Isolation of *Pseudomonas aeruginosa* from ewes milk of clinical and subclinical mastitis in Al-Diwaniyah city

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Abstract:

The aims of present study were to isolate *Pseudomonas aeruginosa* from clinical and subclinical mastitic milk of sheep in AL-Diwaniyah city/ Iraq .as well as to detect the susceptibility of isolated strains to antibiotics, and to evaluate the incidence of the infection among examined ewes. For this purpose ,seventy five (75) milk samples which classified as six samples and sixty nine samples from clinical and apparently healthy cows(subclinical) respectively were collected, and the subclinical samples were examined firstly by CMT ,then clinical samples and positive CMT milk samples examined bacteriologically .Total mastitis percentage 52 (69.3%) that included clinical mastitis 11.5% and subclinical mastitis 88.4%.

Pseudomonas aeruginosa were isolated (40.3%) ,which as 33.3% and 27.5% from clinical and subclinical mastitic milk samples .

The isolated *P. aeruginosa* produces characteristics colonies in selective agar(Cetrimid agar). The isolates produce β-hemolysis on blood agar and by Gram's staining, the morphology of isolated *P. aeruginosa* showed Gram-negative, medium rod shaped appearance. the isolates doesn't ferment lactose.

By discs diffusion methods ,the antibiotic sensitivity test of the isolates of *P. aeruginosa* was performed against to Amikacin, , Gentamicin , Ciprofloxacin ,Tobramicin and Cefazidime .The higher sensitivity was found with Ciprofloxacin (100%) and Gentamicin (50%) , but the highly resistance to Tobramicin (50%), Amikacin (25%) and Cefazidime (25%).

From the above results it may be concluded that mastitis of ewes may be caused by *P. aeruginosa* . Our results also revealed that Ciprofloxacin the best drug of choice for the treatment of *P. aeruginosa* mastitis infection in ewes.

Keywords: *Pseudomonas aeruginosa*, mastitis , susceptibility, antibiotic.

عزل الزوائف الزنجارية من حليب ألاغنام المصابة بالتهاب الضرع السريري وتحت السريري في مدينة الديوانية

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الخلاصة

هدفت الدراسة الى عزل الزوائف الزنجارية من ٧٥عينة حليب تتكون من ٦ عينات ماخوذة من اغنام مصابة بالتهاب الضرع السريري و ٦٩عينة جمعت من اغنام تبدو ضاهريا سليمة(التهاب الضرع تحت السريري) في مدينة الديوانية وقد فحصت العينات تحت السريرية باستخدام اختبار كاليفورنيا وتم اعتماد طريقة الزرع الجرثومي على الاوساط الغنية والتفريقية وكذلك صبغة كرام اضافة الى الاختبارات البايوكيميائية الخاصة بتشخيص الزوائف مثل الكاتليز والاوكسيديز والاندول وتخمير الالكتوز وتحليل الدم . حيث وجد ان نسبة الاصابة الكلية لالتهاب الضرع ٦٩,٣% موزعة على التهاب ضرع سريري وبنسبة ١١،٥% و٨٨،٤% كالتهاب تحت سريري .وتم تشيخص جراثيم الزوائف سالبة لصبغة كرام ومحللة للدم ومن نوع بيتا على وسط الدم .

وقد عزلت الزوائف بنسبة ٤٠،٣% والتي كانت ٣٣،٣% ، ٢٧،٥% من عينات الالتهاب السريري وتحت السريري على التوالي . وكذلك تم استخدام طريقة الانتشار باستخدام اقراص المضادات الحياتية لدراسة مدى حساسيتها للمضادات الحيوية ،فقد ظهر ان السايبروفلوكساسين حساس للعزلات وبمعدل ١٠٠% في حين اظهرت الزوائف المعزولة اعلى مقاومة ضد التوبرامايسين ٥٠% في حين سجلت ٧٥% من العزلات حساسية ضد كل من السيفازيديم والاميكاسين .

Introduction :

Ovine mastitis is the most widely and costly disease affecting the dairy industry. It causes losses of about two billion dollars per year in the United States (1). The majority of losses is occurring due to reduced milk production and bad quality milk, drugs cost and veterinary services , as well as increased culling rate and reduced reproductive efficiency (2). Mastitis or udder infection can classified as clinical and subclinical (3) Clinical mastitis is characterized by an abnormal secretion containing clots or flakes (4, 5) , accompanied by swelling, hardness and increased temperature and it also may be accompanied by depression, loss of appetite, dehydration and fever (6) , but in subclinical mastitis no apparent changes in the udder or milk are expected and the subclinical mastitis and can be detected by appropriate tests (3, 7).

