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Some studies on virulence factors of *Pseudomonas aeruginosa* isolated from mastitic buffalo milk and related antibiotic resistant genes

***Abeer, A.E. Mohamed and Ola A. Abd El-Fattah* ***

*Buffalo Diseases Research Department, **Giza Lab-
Animal Health Research Institute, Dokki,. Agricultural Research Center (ARC), Egypt

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Abstract

Bacteria causing mastitis in buffalo were isolated from 134 buffalo milk samples in dairy farms at Giza Governorate. This study showed that *Staphylococcus aureus* was the most prevalent causative agents for bovine mastitis (42.54%) followed by *Streptococcus* spp. (26.87%) and *Escherichia coli* (24.63%), *Pseudomonas aeruginosa* (8.96%) and *Klebsiella* spp.(1.49%), while the most frequent mixed infection was *S.aureus* and *E.coli* (11.94%). *Pseudomonas aeruginosa* isolates were examined for susceptibility test against some antibiotics, detection of different virulent genes (*opr*L, *las*I, and *exo*S) and some antibiotic resistant genes **(***arr* and *bla*TEM) by polymerase chain reaction (PCR) technique. Results of this study showed all isolates produced haemolytic activity on blood agar plates, 9 (75%) isolates showed lecithinase enzyme activity on egg yolk agar media and 7 (58.33%) were biofilm producers as per Congo red agar method. The antibiotic sensitivity test was performed by disc diffusion methods, the highest resistance was found to ampicillin (83.33%), cefotaxime (75%) and erythromycin (75%) followed by ceftazidime (66.67%), amikacin (66.67%), tetracycline (66.67%), imipenem (58.33%), cephalothin (58.33%), gentamicin (58.33%) and tobramycin (58.33%), while, highly sensitive to ciprofloxacin (100%), followed by norfloxacin(83.33%), levofloxacin (75%) and colistin (66.67%). Results of PCR technique showed that virulent genes including *exo*S and *opr*L genes were amplified from all (5) tested isolates, while 2 of isolates harbored *las*I gene. Also all *P. aeruginosa* isolates showed positive amplification of *bla*TEM and *arr* antibiotic resistant genes.

Keyword :*Pseudomonas aeruginosa , virulence factor genes, buffalo, PCR.*

Introduction

Bovine mastitis continues to be a complex disease associated with significant economic loss in dairy industries worldwide (**Pang *et al.,* 2019**). *Pseudomonas aeruginosa* is an opportunistic pathogen responsible for causing a wide variety of acute and chronic infections with significant levels of morbidity and mortality. The presence of unsanitary housing and bedding conditions can contribute to occasional outbreaks of *P. aeruginosa* infections. These infections are very hard to eradicate because of the expression of numerous virulence factors and the intrinsic resistance against antibiotics **(Malhotra *et al.,* 2019).**

P. aeruginosa is one of the common cause of mastitis **(Mcvey *et al.,* 2013),** this bacterium present a difficult challenge, as it tends to protect itself from antibiotics and white blood cells in layers of slim (**Morita *et al.,* 2014**).

The resistance to antimicrobial drugs has increased in recent years due to the wrong use of antibiotics which led to resistance to actually used antimicrobial agent is of concern to public health officials**.** Also reinfection is also common, so control of *P. aeruginosa* mastitis outbreaks generally requires culling of chronically infected animals, as well as identification and elimination of the source of organisms.

