Isolation of *Pseudomonas aeruginosa* and molecular detection of bla-OXA gene of the bacteria from milk of mastitis cattle and from the wounds of the udder

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Abstract

The study aimed to isolate, identify (*Pseudomonas aeruginosa*), test the susceptibility of *P. aeruginosa* isolates against some antibiotics (class A- penicillineses and D-cloxacillin-hydrolyzing enzymes (OXA)), and detection the virulence factor (Beta lactemase -OXA gene) by PCR technique. Twenty two isolates of *P. aeruginosa* (8 from the milk and 14 swabs of wound) were obtained from 70 cattle have mastitis and wounds on udder, by using nutrient agar and MacConkey agar. The antibiotic sensitivity test was performed by disc diffusion methods using four antibiotics (oxacillin, cefotaxime, ticarcillin, and impenem). Among the 4 antibiotics tested, the highest resistance was found with oxacillin, cefotaxime (100%, and 60%) respectively, and the lowest resistance rate was to the ticarcillin, and impenem (39%, and 55%) respectively. PCR were performed for all the resistant strains where the frequency of bla-OXA gene have product (618bp) to 22 strains with multidrug resistance of *P. aeruginosa* infection to cattle suffering mastitis.
Key words: Mastitis, bla-OXA, *Pseudomonas aeruginosa*, wounds, cattle.

عزل وتشخيص جزئي لجين البيتا- لاكتميزالاوكسا لجرثومة الزوائف الزنجارية *Pseudomonas aeruginosa* من الابقار المصابة بالالتهاب الضرع وجروح الضرع

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الخلاصة

هدفت الدراسة إلى عزل وتشخيص بكتيريا الزوائف الزنجارية وفحص حساسية العزلات لبعض للمضادات الشائعة الاستخدام لصنف A- penicillineses و انزيم(D-cloxacillin-hydrolyzing (OXA) والكشف عن عامل الضراوة (جين البيتا-لاكتميز) بواسطة تقنية سلسلة التفاعل البلمرة . تم عزل الجرثومة من 22 عينة (8 عزلات من الحليب و14 عزلة لمسحات الجروح) تم جمعها من 70 بقرة مصابة بالتهاب ضرع وجروحه وقد استخدم وسط الاكار المغذي ووسط الماكونكي للعزل. اختبار الحساسية للمضادات الحياتية لعزلات الزوائف الزنجارية والتي تمت بطريقة نشر الاقراص، واختبرت اربعة مضادات حياتية ، حيث كانت مقاومة العزلات عالية بوجود (الاوكساسلين والسيفوتوكسيم) (100% ، 60%) على التوالي، واظهرت اقل نسبة مقاومة ل(التكراسلين ، الامبينيم) (39%، 55%) على التوالي. بينت نتائجنا في اختبار سلسلة التفاعل المتبلمرة أن جميع العتر المعزولة للزوائف الزنجارية (22 عزلة) بأنها تمتلك جين المقاومة الدوائية المتعدد (انزيم البيتا –لاكتميز اوكسا) ذات الوزن الجزيئي (618 زوج قاعدي) لأبقار تعاني من التهاب الضرع .
الكلمات المفتاحية: التهاب الضرع ، البيتا لاكتميز، الزوائف الزنجارية ، الجروح ، الابقار.

Introduction

Pseudomonas aeruginosa is a motile gram-negative rod bacteria from the family of Pseudomonadaceae. It is a frequently isolated from clinical specimens and computation for a significant proportion of nosocomial infections (1), it is considered an opportunistic pathogen which eventual disease usually after stressing, debilitating situation, or teat injuries, and the organism is able to attack and alive for several weeks on

