels of nitric oxide (NO). Similar reports by **(Notebaert *et al*., 2008)** indicated that NO was increased during infection**.** There was a significant increase in plasma NO in dairy cows with acute clinical mastitis **(Ibrahim *et al*., 2016).**

Conclusion

In conclusion, in this research, mastitis due to *P. aeruginosa* is considered a great economical problem in sheep industry especially in dairy ewe farms. A close association was declared between the degree of the pathogenicity and the intrinsic virulence factors of *P. aeruginosa* isolates. These organisms can adversely affect the outcome of mastitis infections. Dairy ewes with clinical and subclinical mastitis act as a substantial factor for the alterations of the liver enzymes, kidney function, oxidant and antioxidant balance in plasma. The oxidative stress is the main factor causing dysfunction of the immune system of the organism impairing the response to inflammatory conditions, contributing to numerous diseases in dairy ewes. Hence, accurate isolation and identification of the bacterial cause of mastitis at the inaugural appearance and periodically CMT and SCC evaluation was recommended to discover subclinical mastitis cases and to accelerate the treatment. Also, strict hygienic measures should be done to minimize the chances for bacterial infections in ewe farms.

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