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Bacteriological studies on *Pseudomonas aeruginosa* causing clinical mastitis of cattle with special reference to the antibiotic resistant genes of the isolates

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Abstract

Pseudomonas aeruginosa consider to be one of the most common causes of cattle mastitis producing economic loses among these animals. In this study a total of 100 milk samples were collected from clinically mastitic cows and subjected to the bacteriological examination for detection of the prevalence of *Pseudomonas aeroginousa*. The results revealed the isolation of (19) *Pseudomonas aeruginosa* strains in an incidence of 19%. Vitek2 system succeeded in providing definitive identification results for *Pseudomonas aeruginosa* by identification card (GN) and detection of its antibiotic sensitivity test using AST-GN.

The exotoxins of *P. aeruginosa* isolated from the mastitic milk samples were produced *in vitro* with ordinary laboratory media (trypticas soya broth), and their toxic effect was tested by I/P injection in mice. The virulence genes of two random isolates of *P. aeruginosa* (*toxA* and *exoS*) were detected by PCR.

The results of antibiotic sensitivity test showed that the isolates were resistance to Pipracillin, Oxicillin and Tetracycline due to the presence of multidrug resistance genes as *blaVIM* gene, but sensitive to Ciprofloxacin, Cefoperazone, Impenem, and Amikacin.

Key words: *Pseudomonas aeruginosa, Cows and Clinical mastitis.*

Introduction

Mastitis in cattle is usually mild, subacute or chronic, but can be clinically severe peracute with a mortality high rate as clinically, there is a severe systemic reaction and acute swelling of the gland with the appearance of clotted milk contain pus, blood, and mucous. *P. aerogenosa* is common in the environment of cattle because of its innate ability to survive for long periods in dry and moist conditions **(Ibrahim *et al.,* 2017).** Mastitis not only affects the physical, chemical, bacteriological, technological and organoleptic properties of milk, but also affects milk quantity. It causes major economic losses

in dairy cattle. *P. aeruginosa* is wide spread in the environment of dairy cows because it requires few nutrients to grow and multiply. Water supply from all sources (wells, troughs, ponds, parlor wash houses, and sprinkler pens are major sources of *P. aeruginosa* in dairy farms. Sudden outbreak of clinical mastitis could be caused within few days in the dairy farms by *P. aeruginosa* however there are any changes in weather and/or management techniques or not. Clots or blood will be part of excessive the outbreak. In addition, many infected cows die despite aggressive treatment. Also many cows that are saved are left unfit for productive use **(Dinaol *et al.,* 2016; Ghassan *et al.,* 2016 and Anjali and Kashyap, 2017)**.

Many genes like *mexR* and *blaVIM,* has been detected in many *P. aeruginosa* isolates. These genes were found to have significant percent of

resistance to β-lactam antibiotics. Also, the immunological and biochemical parameters were affected **(Awad *et al.,* 2017).**

Purified exotoxin A had negative effects on mice as it is highly toxic for mice and causing very high mortality percentages in them. This exotoxin A acts as a major systemic virulence factor for the previous strains which causes specific protein biosynthesis and cell death.

Exotoxin A is responsible for local tissue damage, bacterial invasion, and (possibly) immunosuppression **(Frantisek *et al.,* 2017)**. *P. aeruginosa* is an opportunistic pathogen that produces several diseases, and considered all over the world as one of the most dangerous organisms which affected animals. *P. aeruginosa* is responsible for a number of cases of mastitis and remains in the udder for a number of years **(Bengtsson *et al.,* 2009)**. *P. aeruginosa* continuous to be a major of resistant microorganism because of its high intrinsic resistance to antibiotic, which has been demonstrated that this intrinsic resistance arises from the combination of unusually restricted to most classes of antibiotics can readily raised **(Wolf and Elsasser, 2009 and Seol *et al.,* 2002)**. Recently a new automated identification system such as Vitek2, accompanied by identification cards can give reliable and rapid identification. In addition, identification of bacteria by Vitek2 system has revealed prominent inter laboratory reproducibility and is quickly being included as a routine method for animal and human microbiological laboratories **(Pincus, 2006).** Here, we report the performance of Vitek2 for *Pseudomonas spp* for testing contemporary isolates with currently used antimicrobial agents and the most up-to-date software and AST cards available from bioMérieux. Comparison of the Microflex LT and Vitek2 systems for routine identification of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Vitek2 (bioMérieux, Durham, NC, USA) is a widely used commercial antimicrobial susceptibility testing system.