A number of virulence factors enable *P.aeruginosa* to adhere to tissue surfaces to damage tissue for dissemination and nutrition supply and to increase its survival rate, some factors help colonization, whereas others aid in bacterial invasion (**Deepak *et al.,* 2013)**. *P. aeruginosa* produces an extracellular toxins, these toxins include haemolysin, lecithinase, protease, elastase, collagenase enzymes, exotoxin A and exoenzyme S (**Foulkes *et al.,* 2019**). Some of these toxic effect or proteins are injected into the cytosol of host cells through the type III secretion system (TTSS) and are involved in the disruption of epithelial surface and cell death. These TTSS proteins include cytotoxin (*Exo*U), ADP-ribosylating enzymes (*Exo*S and *Exo*T) and adenylcyclase (*Exo*Y). ExoT and ExoY are involved in adhesion, phagocytosis and systemic spread of the bacterial cells together with *Exo*S. *Exo*S and *Exo*U are known to determine specific patterns of pathogenesis (**Engel and Balachandran, 2009**), where *exo*S a bifunctional enzyme induces apoptosis of epithelial cells and macrophages and inhibits macrophages phagocytic activity, so these TTSS-expressing strains are capable of increasing the severity of disease (**Hasannejad-Bibalan *et al.*, 2020)**. Exoenzyme S (*exo*S) considers one of major fatal weapons which causes significant tissue damage in lung, burn and wound infections and remain associated with mastitis infection in bovine **(Narayanan, 2013).**

Usually, antibiotics are effective in treating bacterial infection; however, *P. aeruginosa* is notorious for its resistance to multiple antibiotics. Because of its low outer membrane permeability **(Pang *et al.,* 2019)**, intrinsic or induced expression of efflux pumps and b-lactamase production*, P. aeruginosa* is uniformly resistant to a wide range of antimicrobials including penicillin, first and second generation cephalosporins, and erythromycin and is also often resistant to chloramphenicol and tetracycline **(Hwang and Yoon, 2019),** this reflected the great hazard to general health of public **(Badr *et al.,* 2020).** In addition to these intrinsic resistances, *P. aeruginosa* can also acquire resistance through horizontal transfer of genetic elements that carry resistance genes as well as chromosomal mutations. So further studies focus on better administration of the existing antibiotic program **(Kumari *et al.,* 2019).**

The present work was designed to highlight on *Pseudomonas aeruginosa* as one of the common cause of mastitis in buffalo and detection of some virulence genes responsible for pathogenesis of *P. aeruginosa* (*opr*L , *las*I and *exo*S) using PCR and to find out the drug resistant genes (*bla*TEM and *arr*) and antibiotic susceptibility patterns.

Materials and Methods

Samples: A total of 134 mastitic milk samples were collected from 75 examined buffaloes in dairy farms at Giza Governorate that showed swollen udder quarters, also, milk showed flakes or clots. Samples were collected under complete hygienic measures in sterile bottles and transported to the lab as quick as possible for bacteriological examination in a sterile ice box **(Quinn *et al*., 2002)**.

Isolation and identification (Carter and Cole, 1990):

Samples were inoculated into sterile nutrient broth tubes and then incubated aerobically for 24 hrs at 37°C. A loopful from these incubated tubes was streaked onto MacConkey agar, sheep blood agar, Barid-Barker medium, EMB, Edward medium and pseudomonas agar medium supplied with Cetramide 20% and Nalidixic acid 1.5%. The inoculated plates were incubated aerobically at 37°C for 24-48 hours. Suspected colonies were subjected to morphological and biochemical characters according to **Cruickshank and McCarteney (1996)**.

Determination of virulence markers of *P.aeruginosa*:

1-Congo red (CR) binding test: All isolates were tested for their growth status on Congo red medium. The reaction was best seen after 18, 24, 48 and 72 h of incubation at 37°C and was then left at room temperature for an additional 2 days (not to exceed 4 days). Black brown colonies were considered positive and different intensities in the dye uptake were expressed as +, ++ and +++. Congo red negative (CR-) isolates did not bind the dye and appeared as white colonies, according to **Berkhoff and Vinal (1986)**.

2-Hemolysis assay: Hemolysis was determined by streaking isolates onto blood agar containing 5% sheep blood and incubated at 37°C for 24 h. A visible zone of haemolysis around growing colonies **(Baily and Scott, 1978)**.

3-Lecithinase production: On egg yolk agar medium, colonies surrounded with wide zone of opalescence which indicate lecithinase production **(Krieg and Holt, 1984)**.