Much different microorganisms have been isolated from .Ovine clinically and subclinically intramammary infections (3). One of important these causative organisms of mastitis is Pseudomonas which colonize ovine teat skin and teat canals, are classed as skin flora opportunists (3).

Pseudomonas aeruginosa (P. aeruginosa) is a motile, gram-negative, rod shape that belongs to the family Pseudomonadaceae . *P. aeruginosa* is a ubiquitous organism frequently isolated from mastitic milk and from contaminated milk samples (8).

On the other hand the massive use of antibiotic in treat intramammary infection as well as livestock abscess and contaminated wounds associated with *P. aeruginosa* lead to appear newly resistance bacteria and failure in antibiotic therapy against *P. aeruginosa* could be recorded (9).

The objectives of this study were to isolates and identify mastitis associated with *Pseudomonas aeruginosa* in ewes in Al-Diwaniyah city and to detect their susceptibility to antimicrobial agents used in commercial intramammary infusion products.

Materials and Methods :

Sample Collection

Seventy five(75) milk sample were collected from sheep as six milk samples from clinically mastitic sheep and sixty nine apparently healthy milk samples according to (9) and subclinical milk samples was submitted to CMT according to (10).

Culture and Identification :

The positive CMT samples (subclinical sample) as well as the clinical mastitic milk samples streaked on nutrient agar and blood agar plates and the plates were incubated at 37 C for 24 hours as described by(11) . Then the characteristic suspected single colonies were subjected to Gram's staining also used some biochemicals tests like catalase + ; oxadase + ; lactose fermintation - ; motility + ; blood heamolysis beta and indole - then sub-cultured in selective media (cetrimide agar) . The pure isolates of *Pseudomonas aeruginosa* were transferred to 1% nutrient agar slant and stored in the refrigerator at 4 C.

P. aeruginosa was identified by biochemical test (catalase + ; oxadase + ; lactose fermintation - ; motility + ; blood heamolysis beta and indole -

) and biochemical tests were performed following the methods described by(12).
Antibiotic Susceptibility Test. the test was performed using disc diffusion according to Kirby-Bauer test method(13) .

Antibiotic discs that used (Gentamicin(10µg) ,Amikacin(30µg) ,Ciprofloxicin(5µg) ,Tobramicin(10µg) ,Cefazidine(30µg) (Oxoid Ltd, Bashingstoke, Hampdhire, England) were used for the test to determine the drug sensitivity pattern. The test was done by culture a colony of the testing organism on Muller Hinton agar and incubated at 37 C for 24 hours. Then the Muller Hinton agar surface were sacked with cultured at least twice. After that the antibiotic discs were placed over the media gently by using the dispenser . Finally plates were incubated at 37 C and examined each plate for the presence of zones of inhibition.

Statistics analysis:

The data was analyzed by using statistical package for the social sciences(SPSS)version 16 software program (2007).

Results and Discussion

Out of seventy five milk samples were collected from clinical and subclinical mastitic sheep, there were 69.3% of ewe suffering from mastitis which 11.5% had clinical mastitis but 88.4% of examined ewes suffering from subclinical mastitis (table 1).

Table 1:Clinical and sub clinical mastitis percentage of examined sheep milk samples.

Total examined milk samples	Mastitic milk samples		Clinical		Subclinical	
	No.	%	No.	%	No.	%
75	52	69.3	6	11.5 a	46	88.4 b

The present study showed ovine mastitis (69.3%) was closely into results by (14)as 64.9% and by (15) as 65% ,while present results was disagreement with (16) and (17) as 54.6% and 52.4% respectively as well as it less than studies by (18) and (19) as 77.5% ,77.7% respectively .

apparently 69 healthy milk samples that examined by CMT give positivity as 66.6%.

Pseudomonas aeruginosa mastitis was recorded in 21/75 mastitic milk samples as 40.3% which classified into clinically as 2/6 (33.3%) and subclinically 19/69 (27.5) (table 2).

Table 2: Percentage of clinical and sub clinical *Pseudomonas aeruginosa* mastitis.

Mastitis form	Milk samples number	*Pseudomonas aeruginosa* mastitis	
		No.	%
Clinical	6	2	33.3 a
Subclinical	69	19	27.5 b
Total	75	21	40.3

The *Pseudomonas aeruginosa* mastitis percentage is supported by reports of (20) and (21) as 40% and 42% respectively .,while it regarding as higher than mastitis caused by *Pseudomonas aeruginosa* 7.9% by (22) and showed to be less than resultant by (23) as 1%.