The aim of the present study is to isolate and identify *P. aeruginosa* from milk samples collected from clinically mastitic cows using Vitek2. The antibiotic susceptibility pattern of the recovered isolates were determined by using vitek2, AST–GN. PCR detections of virulence and resistance genes were also applied on MDR isolates.

Materials and Methods

Samples

A total of 100 milk samples were collected from clinically mastitic cows (70 samples were watery contains flakes and clots + and 30 samples with pus) from governmental and private farms under complete hygienic measure in sterilized bottles and transferred to the laboratory in ice box according to (**Quinn *et al.,* 2002**).

Pseudomonas aeruginosa isolation and identification

Milk samples were centrifuged at 3000 rpm. A loopful from the sedement was cultured onto blood agar and MacConkey agar plates. Then, they incubated aerobically at 37 °C for 24 h. The growing suspected colonies were identified morphologically and biochemically **(Quinn and Markey, 2003).**

Pseudomonas aeruginosa identification by Vitek2 compact system

Pseudomonas aeruginosa identification was done using Vitek2 compact system (bioMérieux, Durham, NC, USA), according to the manufacturer's instructions, using the GN card which is a complete system for routine identification testing of most clinically significant Gram-Negative organisms **(Chatzigeorgiou *et al.,* 2011).**

Morphologically similar colonies were transferred to the 0.45 % saline to prepare the organism suspension with a density equivalent to a 0.50 to 0.63 McFarland using a calibrated VITEK® 2 DensiCHEK™ Plus. Then, the last suspension used to fill the test cards for Vitek2 instrument.

3- *In vitro* antibiotic sensitivity test of *Pseudomonas aeruginosa* by Vitek2

The *in vitro* antibiotic sensitivity test of *P. aeruginosa* identification by Vitek2 compact system and antimicrobial susceptibility test

(AST-GN card) were done according to the manufactures' instructions **(Chatzigeorgiou *et al.*, 2011).** For each isolate, the identification AST-GN cards were inoculated with the microorganism suspension. The card identified 47 different 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 reintroduced into the station, the organism suspension was forced through the transfer tube into micro-channels that fill all the test wells. The antibiotic sensitivity test of *Pseudomonas aeruginosa* was done against Amikacin, Cefotaxime, Ciprofloxacin, Ceftriaxone, Cefoperazone, Ceftazidime, Gentamicin, Impenem, Oxacillin, Piperacillin, and Tetracycline.

4- Virulence test of isolated strains

Laboratory animal used in virulence test of the isolated strains

Mice used were of both sexes, weighed 15-20 g each. They were housed in clean cages and fed a clean diet and water; fecal specimens were obtained before the study and examined to ensure the absence of *P. aeruginosa*. They classified into (4) groups; 5 mice each, 3 groups for each dilution as will be explained and one is as control group.

Preparation of toxin using trypticase soya broth (Wolf and Elsasser, 2009)

The toxin was produced with the same techniques described for the production of the serum toxin with the use of trypticase soy broth instead of serum**.**

Virulence test of the isolated strains (Quinn *et al.*, 2002).

The virulence test of *P. aeruginosa* isolate was done by injecting 0.2 ml of 1/1, 1/10 and 1/100 dilution of 18 hours old soya broth culture interrpertonially, 5 mice per dilution. Mortalities of experimentally injected mice were recorded to choice the highest virulent strain to prepare exotoxin A.

PCR detection of *toxA, exoS*, and *blaVIM* genes in *Pseudomonas aeroginousa*

DNA extraction from the isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was added to 10 µl of the proteinase K and 200 µl of the lysis buffer and incubated at 56˚C for 10 min. Then, 200 µl of 100% ethyl alcohol was added to the lysate. After washing and centrifuging the sample, 100 µl of elution buffer that provided by the kit was used to elute the nucleic acid.

PCR amplification

Primers were used for PCR amplification for *toxA* and *exoS* genes. These sets of primers were utilized for the PCR reaction and for the analysis using forward primer and reverse PCR primers for *toxA* gene according to **(Alzubaidy *et al.*, 2014**) and for *exoS* gene according to **(Ajayi *et al.*, 2003)** as shown in table (1). While for *blaVIM* resistant gene according to (**Sánchez *et al.*, 2002).** These primers were utilized in a 25 µl reaction containing 12.5 µl of PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of nuclease-free water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem thermal cycler. Cycling conditions of the different primers during the PCR amplification as shown in table (2) following the manufacturer's recommendations.