Antibacterial sensitivity testing:
The disk diffusion technique was applied according to **Fingold and Martin (1982).** Antibiotic discs are ampicillin (10 µg), imipenem (10µg), cephalothin (30µg), cefotaxime (30 µg), ceftazidime (30 µg), amikacin (30µg), gentamycin (10µg), tobramycin (10µg), tetracycline (30µg), colistin (10 µg), erythromycin (10µg), ciprofloxacin (5µg), norfloxacin (10µg) and levofloxacin (5 µg). The interpretation of inhibition zones of test culture was according to the **Clinical and Laboratory Standards Institute (CLSI, 2018)**.

Molecular examination of (5) *Pseudomonas aeruginosa*: According to **Sambrook *et al.* (1989),** PCR assays were developed with specific primers for the detection of different virulent genes (*opr*L, *las*I, and *exo*S) of (5) *P. aeruginosa* and some antibiotic genes **(***arr* and *bla*TEM).
DNA extraction: DNA extraction from samples 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 incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: Primers used were supplied from Metabion (Germany) are listed in table (1).

Amplification: Primers were utilized in a 25-µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 p mol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

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 PCR products were loaded in each gel slot. Generuler 100 bp ladder (Fermentas, Thermo Scientific, Germany) and gel pilot 100 bp ladder (Qiagen, Gmbh, 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 (1). Primers sequences, target genes, amplicon sizes and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
arr	AGCG-CATCACCCCCA GCAAC CGCCAAGTGCG AGCCACTGA	686 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Jones *et al.* (2013)**
oprL	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Xu *et al.* (2004)**
blaTEM	ATCAGCAA-TAAACCAGC CCCCGAA-GAACGTTTTC	516 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Colom *et al.* (2003)**
lasI	ATGATCG-TACAAATTGGT CGGC GTCATGAAAC-CGCCAGTCG	606 bp	94˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Bratu *et al.* (2006)**
exoS	GCGAGGTCAG-CAGAGTATCG TTCGGCGTCAC-TGTGGATGC	118 bp	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Winstanley *et al.* (2005)**

Results and Discussion

The incidence of each bacterial species isolated and recognized from clinical mastitic milk samples of buffaloes are presented in Table (2), revealed that 134 clinical mastitic milk samples obtained from 75 mastitic buffaloes and *Staphylococcus aureus* was the most prevalent causative agents for bovine mastitis (42.54%) followed by *Streptococcus* spp. (26.87%) and *Escherichia coli* (24.63%), while the most frequent mixed infection was *S.aureus* and *E.coli* (11.94%), on contrary 16 (11.94%) were negative for bacteriological examination. Genus *staphylococcus* was considered the main etiological agent, being of great epidemiological importance in buffalo mastitis **(Vásquez-García *et al.,* 2017)**. Our findings are nearly similar to results regarding the incidence of individual bacterial species recorded by **Hameed *et al.* (2008),** who stated that, the most predominant species recorded in mastitic buffaloes was *S. aureus* and its dominancy was noted (53.85%), followed by *Streptococcus* spp. (26.9%) and *E. coli* (15.8%). Also **Ali *et al.* (2008)** reported the percentage of bacterial growth obtained from mastitic buffalo's milk samples were (49.53%) for *S.aureus,* (26.9%) for *Streptococcus* spp*.,* while lower incidence (15.8%) for *E.coli.* On the other hand, **Park *et al.* (2007)** determined lower incidence of various bacterial species in bovine mastitis during their study, where they recorded the incidence of *S. aureus* in 1.59%, *Streptococcus* spp. in 0.83%, *E. coli* in 1.56% and *P. aeruginosa* in 1.59% samples. Incidence of mixed infection was reported by **Tarek *et al.* (2006**) who recorded that the most predominant isolates in buffalo milk were *S.aureus* +other *Streptococcus* spp. + *E.coli.* In this survey, 86 (64.18%) were found with pure infection while 32 (23.88%) were determined with mixed infections. The data regarding the incidence of pure and mixed infections in clinical mastitic milk samples are in agreement to that of **Baloch *et al.* (2011)** who recorded that 78.57% milk samples with pure infection while 21.42% samples with mixed infections from mastitic buffalo's milk samples, soit is clear from the present investigation on clinical mastitisin buffaloes that pure infection was common as compared to mixed infections. This variation may be due to seasons, manage mental conditions at the farm and difference in sample handling in the laboratory.