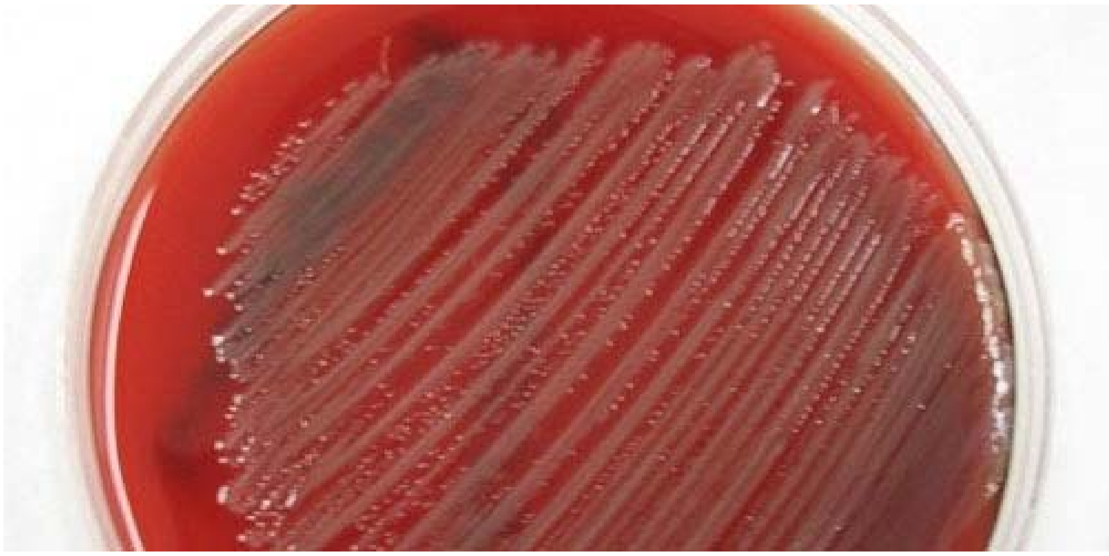
Clinical *Pseudomonas aeruginosa* mastitis is highly significant than subclinical ,which that in similar to reports by (24) ,but incontrast with (25) which showed non-effect of mastitis form on *Pseudomonas aeruginosa* isolation from milk samples.

The variations in percentages of clinical ,subclinical and *Pseudomonas aeruginosa* mastitis between present and other studies could be due to many causes as the season on study ,type of animals housing ,animal breed , age ,general conditions and nutritional status as well as milking hygiene , different degree of sanitary measurement that applied in the dairy ewes.

P. aeruginosa were isolated by using enrich and selective media which were confirmed primarily based on characteristics colony morphology in agars , Gram's staining technique and biochemicals tests.

P. aeruginosa produces circular mucoid smooth colonies with sweat grape odor in nutrient agar ,also produced β-hemolysis on blood agar(figure 1) then subculured on selective media called cetrimide (figure2) , but did not ferment lactose sugar on macconky agar.

. These characteristics colonies were similar with finding of (26)and (27). The isolated *P. aeruginosa* doesn't produce any characteristics pigment on nutrient agar and some atypical strain of *P. aeruginosa* may not produce pigment on agar has been reported by some investigators (28,26) the non-pigmented also reported from different parts of the world (28, 29).

