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Isolation and identification of pathogenic *Pseudomonas aeruginosa* from cow mastitis and detect some virulence factor using specific genetic marker

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Abstract:
This study performed to investigate distribution *tox A* و *exo S* gene among *Pseudomonas aeruginosa* isolated from milk in cows with mastitis using PCR technique.
82 sample from cow wound suffer from different region of Thi Qar city contaminated from period 12 july 2017-february 2018, *Pseudomonas aeruginosa* were isolated using cultured, microscopically and biochemical characteristics. The result showed that 35 isolate blong to *Pseudomonas aeruginosa* these tested for their antibiotic sensitivity the isolate showed (100%) resistance to Ampicillin, oxacillin and (71.42%,57.14%,37.14%) resisitant to cefotamine, impenem, ticarcillin) respectively. The ability of isolate to produce virulence factor also tested all isolate showed the ability to produce Haemolysin, phospholipase and only 54.28% of isolate can produce lecithinase and only 34.28% can produce alkaline protease.
PCR results showed that all 35(100 %) *Pseudomonas aeruginosa* was found carry the virulence gene *TxoA* and 19 (54.23%) found carry the virulence gene *ExoS.*
Key word: *Pseudomonas aeruginosa, TxoA, ExoS*.

Introduction:

Mastitis is one of the most economically important disease affecting the worldwide dairy industry with a huge financial loss (Tenhagen *et al*.,2006; Roesch.,2006) *Pseudomonas aeruginosa* one of many major pathogens associated with mastitis especially subclinical mastitis. (singh *et al*.,2005)
Pseudomonas aeruginosa is a non-fermentative, aerobic, Gram-negative rod that normally lives in moist environments. It has minimal nutrition requirements while being able to use several organic compounds for growth. This metabolic versatility contributes to a broad ecological adaptability and distribution, and reflects a genome of larger size and complexity compared with that of many other bacterial species (Goldberg,2000; Stover,2000).
Pseudomonas aeruginosa, characterized by it's high genetic plasticity and ability to adapting various environments. These bacterial species are frequently isolate from soil and water or colonize in humans, insect's plants and animals. The Pseudomonas *aeruginosa* may be caused food poisoning and also caused mastitis in cattle., the bacterium and has many virulence factors. (Edit et al.,2016; Fadhil *et al*.,2016; Streeter *et al*., 2016; Mitov *et al*., 2010)

The pathogenicity of *Pseudomonas aeruginosa* is may be because these bacteria possess many pathogenic factors, one of them exotoxin A(toxA) and exoenzyme S (exoS) these two are the major fatal weapon., associated with mastitis (especially subclinical) infection (Nrayanan *et al*.,2010) in bovines.

For that the aim of this study is to detection and characterization of exotoxin A(toxA) and exoenzyme S (exoS) in *Pseudomonas aeruginosa* isolates from milk sample isolated from bovines with mastitis from different region of ThiQar city.

Material and methods:

Sample collection:

The study was carried out through 12 July 2017 to 1 February 2018 in this study (82) samples collected from cattle with mastitis different region of Thi Qar Government, the California test is used with clinical sign to determine somatic cell amount in milk. milk from quarter that react to the CMT indicates intrammamary infection (Sears, 2002)

Isolation of *Pseudomonas aeruginosa*:

After milk samples were collected it transfer to the laboratory, the samples were identified based on the morphological and microscopically characteristics of the colonies culture media as well as biochemical tests

following the methods described in McFadden., (2000) and molecular identification.

Antibiotic sensitivity test:

Antibiotic susceptibility test was performed using disk diffusion method on Mueller Hinton Agar according to (Vandepitte *et al*., 2003) Imipenem (IPM) (10mg), cefotaxim(CTX)30 mg, Ticarcillin (Carboxypencillin (75mg), Oxacillin (30 mg) The results were recorded as resistant or susceptible by measuring The inhibition zone by ruler on the under-surface of the plate without opening the lid, and the result was compared with the standard diameter of inhibition zones for each antibiotic according to (NCCLS).