Table (2). Occurrence of bacterial species recovered from (134) clinical mastitic milk samples of (75) mastiticbuffaloes.

Isolated bacteria		No.	%	No.	%
Single infection	***S.aureus***	39	29.10	86	64.18
	***Streptococcus* spp.**	22	16.42		
	E.coli	17	12.69		
	P.aeruginosa	6	4.48		
	***Klebsiella* spp.**	2	1.49		
Mixed infection	***S.aureus* + *E.coli***	16	11.94	32	23.88
	Streptococcus* spp.+ *P.aeruginosa	4	2.99		
	S.aureus* + *P.aeruginosa	2	1.49		
	***Coagulase Negatine Staphylococcus* spp. + *Streptococcus* spp.**	10	7.46		
Negative for bacteriological examination		16	11.94	16	11.94
Total number of samples		134	100	134	100

Table (3) observed that, out of 134 mastitic milk samples, 12 (8.96%) samples were positive for *Pseudomonas aeruginosa*. Incidence of *P. aeruginosa* isolated from mastitic buffaloes observed by **Sharma and Sindhu (2007)** in the State of Haryana (India) and that obtained by **Vásquez-García *et al.* (2017)** from 4 dairy farms in Ŝao Paulo State, Brazil, are very close to the incidence noted in the present investigation. While lower results (1.42%) and (3%) from mastitic buffaloes were obtained by **Baloch *et al*. (2011) and Kavitha *et al*. (2016).** These differences may be attributed to environmental aspects such as poor hygiene, poor husbandry, overcrowding, bad ventilation, poor milking technique and malfunction of milking machines in dairy farms. Although the incidence of *P.aeruginosa* was low, it was important to highlight on it, as it is very difficult to be diagnosed, which may lead to recurring chronic clinical infections resulting into irreparable damage of mammary gland of infected buffalo, thus culling or elimination cost is also involved in the list of economic losses **(Raboisson *et al.*, 2020)**. Signs of mastitis caused by *P.aeruginosa* may include infections that are totally resistant to antibiotic therapy, toxic mastitis and multiple quarter infections.

Table (3): Occurrence of *P.aeruginosa* isolated from buffalo's mastitic milk samples and its virulence factors.

Samples	Number of samples		Incidence of virulence factors of (12)*P.aeruginosa*					
			Haemolysin		Lecithinase		Congo red	
	No. of positive *P.aeruginosa*	%	No.	%	No.	%	No.	%
Milk samples	12	8.96	12	100	9	75	7	58.33

All isolates were haemolytic on blood agar (Table 3), these results coincident with results obtained by **Ammar *et al.* (2016).** While, **Younis *et al.* (2015)** reported that 80% of *P. aeruginosa* strains produced haemolysin enzyme on blood agar media. *P. aeruginosa* produces many factors that may contribute to its virulence as β-haemolysin, which is a sphinomyelinase that is highly active against bovine erythrocytes, where it had the cytotoxic effect and causes damage of bovine mammary epithelial cell **(Wang *et al.*, 2011)**.

Lecithinase enzyme and its activity was detected, they act synergistically to break down lipids and lecithin where in Table (3), 9 (75%) of *P. aeruginosa* strains recorded to be lecithinase positive, this was nearly similar results obtained by **Younis *et al.* (2015)**. Lecithinase producing bacteria act on lecithin and produce phosphorous and choline with precipitation of fat. *P.aeruginosa* found to produce 2 phospholipases or phospholecithinases which are hemolytic and act as an important virulent factor. **Bharadwaj and Gopinath (2016)** concluded that, isolates were found to exhibit both lipase and lecithinase activity may play a crucial role in causing infections.

