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Prevalence of pseudomonas aeroginosa causing of ewes mastitis with special reference to the virulence and antibiotic resistance genes of the isolates

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Abstract

Mastitis is a major problem affected dairy animals caused by several pathogens one of which is pseudomonas aeroginosa resulting in great economic loss. In this study a total of 100 milk samples of which 20 from clinical mastitic ewes and 80 apparently healthy one (for detection of subclinical mastitis by California mastitic test and somatic cell count)were collected and examined for detection of pseudomonas aeroginosa , prevalence, antibiogram of isolates as well as detection of some virulence and antibiotic resistance genes . the results showed that pseudomonas aeroginosa isolated from 20 samples of which 7 clinical mastitic milk and 13 from subclinical mastitic milk.

The antibiotic resistance profile of *P. aeruginosa* isolates showed wide range of resistance (85%-100%) against most tested antibiotics however, gentamycine and ciprofloxacin recorded the highest sensitivity rates (63% and 55%), respectively. Some virulence genes of *P. aeruginosa* isolates (*las I, tox A,* and *opr L*) were detected with PCR five out of seven tested isolates, meanwhile the *tet A* and *sul1* antibiotic resistance genes were found in all *P. aeruginosa seven tested* isolates.

Keywords*: Mastitis, P. aeruginosa, antibiotic sensitivity , virulence gene , pcr*

Introduction

Mastitis is a major health problem in the dairy sheep and goat 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 **(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).** Also**,** California mastitis test (CMT) is used for diagnosis of both forms of mastitis as a special field screening test **(Al-Khazay and Kshash, 2014).**

*Pseudomonas aeruginosa*is occasionally involved in enzootic or epizootic outbreaks of mastitis in small ruminants (**Sela *et al*., 2007**).

*Pseudomonas aeruginosa*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 multifa torial process. They have many cell associated factors such as lipopolysaccharide (LPS), flagellum, and pilus and non-pilusadhesins, 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 Aencoded by the *toxA*gene which 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)**.

The objective of the study was to isolate and identify most common bacteria causing mastitis in ewes and to detect some virulence and

antibiotic resistance genes of *Pseudomonas aeruginosa* isolates

Materials and Methods

I- Animals and sample collection : The current study was carried out in five sheep private farms at Ismailia Governorate, Egypt. Their age ranged from 3-5 years. Eighty milk samples of ewes free of any signs of clinical mastitis or any other palpable udder lesions (subclinical) were collected. In addition, 20 milk samples were collected from ewes showing the clinical signs of mastitis. A history of stopping 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 were pyrexia, watery nasal discharge, cough, cessation the motility of rumen, hard painful swelling of left quarter of udder and injury noticed on the leftteat. each sample was collected in a separate sterile container , and disinfection with 70% alcohol after teat and udder cleaning , identified and kept in an ice box and send to the laboratory without delay.

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 furtherexamination.

Application of field California Mastitis test (CMT): It is used for field diagnosis of subclinical forms of mastitis cases in 5 dairy ewes' farms at Ismailia Governorate as a special field screening test **(Al-Khazay and Kshash, 2014).** The CMT was scored on a scale from 0 to 4 as shown in **table(1)**

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)** as shown in **Table (1)**

Table (1). The viscosity degree and somatic cell count evaluate on 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 oneplate.	1500-5000
4 (+++)	Forms ropey, viscous mixture, significantly visible clot, pouring mixture falls of theplate	>5000
0: (-) Negative reaction, 1(-): trace, 2: (+) slightly positive, 3: (++) moderately positive, 4 (+++) strongly positive.		

Bacterial isolation and identification Quinn *et al.* (2002): Ten ml of each milk sample (clinically mastitic and +ve CMT) transferred to sterile test tube and incubated at 37ºC for 24h aerobically then centrifuged at 3000 r.p.m for15 minutes, the supernatant discard and the sediment were streaked onto plates of sheep blood agar and pseudomonas agar media, the plates were incubated at 37ºC. Aerobically for 24 h suspected colonies of pseudomonas aeroginosa were examined for culture, morphological character and purified, picked up and cultured in slant agar for identified by vitek 2 compact system .