Determination of some virulence factors:

The *Pseudomonas aeruginosa* isolates in present study were test for their ability to produce gelatinase, hemolysin, Alkaline protease and lecithinase following the method described in (Muerer 2003; De La Maza et *al*.,1997; Collee et al., 1996).

PCR reaction: -

Bacterial DNA extraction and PCR Method:

1-DNA extraction: The bacterial isolates nucleic acid extraction by using commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA. depend on the manufacturing instructions

2-Nanodrop: The extracted DNA was estimated by nanodrop device at 260/280nm, and then kept at deep freezer for used in PCR method.

3-Primer: specific primer encodes used to detect the *Exos* and *ToxA* genes which code for exoenzyme S and exotoxin according to Khan and Cerniglia)1994) and Mitove *et al* (2010) as appear in table (1).

Table NO.1 specific primer encode used to detect the exoS and 2s322

Primer name	Sequence primer	The expected volume	Company
tox A	F:GACAACGCCCTCAGCATCACCAGC R:CGCTGGCCCATTCGCTCCAGCGCT	396	Alpha DNA
exoS	F: CTT GAA GGG ACT CGA CAA GG R: TTC AGG TCC GCG TAG TGA AT	504	Alpha DNA

tox A: Exotoxin, exoS: exoenzyme

4- (PCR master mixture) preparation: Master reaction mix prepared by mixed reaction component in Eppendorff tube (1.5 ml) showed in table (2).

Table NO. (2) Master reaction mix reaction component

Componants	Volume
D.W.	15.7µl
PCRBuffer 1X	5µl
dNTPs	0.5 µl
ETA-F	0.8 µl
ETA-R	0.7µl
Taq DNA Polymerase (1.5U)	0.3 µl
DNA	2.0 µl
Volume	25 µl

5- PCR thermocycler conditions:

The amplification according to Khan and Cerniglia (1994) as follow: denaturation at 94°C for 60 second ,60 secs at 68° C for annealing ,60 secs at 72° C for extension of connected primer and final extension at 72° C for 7min.

After amplification, 10 µl sample was subjected to electrophoresis with molecular size marker for 1-1.5 hours, stained with ethidium bromide (Sigma) and detected by UV transillumination.

For detection of *exoS* same step were performed except the differences in quantity added from primer and water and in thermal cycler program explain in table (3) (Mitov *et al* ,2010)

Table NO. (3) The material and conditions used for the PCR namplification of *exoenzyme S* virulence genes in *P. aeruginosa* strains.

Gene	Volume of Water added	Amount of primer F	Amont of primer R	Cycling program		
				Temperature	Time	No. of cycle
Exo S	15.4micro	1.0 micro	0.8 micro	94	5 min	1
				94	35 second	30
				60	45 second	
				72	45 second	
				72	7 min	1

Results:

Isolation and Identification of *Pseudomonas aeruginosa*:

Out of 82 mastitic sample from cattle, 35 that represent (42.6%) *Pseudomonas aeruginosa* isolates were recovered.

The colony appears circular mucoid smooth with sweet grape odor on nutrient agar., gave beta hemolysis on blood agar but not fermented lactose sugar on MaCconky., the suspected colony was re culture to do more tests, the *p. aeruginosa* isolate was depending on different biochemical test.

The *P. aeruginosa* isolated in this study showed high resistance to oxacillin, ampicillin, cefotamine (100%,100%,71.14%) and moderate resistance to impenem (57.14%) and less resistance in (35.71 %) to ticarcillin table (4).

Table (4) the resistance of *P. aeuroginosa* antibiotics aganists some antibiotics

antbiotics / **No.**	**Oxacillin**	**ampicillin**	**cefotamine**	**impenem**	**Ticarcillin**
resistance isolate	**35(100%)**	**35(100%)**	**25(71.42%)**	**20(57.14%)**	**13(37.14%)**
sensitive isolate	**0**	**0**	**10(28.57%)**	**15(42.8%)**	**22(62.85.28%)**

Determination of some virulence factor produce by *P. aeuroginosa*:

Distribution of Virulence factors in clinical isolates of *Pseudomonas aeruginosa* showed all isolates were (100%) positive for producing hemolysin and Gelatinase, 54.28% Positivity for Lecithinase and 34.28% were positive for Alkaline protease (Table 5).