Results in Table (3) showed that, out of 12 *P. aeruginosa* isolates, 7 (58.33 %) were biofilm producers as per Congo red agar method. Very high rates of biofilm production in comparison to our study were reported by **Rewatkar and Wadher (2013)** among the strains of *P. aeruginosa* (90%) by Congo red agar method, while only 13 (15.29%) were biofilm producers as per Congo red agar method reported by **Baniya *et al.* (2017)**. **Ghafoor *et al.* (2011)** used the Congo red binding assay to assess the ability of *P. aeruginosa* mutants to produce Pel polysaccharide (an exopolysaccharides that contribute to the formation of biofilms in this organism). Although the specificity of this Congo red binding assay has not been established, it has been widely used as an indicator of Pel production **(Vasseur *et al.*, 2007).**

Table (4). Antibacterial sensitivity test among *P. aeruginosa* isolates.

Antimicrobial agents	Sensitive		Resistant	
	No.	%	No.	%
β-lactams (penicillin)				
Ampicillin (AM, 10µg)	2	16.67	10	83.33
β-lactams (Carbapenems)				
Imipenem (IPM, 10µg)	5	41.67	7	58.33
β-lactams (Cephalosporine)				
Cephalothin (KF, 30µg)	5	41.67	7	58.33
Cefotaxime (CTX; 30 µg)	3	25	9	75
Ceftazidime (CAZ, 30 µg)	4	33.33	8	66.67
Aminoglycosides				
Amikacin (KA,30µg)	4	33.33	8	66.67
Gentamicin (CN, 10µg)	5	41.67	7	58.33
Tobramycin (TOB,10µg)	5	41.67	7	58.33
Tetracyclin				
Tetracycline (TE, 30µg)	4	33.33	8	66.67
Polypeptides (Polymyxins)				
Colistin (CT, 2µg)	8	66.67	4	33.33
Macrolides				
Erythromycin (E,10µg)	3	25	9	75
Flouroquinolone				
Ciprofloxacin (CIP, 5µg)	12	100	-	-
Norfloxacin (NF, 10 µg)	10	83.33	2	16.67
Levofloxacin (LEV, 5 µg)	9	75	3	25

In the present study, the antibiogram sensitivity pattern of *P. aeruginosa* isolates was investigated and reported in Table (4). Antibiotic selection for treatment is often a great problem due to antibiotic resistance of *P. aeruginosa* to a broad range of antibiotics. The results of in vitro sensitivity testing of 12 isolates of *P. aeruginosa*, the highest resistance was found to ampicillin (83.33%), cefotaxime (75%) and erythromycin (75%) followed by ceftazidime (66.67%), amikacin (66.67%), tetracycline (66.67%), imipenem (58.33%), cephalothin (58.33%), gentamicin (58.33%) and tobramycin (58.33%), while, highly sensitive to Ciprofloxacin (100%), followed by norfloxacin (83.33%), levofloxacin (75%) and colistin (66.67%). All isolates were considered multi-drug-resistant (MDR) as they were intermediately or fully resistant to at least three different classes of antimicrobial agents (**Tavajjohi *et al*., 2011**). These findings agreed with the other previous study **(Dapgh *et al.,* 2019)** which illustrated that the organism is completely resistant to ampicillin, erythromycin, tetracycline and tobramycin but sensitive to ciprofloxacin and norfloxacin. Also**, Awad *et al.* (2017)** found isolates of *P. aeruginosa*, isolated from mastitic milk, were resistant to cefotaxime and ampicillin. These findings agreed with the other previous studies which illustrated that the organism is resistant to all used antibiotics except quinolones **(Liew *et al.,* 2019)**. On the other hand, data obtained by other studies (**Park *et al.*, 2014; Ghazy *et al* ., 2015**; **Tartor and El-naenaeey, 2016)** reported that ciprofloxacin showed higher activity against *P. aeruginosa* isolated from bovine mastitis, therefore in our study, ciprofloxacin considered the antibiotic of choice against *P. aeruginosa* strains isolated from mastitic buffaloes.