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, 20 µl of the products was loaded in each gel slot. A gelpilot 100bp and 100bp plus DNA Ladders (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Table (1). Target genes, primers sequences, and amplicons sizes.

Target gene	Primers sequences Forward (5`-3`) Reverse (3`-5`)	Amplified segment (bp)	Reference
toxA	F:GACAACGCCCTCAGCATCACCAGC	396	**Al-zubaidy *et al.*, (2014)**
	R:CGCTGGCCCATTCGCTCCAGCGCT		
exoS	F:GCGAGGTCAGCAGAGTATCG	118	**Ajayi *et al*., (2003)**
	R:TTCGGCGTCACTGTGGATGC		
blaVIM	TTTGGTCGCATATCGCAACG	500	**Sánchez *et al*., (2002)**
	CCATTCAGCCAGATCGGCAT		

Table (2). Cycling conditions of each primer during PCR.

Target Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
toxA	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
exoS	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
blaVIM	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

Results

Prevalence of *P. aeruginosa* isolated from milk samples:-

Nineteen strains of *P. aeruginosa* were isolated from hundred of clinically mastitic cattle milk samples in an incidence of 19% as shown in table (3).

Table (3). Prevalence of *P. aeruginosa* isolated from clinically mastitic milk .

Total examined mastitic milk samples	Pseudomonas isolates	
	No.	%
100	19	19

Biochemical identification of *P. aeruginosa* isolates by Vitek2 system is shown in table (4)

Table (4). Biochemical details of *P. aeruginosa* using Vitek 2 compact system.

2	APPA	-	3	ADO	-	4	PyrA	-	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	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Virulence test of *P. aeruginosa* isolates in mice:

Pseudomonas aeruginosa isolates live broth culture containing exotoxins) (19 strains isolated from masititic milk samples) was highly lethal to mice, where mice, died within 4-6 hours after injection at dilution 1/1. However, it took three days after the injection at dilution 1/10. While, at the dilution 1/100 only 2 mice died out of five mice within five days. On the other hand, the control group showed no death cases as shown in table (5).

Table (5). Virulence effect of the highest virulent *P. aeruginosa* (isolated from mastitic milk samples) in each group of mice.

Strain	Dilution in broth culture	Mortality of mice /day*					Total number of dead mice
		1 day	2 day	3 day	4 day	5 day	
P. aeruginosa	1/1	5/5	-	-	-	-	5
	1/10	2/5	4/5	5/5	-	-	5
	1/100	0/5	1/5	2/5	2/5	2/5	2
Control mice	Sterile broth	0/5	0/5	0/5	0/5	0/5	0

***** Expressed as the number of mice that died/ Number of mice used.

The results of antibiotic sensitivity test

The results showed that the isolates were resistant to Oxacillin, Piperacillin, and Tetracycline. While these isolates were sensitive to Amikacin, Ceftazidime, Cefoperazone, Ciprofloxacin, and Impenem with different percentages as shown in table (6).

Table (6). Antibiogram pattern of *P. aeruginosa* isolates against different antibiotics.