solid surfaces (2), also has been identified as an animal pathogen and as the accidental cause of bovine mastitis (3). It has the ability of causing mastitis in dairy cow, this bacterium present a difficult challenge, as it tends to protect itself from antibiotics and white blood cells in layers of slim (4). The resistance to antimicrobial drugs has increased in recent years. The wrong use of antibiotics in humans (to treat infections), and in animals (to advance growth and prevent colonization by pathogenic bacteria) has led to resistance to actually used antimicrobial agent is of concern to public health officials (5). Extended-spectrum beta-lactamases (ESBLs) that can confer resistance to cephalosporins are common in Enterobacteriaceae and have spread worldwide. Various classes; A ESBLs, such as (TEM-,SHV-,VEB-, and PER-type ESPLs), and class D ESBLs such as (OXA-type ESBLs) have been identified in *P. aeruginosa* (6). The categorization of β-lactamase enzymes involves the use of classification according to functional mechanism (Serine-β-Lactamases) included two classes; Class A-penicillineses e.g. (Broad-spectrum β-lactamases: TEM-1, TEM-2, SHV-1 have Substrates Ticarcillin (carboxypenicillins) Narrow-spectrum cefot-axime (cephalosporins), and Carbapen-emases: (KPC-1, KPC2 and KPC-3; GES-1 and GES-2) Substrates of the expended-spectrum-β-lactemases group plus (imipen-em) (carbapenems).

Class D-cloxacillin-hydrolyzing enzymes (OXA) e.g. (Expended- spectrum- β-lactamses (ESBL): TEM family and SHV-family. Most of OXA family Substrates of the broad-spectrum group β-lactameses plus (oxacillin) (7).

The study aimed to isolate *P. aeruginosa* and detection of virulence factor beta-lactem antibiotic resistance beta- lactamas (OXA) gene of *P. aeruginosa* isolated from milk and wound from udder of dairy cattle accompanying mastitis.

Materials and methods

1.Sample collection

(140) milk and wound swab samples were collected (70 milk samples and 70 wound samples) from (70) animals have mastitis accompanied with skin wound on udder. The milk sample were collected aseptically from the affected udder in10 ml sterile plastic vials from each animal, and the swabs of skin wound of the udder of dairy cattle accompanying mastitis were collected by using sterile transport media swabs .

2.Culture and identification

The samples were streaked on nutrient agar plates and the plates were incubated at 37 C° for 24 hours as described by (7). Then the characteristic suspected single colonies were subjected to Gram's staining then sub-cultured on MacConkey agars and blood agars. 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 (sugar fermentation test) and biochemical tests were performed following the methods described in (8).

2.Antibiotic sensitivity test

The antibiotic discs were used included, Imipenem (IPM) (Carbapenem) (10mg), Ticarcillin (Carboxypencillin) (75 mg), Cefotaxim (CTX) (Cephalosporin) (30mg), Oxacillin (ESBL)(30 mg) (Hi media India). Antimicrobial susceptibility tests were performed by the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) guide lines . *Pseudomonas aeruginosa* PTcc 1310 was used as quality control strain in susceptibility isolates were defined as these showed resistant to classes of anti-pseudomonas agent (A-penicillineses, D-cloxacillin-hydrolyzing enzymes)

3.Genomic DNA extraction

Fresh bacterial genomic DNA of *P. aerginosa* was extracted from 1ml nutrient broth samples in 1.5ml microcenterfuge tubes by using (Presto TM mini g DNA Bacteria Kit, Geneaid. USA), where the extraction was done according to company instruction. After that, the extract gDNA was checked by nanodrop spectrophotometer, than store in -20C^{o} at refrigerator until perform PCR.

4.PCR reaction

PCR was used to detect bla OXA gene in the multidrug resistance bacterial strain

utilizing the following primers: F:ATATCTCACTGTTGCATCTCC, R:AAACCCTTCAAACCATCC (16S rRNA, 618 bp) (Karami and Hannoun 2008).

PCR was carried out with 5µl of the template DNA, 12 PCR water Bioneer (south Korea). Amplification was carried out in thermocycler (Eppendorf mastercycler ®) (bioneer-south korea). Agarose gel electrophorsis (1%) of PCR products was carried out using mM Tris-Borate- EDTA (TBE) buffer at 70V for 2hour, and the DNA bands were stained with ethidium bromid (sinaclon iran) 100bp DNA ladder was used to confirm the size specific bla –OXA gene. Simultaneously, appositive control was used for bla OXA gene. The reaction conditions were as following predenaturation at 94C° for 4 minute, annealing 55 C° for 30 minute, with a final extension step 72C°.