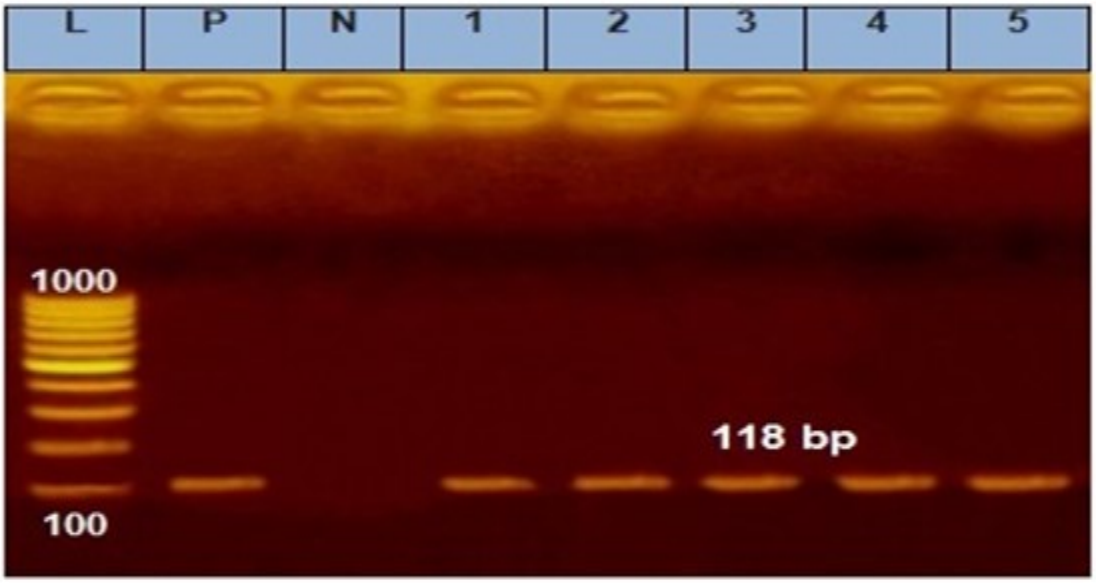

Figure (1). Agarose photo on electrophoresis of PCR assay represents detection of ***exoS*** gene in *P. aeruginosa* isolates. Lane L: DNA Ladder (100-1000 bp). Lane P: Positive control. Lane N: Negative control. Lanes 1-5: *P. aeruginosa* isolates showing presence of ***exoS*** gene.

PCR was used on (5) strains for detection of *P. aeruginosa* exoenzyme S (*exo*S gene). The obtained results in figure (1) demonstrated that all strains were positive for *exo*S gene. Moreover, our results match with that obtained by **Younis *et al.* (2015), Neama (2017) and Banerjee *et al*. (2017)** who mentioned that, *P.aeruginosa* strains isolated from mastitic milk were positive for *exo*S production. *ExoS* gene is translated into protein products (toxin) related to type III secretion systems (TTSS). These TTSS-expressing strains are capable of increasing the severity of disease and have the ability to affect the SCCs and milk components by damaging mammary epithelial cells in the case of an intramammary infection. However, only a few studies have investigated the TTSS of *P. aeruginosa* isolated from bovine milk **(Szmolka *et al.,* 2012).**

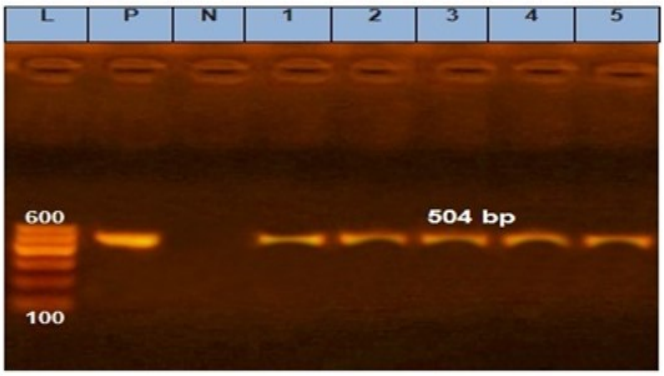

Figure (2). Agarose photo on electrophoresis of PCR assay represents detection of *opr*L gene in *P. aeruginosa* isolates. Lane L: DNA Ladder (100-600 bp). Lane P: Positive control. Lane N: Negative control. Lanes 1-5: *P. aeruginosa* isolates showing presence of *opr*L gene.