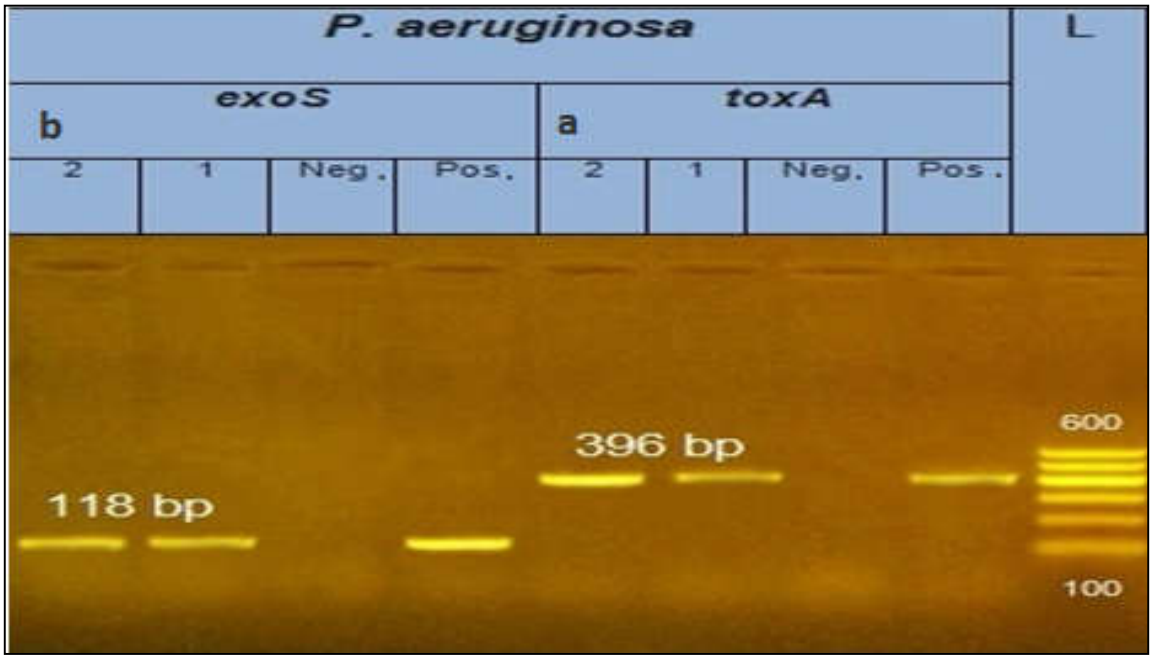
Antibiotic	Strength potency	Sensitive		Resistant	
		No.	%*	No.	%*
Amikacin	30 µg	17	89.47	2	11.76
Ceftazidime	30µg	14	73.68	5	29.42
Cefotaxime	30µg	7	36.84	12	70.58
Cefoperazone	50µg	16	84.21	3	17.65
Ceftriaxone	30µg	13	68.42	6	35.29
Ciprofloxacin	5µg	14	73.68	5	29.42
Gentamicin	120µg	12	63.16	7	41.18
Impenem	10µg	16	84.21	3	17.65
Oxacillin	1µg	6	31.58	13	76.47
Piperacillin	100µg	5	26.32	14	82.35
Tetracycline	30µg	3	15.79	16	94.12

*Percentages were calculated according to the No. of tested isolates (19).

Detection of *Pseudomonas aeruginosa* virulence and antibiotic resistant genes using PCR

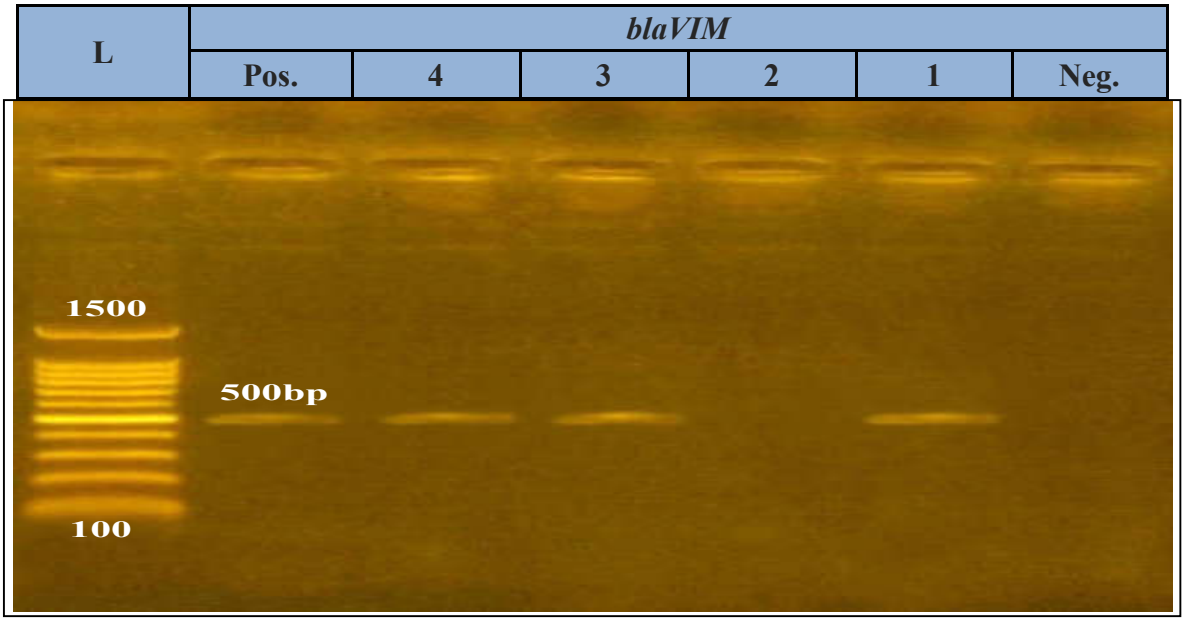
The *Pseudomonas aeruginosa* virulence genes based on *exoS*, and *toxA* primers which revealed to *exoS* and *toxA* virulence genes of *Pseudomonas aeruginosa* which give amplicon at 118bp for *exoS* gene and 396bp for *toxA* gene as in Fig (1). On the other hand, detection of the antibiotic resistant gene of the tested *Pseudomonas aeruginosa* isolates by PCR showed that (3) isolates of the (4) tested ones were positive for *blaVIM* gene give amplicon at 500bp as shown in Fig (2).