Identification by Vitek2 compact system and were done according to the manufacture' instruction (BioMe'rieux, 2006) as following:

When used with VITEK® 2 instrumentation, the GN card is a complete system for routine identification testing of most clinically significant Gram-negative organisms.**(Chatzigeorg-iou *et al.*, 2011)**

Procedure

1.Use a sterile stick or swab to transfer a sufficient number of morphologically similar colonies to the saline tube prepared in step

2. Prepare a homogenous organism suspension with a density equivalent to a McFarland No. 0.50 to 0.63 using acalibrated VITEK® 2 Densi CHEK™ Plus.

Note: Age of suspension must not exceed 30 minutes before inoculating card. Place the suspension tube and GP card in the cassette. Refer to the appropriate Instrument User Manual for instructions on data entry and how to load the cassette into the instrument

Inoculation Identification cardsare inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can to 10 tests .The filled cassette is placed either manually (VITEK 2 compact) or transported automatically (VITEK 2 and VITEK 2 XL) into a vacuum chamber station. After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells.

Card Sealing and Incubation

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. The carousel incubator can accommodate up to 30 or up to 60 cards. All card types are incubated on-line at 35.5 + 1.0ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data are collected at 15-minute intervals during the entire incubation period.

Identification of antibiotic sensitivity by vitek2:

Identification by Vitek2 compact system and antimicrobial susceptibility test (AST-GN card) were done according to the manufacture' s instructions following **(*Chatzigeorgiou et al.*, 2011)**

Suspension preparation:

A sterile swab was used to transfer a sufficient number of colonies of pure culture and to suspend the organism in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, PH 4.5 to 7.0) in a 12x75 mm clear plastic (polystyrene) test tube. The turbidity was adjusted to the equivalent of a 2.7-3.3 McFarland turbidity with a VITEK2 instrument DensiCheek**(Chatzigeorgiou *et al.*, 2011)**. In case of AST, 280ul of microorganism suspension were transferred into 3.0 ml saline polystyrene test tube.

Inoculation:

For each isolate, the identification AST-GN cards were inoculated with the microorganism suspension. The card identified different 47 biochemical tests. A test tube containing the microorganism suspension was placed into a special rack (cassette). The filled cassette was placed into a vacuum chamber station. After the vacuum is applied and air is re-introduced into the station, the organism suspension was forced through the transfer tube into micro-channels that fill all the test wells.

Card sealing and incubation

inoculated cards were passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. All cards were incubated on line at 35.5^{o}C± 1.0 C for approximately 6 hours. During incubation, the cards were read every 15 min. The final results were obtained automatically printed within 6-8 hours.

All used cards were automatically dispensed into waste container.

Table 14:Biochemical details of *Pseudomonas aeruginosa*																	
2	AP-PA	-	3	ADO	-	4	Py-rA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTP	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	+
23	proA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTER	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATK	+	41	AG-LU	-	42	SUCT	+	43	NA-GA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IM-LTa	-	62	ELLM	-	64	ILA-Ta	-			

Antimicrobial testing of *Pseudomonas aeruginosa* isolates: The identified *P. aeruginosa* isolates were tested against a panel of eleven commercial antimicrobial agents: gentamycine (120µg), ciprofloxacin (5µg), norfloxacin (10µg), enrofloxacin (5µg), ceftriaxone (5 µg), amoxicillin+clavulanic acid (10µg), erythromycin (15µg), sulphamethoxazole (25µg), streptomycin (10µg), trimethoprim (30µg) and tetracycline (10µg) 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:

DNA extraction :DNA extraction from samples was performed using QLA amp DNA mini kits (Qiagen, Germany, GmbH)