Figure (2) showed that, all strains were positive for *opr*L (outer membrane protein) gene and these results were agreed with that obtained by **Mohammed *et al.* (2015)** and **Tartor and El-naenaeey (2016),** while **Neama (2017)** stated that, 50% of *P.aeruginosa* strains from different fields in Al-Diwanyiaprovinc were positive for *opr*L gene. *Opr*L is an outer membrane lipoprotein which which is essential for the invasion of epithelial cells and implicated in efflux transport systems affecting cell permeability so there is a strong relationship between the detection of oprL and phenotypic antibiotic resistance that reported by **Lavenir *et al.*, (2007).** PCR targeting the *opr*L gene, is a useful technique in the detection of *P. aeruginosa* **(Silva *et al.*, 2014)** and the important reason for antimicrobial resistance to *P.aeruginosa* was impermeability which belongs to the outer membrane lipoprotein (oprL gene) that implicated in efflux transport systems and affects cell permeability **(De Vos *et al*., 1997).**

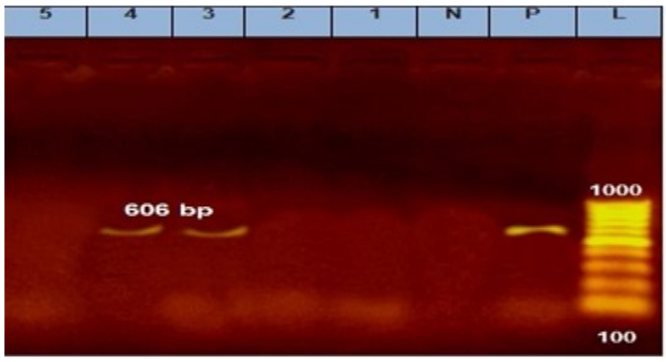

Figure (3). Agarose photo on electrophoresis of PCR assay represents detection of *las*I gene in *P. aeruginosa* isolates. Lane L DNA Ladder (100-1000 bp). Lane P: Positive control. Lane N: Negative control. Lanes 3 and 4: *P. aeruginosa* isolates showing presence of *las*I gene.

The observed results in figure (3) demonstrated that 2 (40%) strains were positive for *las*I gene. These findings are in close relation with that reported by **Mohammed *et al*. (2015).** Our percentage of *las*I gene was less than that was detected by **Shahat *et al.* (2019)** who succeeded in the amplification of *las*I gene in 71.42% all isolates of *P.aeruginosa*, while **Alshalah *et al.,* (2017)** detect the gene in all clinical isolates of *P.aeruginosa*. In the las system, the autoinducer synthase, *las*I triggers the *las*R-encoded transcriptional activator to induce virulence genes **(Habeeb *et al.,* 2012)**. This system is also implicated in the formation and development of the biofilm. The expression of *las* system relies on environmental stimuli such as iron, osmolarity, nitrogen and oxygen availability. These genes are expressed only when a high cell density is achieved. This controlled transcription of genes, in the course of a rapid adaptation to environmental challenge, is essential for bacterial survival and for the promotion of chronic infection. In addition to, **Nikbin *et al*. (2012)** explained that, the possession of *P. aeruginosa* for several virulence genes make it a reason for various levels of virulence and pathogenicity.

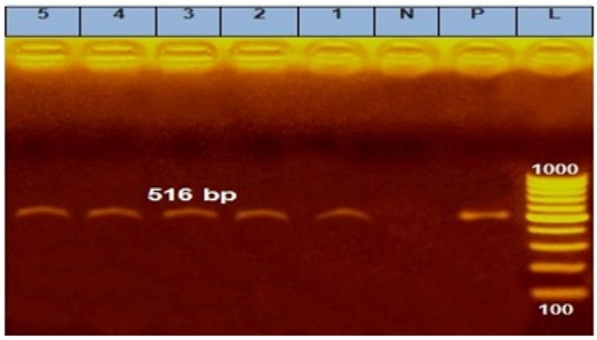

Figure (4). Agarose photo on electrophoresis of PCR assay represents detection of *bla*TEM gene in *P. aeruginosa* isolates. Lane L: DNA Ladder (100-1000 bp). Lane P: Positive control. Lane N: Negative control. Lanes 1-5: *P. aeruginosa* isolates showing presence of *bla*TEM gene.