Figure (1). Agarose gel electrophoresis of PCR amplification of *P. aeruginosa*.



(a) exotoxin A (*toxA*) gene, (b) exotoxin S (*exoS*) gene, Lane L: 100-600bp DNA Ladder, Neg.: negative control, Pos.: positive control, Lane a1 and a2 are *P. aeruginosa* positive (at 396bp), and Lane b1and b2: *P. aeruginosa* positive (at 118bp).

Figure (2). Agarose gel electrophoresis of multiplex PCR amplification of *Pseudiomonas aeruginosa* extracted DNA for *blaVIM* gene.



L: represents the molecular size marker (100-1500bp DNA ladder). Neg.: negative control, Pos.: positive control of *bla*VIM (500bp), Lanes: 1, 3 and 4 are positive for *bla*VIM gene.

Discussion

Mastitis caused by *P. aeruginosa* is rare in cattle but its isolation from environment is common. This infection usually originates from contaminated water used for udder washing. This pathogen colonizes in milking equipment; enter teats in milking intervals and causes mastitis under stressful conditions and poor hygiene. Clinical mastitis due to *P. aeruginosa* is often severe with gangrene and death. Its subclinical and recurrent mild clinical mastitis can also be seen **(Park *et al*., 2014)**. In this study, *P. aeruginosa* was isolated from clinically mastitic cows in a prevalence rate of 19% as shown in table 3 which agreed with **(Bancijee *et al.,* 2017)**.

Mice can be used as a model in studying the toxicity of exotoxin A in laboratory. In case of *P. aeruginosa* infection, the response of the host to the toxin is different, in this study the host was subjected only to the purified exotoxin whereas during an infection the host is exposed to numerous toxic products, each inducing various biochemical and pharmacological effecting it. Moreover, the host was exposed to relatively large amount of toxin in very short time. The work presented here represents a first attempt to define the biological effect of the large amount of toxin which is one of the factors that may play important role in pathogenicity **(Anjali and Kashyap, 2017)**.

Rapid identification by Vitek2 accompanied using Gram negative card is based on established biochemical methods and newly developed substrates measuring carbon source utilization enzymatic activities and resistance. There are 47 biochemical tests and one negative control well; Final results are available in approximately 10 hours or less **(Biomerieux user guide, 2006 and Chatzigeorgiou *et al.,* 2011).**

The results regarding to Vitek2 system identification was in agreement with which mentioned by many laboratories worldwide have adopted the Vitek automated system for the detection of gram negative strains in routine clinical microbiology because it is rapid and fast **(Bancijee *et al.,* 2017).**

Several advantages of the Vitek2 system can be mentioned. First, it is a closed system that can avoid unwanted cross-contamination or environmental contamination. Second, if a specimen card is misplaced on its cartridge, Vitek2 system can detect it. So, it owns a dependable recheck system during its operation. Third, the Vitek2 system is able to handle dozens of specimens automatically at the same time. It is also easy for laboratory staff to prepare and load bacterial specimens. The decreased turnaround and hand-on times greatly improve the efficiencies of routine clinical laboratories. In conclusion, the Vitek2 system gave rapid, reliable, and highly reproducible results **(Ling *et al.,* 2001).**