With manifacttions from the manifactures recommendations. Briefly , 200 µl of the sample suspension was incubated 10µl of proteinase K and 200µl of the lysis buffer at 56ºC for 10 Min . After incubation , 200µl of 100 %ethanol was added to the lysate . The sample was then washed and centrifuged following the manufacture recommendations .Naclic acid was eluted with elution buffer provided in kits **(Sambrook *et al*., 1989)**

PCR amplification: As shown in table (2), the primers for the selected virulence (Las I, Tox A, and opr L) and antibiotics resistant genes (Tet A and Sul 1) of seven *Pseudomonasaeruginosa* isolates 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).**

Oligonucleotide primers were supplied from metabolism Germany are listed in table (2)

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 TTCGGC	Xu *et al*. (2004)
R		CTT CTT CAG CTC GAC GCG ACG	
l ToxA	F	GACAACGCCCTCAGCATCACCAGC	Matar *et al. (* 2002)
R		CGCTGGCCCATTCGCTCCAGCGCT	
TetA	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

Protein electrophoresis: It was done using SDS-Polyacrylamideg elelectrophoresis according to
Laemmli, (1970) in animal health research institute**.**

Results

The prevalence rate of subclinical mastitis (SCM) in examined ewes' farms: In the present study, SCM cases were recorded in 45 from total eighty apparently healthy animals with a percentage of (56.25%). SCM milk samples of apparently healthy animals were recorded as of grade 3 (++) and 4(+++)in farm using CMT evaluation

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 **(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	35/80	200×103	450×103	267.2×103±12.3×103
Infected animals somatic cell count ≥500.000	45/80	500×103	900×103	700×103±20.5×103

Bacteriological isolation and identification results: The bacteriological examination of total 65 mastitis milk samples (20 from CM and 45 from SCM) showed that *P.aeruginosa* was isolated from 20 samples as (7) from clinically mastitic milk and (13) from subclinical mastitic milk in an incidence of(35%, 28.9% respectively with over all incidence of (20%)

Table (5). The prevalence of subclinical mastitis in examined milk samples

No of examined milk samples	CMT +VE &SCC		-VE	
	No.	%	No.	%
80	45	36	35	28

as shown in **(Table 6)**.

Table (6). Prevalence of pseudomonas aeroginosa in the examined samples

Animal case	No sample	+ve	
		No	%
Clinical	20	7	35
Subclinical	45	13	28.9
Total	65	20	30.7

% calculated according to the no. examined samples in each item

Antibiotic sensitivity patterns of *P. aeruginosa* isolates: The antibiotic susceptibility testing of twenty recovered *P. aeruginosa* isolates from milk samples exhibited high resistance rate against most used antibiotics. All *P. aeruginosa* isolates showed 100% resistance to Amoxicillin+clavulanic acid, Erythromycin, Sulphamethoxazole, Streptomycin, Trimethoprim and tetracycline However, only gentamycine and ciprofloxacin were the most effective drugs with percentages of 65% and 55%, respectively **(Table, 7)**

Table (7): Antibiotic sensitivity testing of the recovered *P. aeruginosa* isolates

Antibiotic/potency	No. of *P. aeruginosa* isolates showing susceptibility pattern (20)	
	Sensitive	Resistant
Gentamicin (120µg)	13 (65%)	7 (35%)
Ciprofloxacin (5µg)	11 (55%)	9 (45%)
Norfloxacin (10µg)	3 (15%	17 (85%)
Enrofloxacin (5µg)	1 (5%)	19 (99%)
Ceftriaxone	1 (5%)	19 (99%)
Amoxicillin+clavulanic acid (10µg)	0	20 (100%)
Erythromycin(15µg)	0	20 (100%)
Sulphamethoxazole (25µg)	0	20 (100%)
Streptomycin (10µg)	0	20 (100%)
Trimethoprim (30µg)	0	20 (100%)
Tetracycline (10µg)	0	20 (100%)

% calculated according to the no. of tested Pseudomonas aeroginosa were isolates (20)

Detection of some virulence and resistant genes of *P. aeruginosa* isolates:

The results of PCR showed the presence of virulence genes of *P. aeruginosa* (*Las I, Tox A,* and *opr L*) in five out of seven examined isolates (**Fig.1; A, B&C**). Meanwhile, the resistant genes (*TetA* and *SUL1*) were found in all seven tested isolates ,where clear bands were shown at 576 bpand433 bp, respectively, as shown in (**Fig.1; D&E**).