Results

The *P. aeruginosa* isolated from milk samples and skin udder wounds were produces circular mucoid smooth colonies with emits sweat grape odor on nutrient agar (Fig. 1). It seen make β-hemolysis on blood agar and grew on MacConkey agar, but did not ferment lactose sugar (Fig. 2). Twenty two (8 milk and 14 udder wounds) samples were positive for *P. aeraginosa* out of 140 samples. Table (1).

The isolated *P.aeruginosa* show highest resistance rate to oxacillin, cefotaxime (100%, and 60%) respectively, and lowest resistance rate to ticarcillin, impenem (39%, 55%) respectively in antibiotic sensitivity test (Fig. 3). Results PCR were performed for twenty two (8 milk and 14 wounds) resistant strains where the frequency of bla-OXA gene (618bp) with multidrug resistance (Fig. 5).

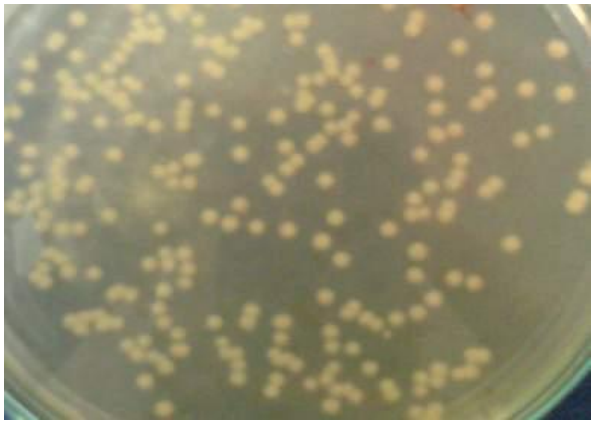

Fig. (1): *P. aeruginosa* on nutrient agar have circular mucoid smooth colonies.

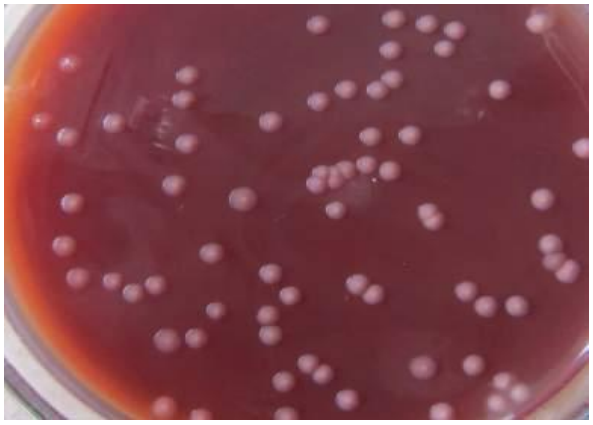

Fig. (2): *P. aeruginosa* grow on Mac-Conkey agar without sugar fermentation.

Table (1): Isolates of *P. aeraginosa* from infected cattle.

	Pseudomonas aeruginosa isolates			
Cattle suffering mastitis	Sample	Positive	Negative	Total
	Milk	8	62	70
	Swab of wound	14	56	70
Total	22		118	140

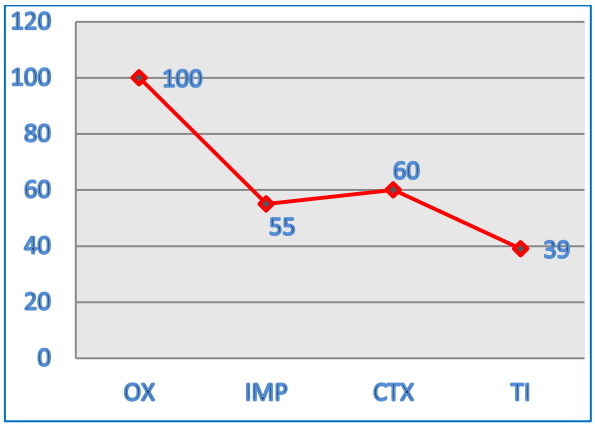

Fig.(3): Resistance range of *P. aeruginosa*. OX; oxacillin, IPM; impenem, CTX; cefotaxim, TI; ticarcillinely.