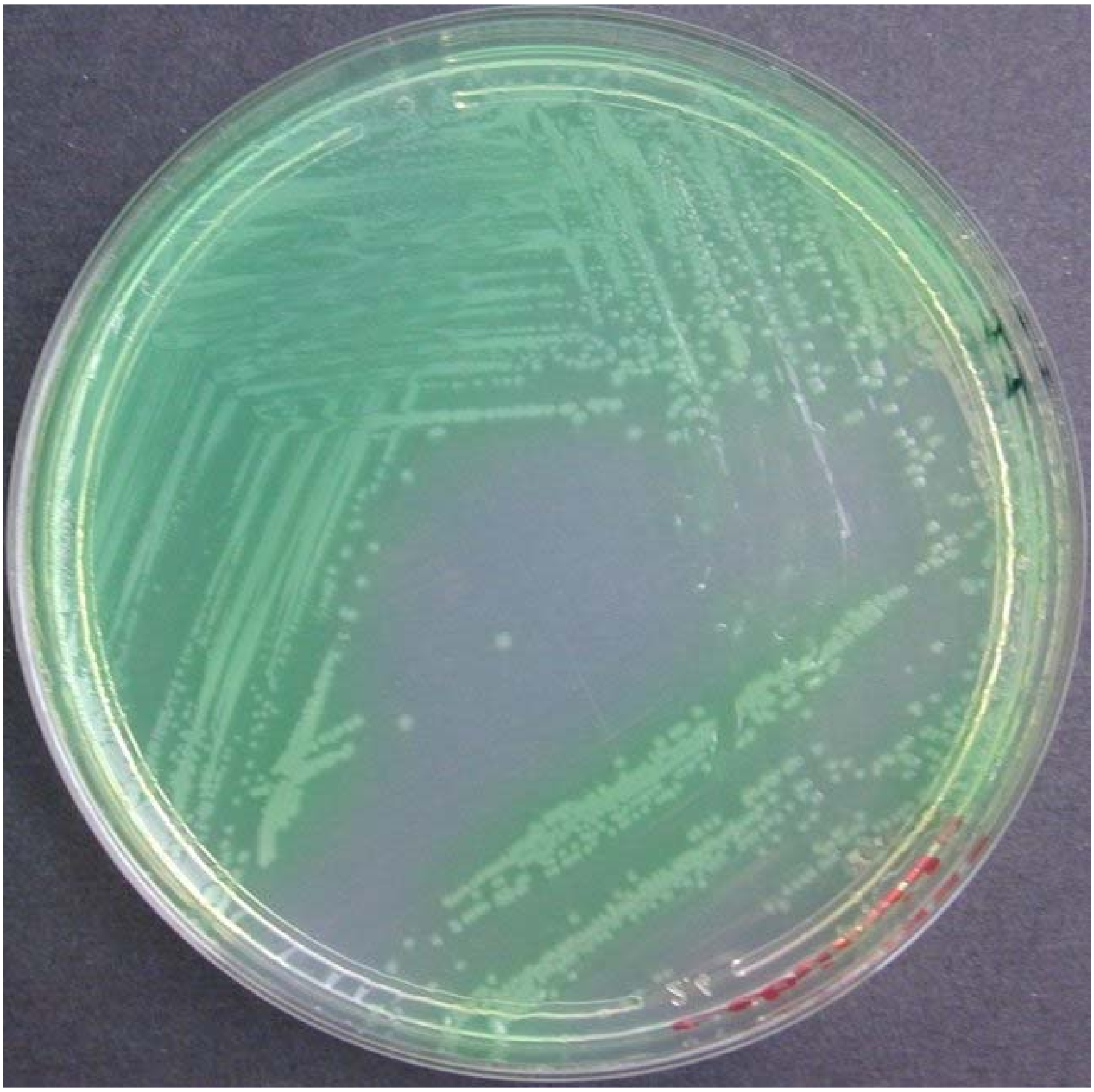


Figure(1) : Show *Pseudomonas aregenosa* colony on blood agar.

. In Gram's staining, the morphology of isolated *P. aeruginosa* showed Gram-negative, pink colored, medium rod shaped appearance . These findings agreed with the findings reported by (30) and (31).

In lactose fermentation test, the isolates of *P. aeruginosa dont* fermented lactose strongly on macconky agar supports the observations of (30)and (11).

To detect the sensitivity of antibiotics against *P. aeruginosa* which is responsible for mastitis and another specific disease. On the other hand this test is done to measure the ability of antibiotics to prevent the growth of bacteria under in-vitro condition or in a suitable environment or outside the body.