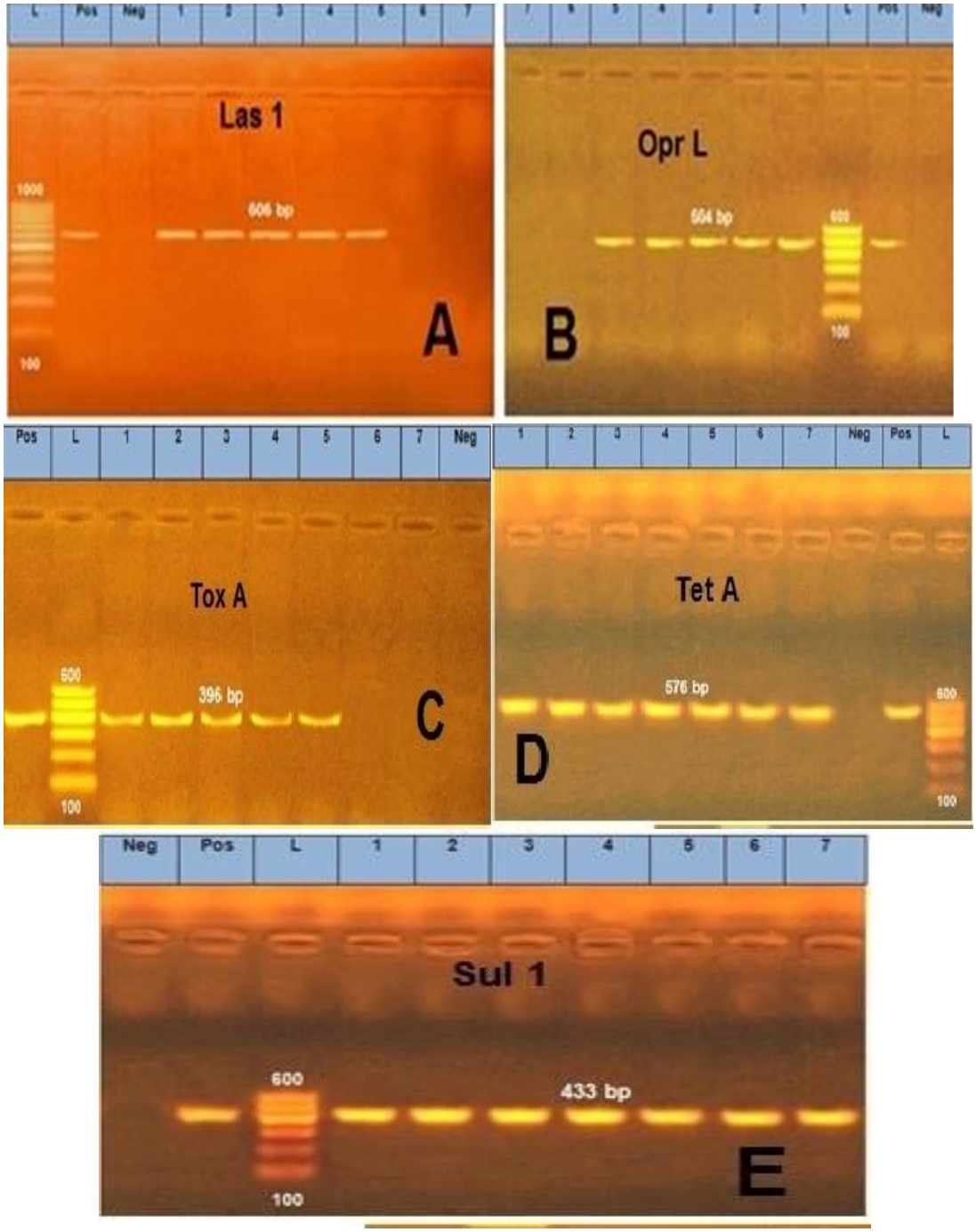

Fig (1): Agarose gel electrophoresis of PCR amplified products of virulent genes**:** *Las I* **(A)**, *Opr L* **(B)** and *Tox A* **(C)** and resistant genes: *Tet*A
(D) and *Sul 1* **(E). Lane L**: DNA molecular size marker (100 bp), **lanes 1-7**:*P.aeruginosa*strains**, lane (Pos):** positive control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

Discussion

Mastitis is considered as one of the most important diseases in dairy ewe industry **(Tormod *et al*., 2007)**. The variationin the incidence 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 this study in 45/80 of cases with a percentage of (56.25%) in different ewes' farm at Ismailia Governorate using CMT evaluation. Similar studies recorded the total percentage of subclinical mastitis in ewes' farms according to CMT in field was 69.3% and 64.9% **(Al-Khazay and Kshash, 2014)**. Also, these results were in accordance with that mentioned by **(Sharma *et al.*, 2010 and Marwa *et al*., 2017)** who declared that 53.73% and 54% of ewe milk samples respectively were positive for subclinical mastitis. **Radostits *et al*., (2000)** attributed higher prevalence of SCM due to late lactation period, also **Khanal and Pandit, (2013)** recorded that the prevalence of 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 increased milk SCC (MSCC) of cows and sheep during the stage of lactation and parity was mainly caused by intramammary infections (IMI). However, when IMI in goats increased MSCC, other non-infections factors such as estrus, season of milking, milk yield and stage of lactation discussed the increase in MSCC (**Raynal-Ljutovac *et al*., 2007***)*. 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).**

Table (6)illustrated that P.aeroginosa was isolated from 20 samples out of 65 tested (30.7%) where 7(35%) from clinically mastitic ewes and 13 (28.9%) from subclinical mastitic ewes .Nearly similar findings were recorded **(Al-Khazay and Kshash, 2014)** who isolated P. aeroginosa in an incidence of 33.3% from clinical and 27.5% from subclinical mastitic 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)**.