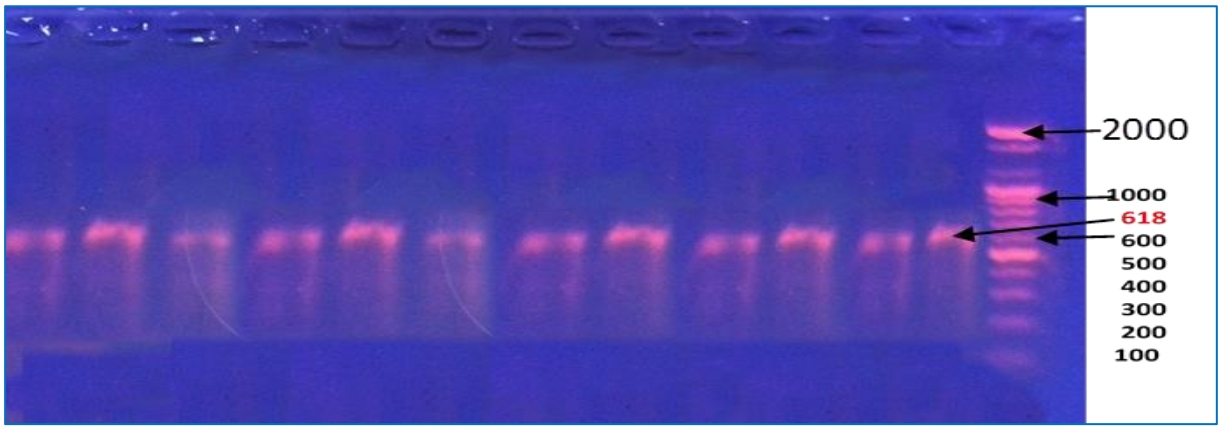

Fig. (5): PCR for the detection of B-lactamas-OXA gene (618bp) of *Pseudomonas aeruginosa*

Discussion

Pseudomonas aeruginosa was isolated by using nutrient agar which was promoted primarily based on characteristics colony morphology in nutrient agar, blood agar and MacConkey agar media and Gram's staining technique, Many strains of *P.* aeruginosa produce various species of pyocins and this pyocin producing strain of *P. aeruginosa* afford pigment on agar media (9). *Pseudomonas aeruginosa* produces circular mucoid smooth colonies with emits sweat grape odor on nutrient agar, these characteristics colonies were similar with finding of (10). 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 (11). Resistance to β-lactam antibiotics in *P. aeruginosa* is a endure problem in the treatment of *P. aeruginosa* infections (12). Permeability of the outer membrane has been suggested as a major contributing factor in the intrinsic resistance of these types (13). In this study highest resistance attribution to oxacillin agreement with (14), therefore the first characterized Class D -lactamases were as well referred to as oxacillinases because their commonly hydrolyze the isoxazolyl-penicillin oxacillin many faster than classical penicillins; i.e. benzylpenicillin. The specification, OXA, of Class D -lactamases, thus refers to their preferred penicillin substrate (15). ESBLs are existence increasingly reported in *P. aeruginosa* worldwide (16, 17); among which OXA type ESBLs have been meeting most commonly (18). The prevalence of resistance IPM impenem (55%) in *P. aeruginosa* in Dewiniya city differs across Iranian studies (21%) (19) and (22%) (20) in Kurdistan Province which may be because of differences in geographic regions. Class A ESBLs are typically identified in *P. aeruginosa* isolates exhibitory resistance to extended-spectrum cephalosporin (ESCs) (21).

Classical ESBLs have evolved from restricted-spectrum class A TEM and SHV β-lactamases although a variety of non-TEM and non-SHV class A ESBLs have been described such as CTX-M, PER, VEB, GES, and BEL (22) and class D ESBLs obtained from narrow-spectrum OXA β-lactamases are also well known (23).

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