Table (5) the production of some virulence factor by by *P. aeuroginosa*

Results	**Virulence factors**

	Hemolysin	Gelatinase	Lecithinase	Alkaline protease
Positive	**35(100%)**	**35(100%)**	**19(54.28%)**	**12(34.28%)**
Negative	**0**	**0**	**16(45.75)**	**25(71.42%)**

Clinical strains were screened for the prevalence of different virulence genes of *P. aeruginosa*. 100% prevalence of *toxA* gene was found in all analyzed strains (Figure 1).and over 80% of isolates were were positive for the presence of *exoS* gene, giving amplification at 60°C at 155 bp (Figure 2).

Table (6) the prevalence of of toxA gene exoS gene among the *p. aeruginosa* isolates

Virulance gene	positive	Negative
ToxA	**35 (100%)**	**0(0%)**
exoS	**31(88.57%)**	**4(11.42%)**

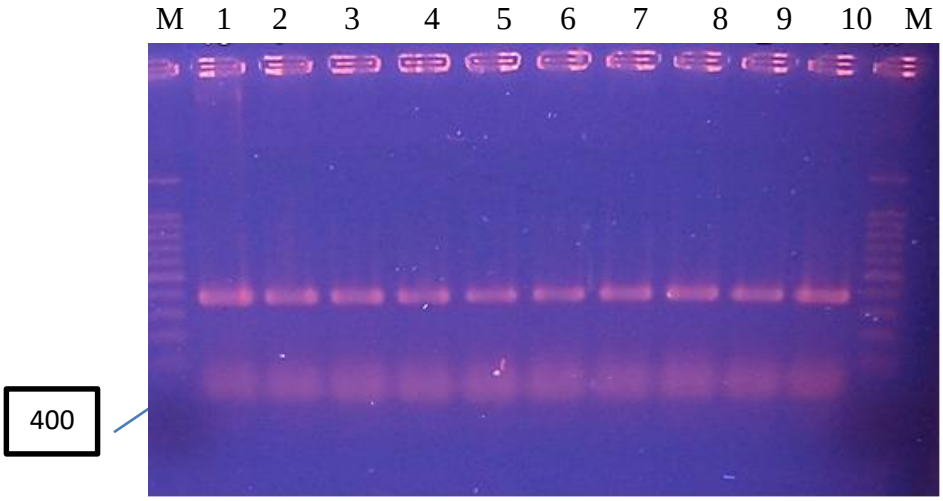

Figure (1): reveal some positive results of exotoxinA toxA gene in *P. aeruginosa* isolated in present study (lane 1 to 10)(M: 100bp DNA ladder)