Antimicrobial therapy is an important tool in the scheme of mastitis control and the 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 **(Muramatsu *et al.,* 2005)**.

In this study, screening of antibiotic sensitivity exhibited high resistance rate of examined *P. aeruginosa* isolates against norfloxacin, enrofloxacin, ceftriaxone, amoxicillin+clavulanic acid, erythromycin, sulphamethoxazole, streptomycin, trimethoprim, tetracycline, meanwhile, gentamycine (65%) and ciprofloxacin (55%) recorded the highest sensitivity rates against *P. aeruginosa* isolates from both clinical and subclinical cases. 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 of5 (D, E) where 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)**. Italso 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 environment 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 permeability **(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 transcription of three proteases genes: elastase (*lasB*) and protease (*LasA)* and alkaline protease 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 71.74% of seven examined isolates from clinical and subclinical milk 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 geographicalsources.

P. aeruginosa expresses a multi resistance to antibiotics and this resistance could be acquiredeitheron (plasmidsortransposons) ornatural. 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 5 (D, E) where clear bands were shown at 433 bp and 576 bp, respectively

Conclusion

In conclusion, in this research, *P. aeruginosa* either in pure or in mixed infections could cause for the incidence of mastitis in dairy ewe farms. A close association was declared between the degree of the pathogenicity and the intrinsic virulence factors of *P. aeruginosa* isolates which could be consequently adversely affected on the outcome of mastitis infections. 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 ewefarms

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