P. aeruginosa produces virulence factors, which give it major roles in causes of diseases, these virulence factors encoding by virulence genes located in the chromosome of *Pseudomonas aerouginosa*. However, this study examined the virulence factors genes included *toxA* that encoding to exotoxin A, and *exoS* that encoding to exoenzyme S (**Nikibin *et al.,* 2012; Khattab *et al*., 2015 and Neamah, 2017)**

In this study, the multidrug resistance of *Pseudomonas aerogenosa* was detected this was in agreement with **(Randall *et al*., 2004).** As *P. aeruginosa* was reputed for its innate resistance to most antimicrobials Intrinsic resistance is attributable to low permeability of its outer membrane, multidrug efflux pumps and the production of chromosomally-encoded β-lactamases. In addition, organisms within biofilms are less susceptible to the action of antimicrobial agents (**Oliverira and Ruesg , 2014**).

This results agreed with (**Rubin *et al*., 2008 and Beier *et al*., 2015)** *in vitro* antibiotic sensitivity test of *Pseudomonas aeruginosa* isolates revealed unique pattern of sensitivity against different drugs. From this study, it was found that the isolates were mostly sensitive (90%) to the drugs like Amikacin, Cefoperazone, and

Impenem followed by Ceftazidime, and Ciprofloxacin all in range (80-70%) and lastly against Ceftriaxone and Gentamicin (70-60%). However, these isolates showed high resistance to Tetracycline, Piperacillin, Oxacillin and Cefotaxime (70-94%) in accordance with **(Abdel-Tawab *et al.,* 2016 and Badamchi *et al.,* 2017)** these results are detailed in table (6) and Fig (2). As well as, *P. aeruginosa* strains were highly resistant to many antibiotics and such phenomenon might be due to R-factors **(Dinaol *et al*., 2016)**. Intrinisic antibiotic resistance in the expression of multi-drug resistance (MDR) pumps is strictly down regulated at least under laboratory growing conditions. However, mutants over expressing MDR determinants are easily selected in the laboratory under antibiotic selective pressure **(Frantisek *et al*., 2017)**. These types of mutant are encountered after post therapeutical**.** This indicates the derepression MDR determinant has an important role in mutationally acquired antibiotic resistance during treatment. *P. aeruginosa* is MDR determinants usually due to mutations in the genes encoding the proteins that regulate the expression of the structural operons. MDR mutant (mainly the nalB mutant) is impaired in terms of virulence found in this study. The emergene of multi-resistance *P. aeruginosa* is becoming a challenging problem in infection control programs. It is almost always predominant in monobacterial as well as polybacterial infection **(Wolf and Elsasser-Beile, 2009 and Gangwal *et al*., 2017)**. The resistant gene for *P. aeruginosa* (*blaVIM*) was detected by PCR agreed with results of **Zhao and Hu, (2015) and Awad *et al*., (2017).** The resistance to antibiotic may be also due to presence of virulence genes *P. aeruginosa* as *exoS* and *toxA* genes in all examined isolates these results are in line with **(Alhede *et al.,* 2014 and Sonbol *et al.,* 2015)**.

Conclusion and Recommendation

1- Mastitis in cows is a problem causing severe losses economically due to losses of milk production either qualitative or quantitative in addition to the mortalities in non-treated animals, treatment cost and consequently reduction in dairy products manufacture. *Pseudomonas aeruginosa* is a main cause for mastitis in cows and cause severe losses among infected animals because it is highly resistant to antibiotic treatment.

2- Vitek2 compact system is a good powerfull, rapid method for identification of *Pseudomonas aeruginosa* isolated from mastitic milk. Also, PCR is a good and accurate powerfull technique for the detection of virulence genes of *Pseudomonas aeruginosa* isolates as well as antibiotic resistance genes.

3- *ToxA* and *exoS* genes of *P. aeruginosa* are responsible to the sever pathogenicity of mastitis. *P. aeruginosa* is highly resistance to antibiotic. This may be due to presence of antiobtic resistant gene as *blaVIM*, and also presence of virulence genes, mutant and miss use of antibiotics.

4- Strict antibiotic policy, establishment of infection control programs, and strict hygienic measures should be applied in dairy farms, which will help to lower the incidence of resistance in *P. aeruginosa* and control this mastitis problem.

5- Owners should avoid miss use of antibiotics which cause increasing resistance of bacterial pathogens to the antibiotics and economic losses.

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