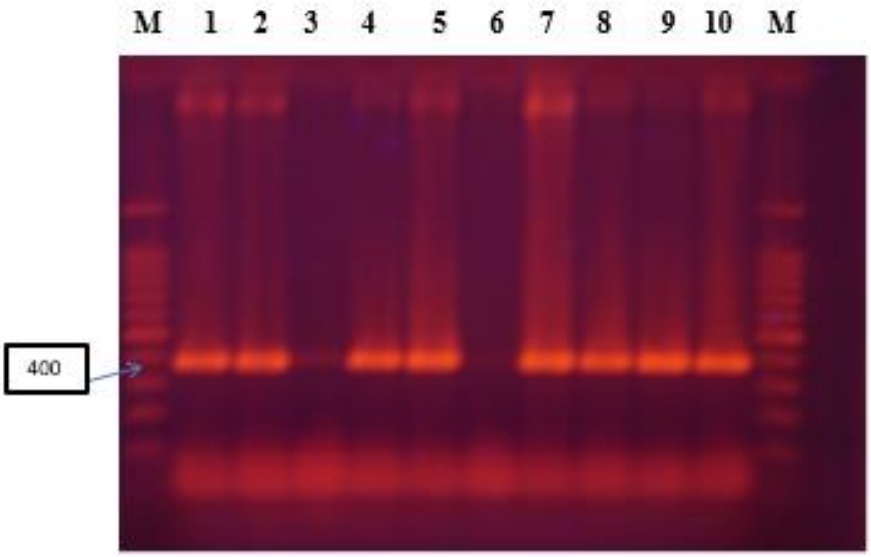

Figure (2) Some positive results of exotoxin exoS gene; M(100bp DNA ladder), Lane (1-2-3-4-6-7-9-10) some positive isolates at 400 bp for exoS gene in *Pseudomonas aeruginosa* isolates lane (5-8) negative results.

Discussion:

All isolates were oxidase and catalase positive after growing on MacConkey, blood and nutrient agars with observed pyocyanin pigment in 84% of isolates. Al-Shamaa *et al*., (2011) revealed that 80% of *P. aeruginsa* produced pyocyanin within 24 hrs when cultured in media contained cetrimide and 4-5 days in modified MacConkey agar and broth. Most isolates have β-hemolytic- mucoid colonies and grapelike odor, the later is due to the production of 2-aminoacetophenon. Rod shaped cells with pink color appeared under microscope when stained with Gram stain. These are a presumptive identification according to (Forbes *et al*., 2007, Kiser *et al*., 2011 and Todar 2011).

Laboratory findings and clinical history offered that *P. aeruginosa* contaminated teat wipes were the motive of the mastitis. The probable sequence of events was that *P. aeruginosa* pollution wipes were rubbed on the teat, and the bacteria deposited at the teat opening were subsequently lead into the teat lumen by the nozzle (Quinn *et al*.,2002).

Percentage of *pseudomonas aureogenosa* that isolated from cow mastitis in this study was (42%) these results agreed with (Ghassan khudhair ismaeel and Hassan Hachim Naser., 2016) also this was near the presentage found by (Neamah A.A.,2017) who found that p. in 26 % of milk sample.

Resistance to b-lactam antibiotics in *p. aeruginosa* is an endure problem in the treatment of *pseudomonas aureogenosa* infection (Cavallo et al.,2000). In this study highest resistance attribution to oxacillin agreement with (Ashraf et al.,2008), The prevalence of resistance IPM impenem (55%) in P. aeruginosa in thi Qar city differs across Iranian studies (21%) (Yousefi et al., 2010) and (22%) (Enayatollah et al.,2012) in Kurdistan Province which may be because of differences in geographic regions.

Different in the values; the prevalence of *pseudomonas areugenosa* and percentage of virulence factors genes depend on several causes including nature of places, immune status of patients, degree of contamination and type and virulence of strain (Khan and Cerniglia.,1994).

Pseudomonas aeruginosa produces many of virulence factors (Morales-Espinosa et *al*.,2012) whose expression is arranged by different systems (Empel et al., 2007) A recent studies reveal *P. aeruginosa* is

most frequent pathogen that formed many of virulence factors example ToxA, exoA, oprL and oprI genes (Vincent.,2000) (Rhonda et al., 2012 (Zourob et al.,2008).

Results of *toxA* gene prevalence revealed that total 35/35(100%) of the isolates while exo s was found in 31/35(88.57%) of the isolates.

The presence of toxic gene like *tox*A (100%) was more than that of *exo*S (88.57%) among all 35 positive *P. aeruginosa* isolates were also reported by Lanotte *et al.* (Lanotte *et al*.,2004) who detected *tox*A (approximately 100%) and *exo*S (84.5%) genes in 162 *P. aeruginosa* isolates. The occurrence of "exotoxin A" (90-100%) and "exoenzyme S" (50-84%) in *P. aeruginosa* strains was also reported by Badr *et al.* (Badr *et al*., 2008), Nikbin *et al.*, (Nikbin *et al*., 2012) and Sharma *et al.* (Sharma *et al.*, 2004).

The finding of present study agreed with were (Neamah,2017) illustrated that this gene was reported in 100% of their isolates which is the same with our percentage. While he finds that the exos found in 75 % of mastitis *P. aeruginosa*.

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