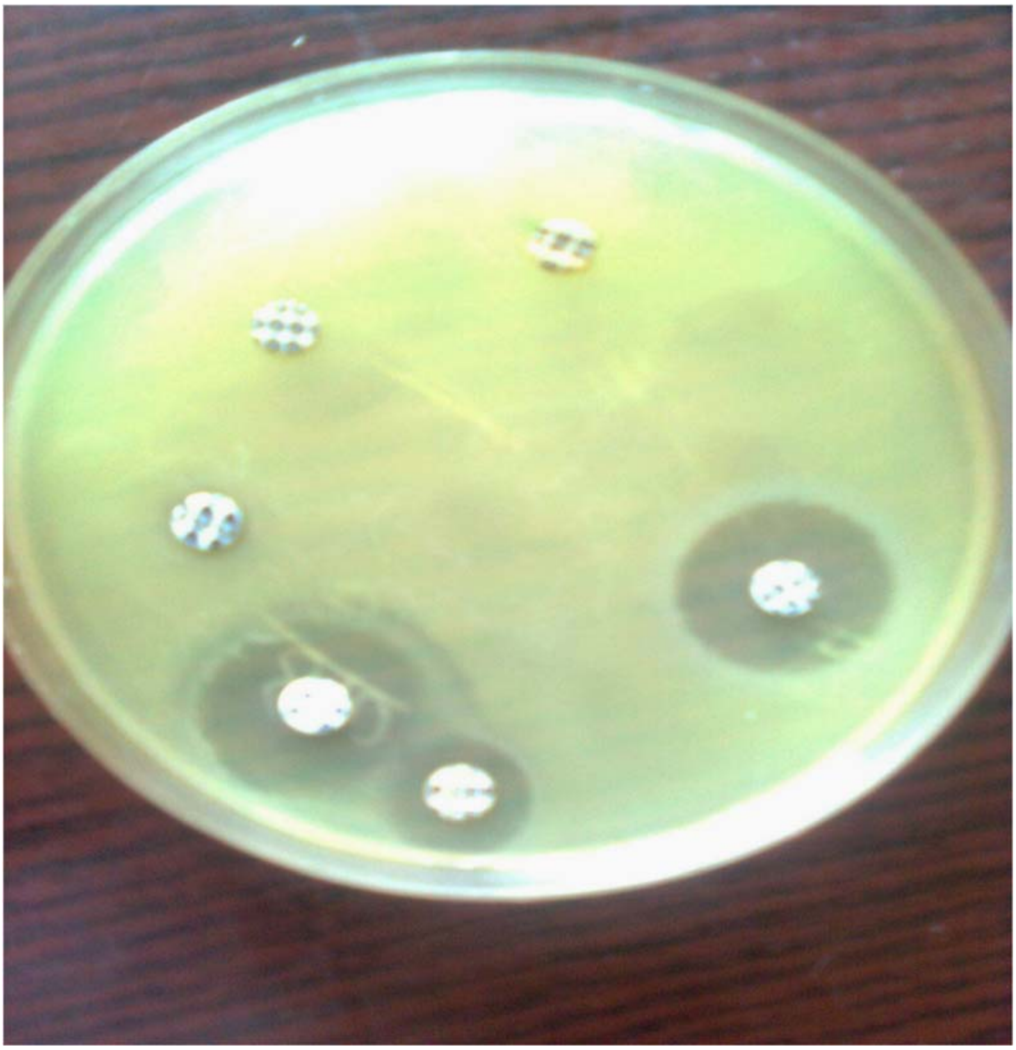
Figure(2) : Show *Pseudomonas aregenosa* colony on cetrimide agar.

The isolates of *P. aeruginosa* were investigated for susceptibility and resistance patterns by disc diffusion method using 5 commonly used antibiotics according to NCCLS . Among the variety of antibiotics tested, the highest resistance was found with Tobramicin and Amikacin (Table 3) (figure3).

Table 3: Antibiotic sensitivity pattern of the isolates of *Pseudomonas aeruginosa.*

Antibiotic Name	Disc Concentration (µg /disc)	Antibiotic Susceptibility results % R	I	S
Gentamicin	10 µg	0	50	50
Ciprofloxacin	5 µg	0	0	100
Amikacin	30 µg	25	0	75
Tobramicin	10 µg	50	25	25
Cefazidine	30 µg	25	0	25

Zone of inhibition (diameter) produced by each antibiotics disc on the agar plate were measured in millimeter (mm) and compare with data of National Committee on Clinical Laboratory Standards (NCCLS), 2007 and A Laboratory Manual for Microbiology, Third edn., by John M. Larkin, *with* some additions by Ruth A. Gyure for WISTR workshop July 2006, Western CT State University. Here: µg = Microgram, , S = Sensitive, I = Intermediately sensitive, R = Resistant.



Figure(3): Show antibiotic sensitivity test on Muller Hinton agar for *Pseudomoans aregenosa* .

These findings were more or less similar to other researchers (27 ,33) and (34) whereas, these authors also concluded that 100% *P. aeruginosa* were resistant to Tobramicin,and 40%of these isolates resistance to Amikacin and Cefazidime. On the other hand, the isolates were intermediately sensitive to Gentamicin and Tobramicin (table 3). These results were nearly comparable with Tripathi *et al.,* (2011) where the authors found that all the isolates of *P. aeruginosa* were sensitive to Gentamicin.

Also isolates of *P. aeruginosa* were highly sensitive to Ciprofloxacin and in less to Cefazidime (table 3). These results of antibiotic sensitivity test were similar with (34) and (35).Therefore, *P. aeruginosa* absolutely sensitive to Ciprofloxacin.

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