Results obtained in figure (4) revealed positive amplification of *bla*TEM gene in all *P. aeruginosa* isolates which showing multidrug resistance. These results were coparable by that obtained by **Bokaeian *et al.* (2014)** that all isolates of *P. aeruginosa* resist to at least one of the antibiotics ceftazidime, ceftriaxone, cefotaxime or aztreonam showing amplified *bla*TEM gene. Also the correlation between β-lactamase production and the presence of antibiotic resistance *bla*TEM gene in *P. aeruginosa* isolates were discussed by previous study (**Rafiee *et al*., 2014**), Resistance to beta-lactam antibiotics is multi-factorial but is mediated mainly by inactivating enzymes called beta-lactamases. These enzymes cleave the amide bond of the beta-lactam ring causing antibiotic inactivation. Resistance of *P. aeruginosa* strains to broad-spectrum cephalosporins may be mediated by extended-spectrum β-lactamases (ESBLs) which allow the bacterium to tolerate against extended–spectrum cephalosporins, such as cefotaxime, ceftriaxone and ceftazidime. ESBLs are undergoing continuous mutations, causing the progression of new enzymes. TEM is one of the major genetic groups of ESBLs amongst clinically important Gram-negative bacteria **(Paterson and Bonomo, 2005)**.

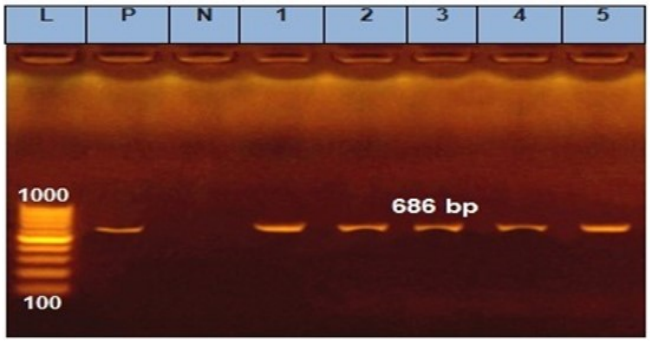

Figure (5). Agarose photo on electrophoresis of PCR assay represents detection of ***arr*** gene in *P. aeruginosa* isolates. Lane L: DNA Ladder (100-1000 bp). Lane P: Positive control. Lane N: Negative control. Lanes 1-5: *P. aeruginosa* isolates showing presence of ***arr*** gene.

Figure (5) showed that, all strains were positive for *arr* (aminoglycoside response regulator) gene. Aminoglycoside uptake and subsequent action within bacterial cells is a complex process that involves Lipopolysaccharides (LPS) binding and outer-membrane permeation, cytoplasmic membrane traversal driven by membrane potential, and ribosome disruption, leading to the production of membrane-damaging mistranslated polypeptides **(Krahn*et al*., 2012)**. In *P. aeruginosa*, a gene, which we designated aminoglycoside response regulator (*arr*), was essential for induction biofilm formation in *P. aeruginosa* and contributed to biofilm-specific aminoglycoside resistance. The *arr* gene is predicted to encoding inner-membrane phosphodiesterase whose substrate is cyclic di-guanosine monophosphate (c-di-GMP), a bacterial second messenger that regulates cell surface adhesiveness **(D'Argenio and Miller, 2004).**

Conclusion and Recommendations

P. aeruginosa could be implicated in mastitic buffalo's milk, and the isolates had numerous virulence markers which play an important role in certain types of infection as well as showed high resistance to commonly used antibiotics. ciprofloxacin is the best drug of choice for the treatment of *P. aeruginosa* infection in this small scale study. A strict antibiotic policy and establishment of infection control programs will help to lower the incidence of resistance in *P. aeruginosa*. The animal's environment should be as clean and dry as possible with no access to manure, mud and pools of stagnant water, also calving area must be clean. Post milking teat dipping with a germicidal dip is recommended. Animals should receive diets which are supplemented with vitamin E and selenium or immunomodulators to reduce incidence of mastitis caused by environmental pathogens.

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