Antibiotic Susceptibility and Molecular Detection of *Pseudomonas aeruginosa* Isolated from Bovine Mastitis

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ABSTRACT

This study aimed to isolate *Pseudomonas aeruginosa* from cattle (bovine) milk with mastitis to characterize its antimicrobial susceptibility against some antibiotics, and to identify aminoglycoside acetyltransferase (*aac-3-Ib*) gene. A total of 100 bovine milk samples were collected randomly from different local cow farms at districts of Wasit governorate, Iraq. Six *P. aeruginosa* isolates were obtained using bacterial culture method and further identified by Analytical Profile Index (API-20E). The antibiotic sensitivity test was performed by the disc diffusion method. Among the 5 antibiotics used, the highest resistance (100%) was found with Nalidixic acid and tetracycline, followed by gentamicin (50%), and the lowest resistance rate (16.6%, and 33.3%) was to the ciprofloxacin and cephalothin, respectively. PCR was performed for all the gentamicin resistant isolates. The frequency of *aac(3)-Ib* gene that had a product of 530 bp was 3 of *P. aeruginosa* isolates. From the findings of the present study, we concluded that *P.aeruginosa* isolated from mastitic bovine have developed resistance against aminoglycosides through presence of the *aac(3)-Ib* gene, and the ciprofloxacin and cephalothin can be taken as good choice of treatment.

Keywords: Antibiotics, Susceptibility, *Pseudomonas aeruginosa*, Bovine, Mastitis

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the top 10 superbugs in the world, causing infections with bad condition in human and animal (1). Due to the spread of antimicrobial-resistant strains, therapeutic options are still severely limited; thus, infection with *P. aeruginosa* remains a life-threatening risk (2). Serious infections, both acute and chronic, are always nosocomial and correlated with compromised host defenses; but, this opportunistic pathogen is increasingly identified as the cause of disease in both livestock and fellow animals, these include otitis and urinary tract infections in dogs and cats, mastitis in dairy cows and goats, hemorrhagic pneumoniae in mink cows and goats, hemorrhagic pneumoniae in mink and foxes, and endometritis in horses. *P. aeruginosa* mastitis in cattle occurs either in dry cows or in very recently calved animals. The high rate of this organism fecal carriage can lead to contamination of the water supply on farms; and the presence of certain types of pyocin in the udder, gut or water may lead to their transmission from one reservoir to another; however, many types did not appear to spread (3, 4). Antimicrobial resistance has increased due to the misuse of antibiotics in humans (for the treatment of infections), and in animals (to promote growth and prevent colonization by disease-causing bacteria). Resistance to the antimicrobial agents actually used has been a concern for public health officials (5, 6).

Aminoglycosides resistance caused by altered enzymatic effect can lead to activation of efflux pumps (7, 8) and activation of 16S rRNA methylases.

There are other mechanisms, such as deformation of certain chemical drugs, enzymes such as

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DOI: https://doi.org/10.30539/iraqijvm.v43i2.536

aminoglycoside phosphoryl transferase (APH) that act on plasmid or chromosome genes. another example (9, 10). The six enzymes produce by six genes (AAC-6'-I), (AAC-6'-II), (AAC-3'-Ia), (AAC-3'-Ib), (AAC-6'-IIb) and (APH-3'-VI) (11) are the most commonly changed enzymes in *P. aeruginosa*, and their substrates are the most common and most important against pseudo aminoglycosides.

Therefore, this study aimed to isolate *P. aeruginosa* from cattle infected with mastitis with detection of aminoglycoside acetyltransferase (*aac-3-Ib*) gene that is responsible for gentamicin resistance among aminoglycoside group.

Materials and Methods

Sample Collection

A total of 100 bovine milk samples were collected aseptically and directly from the udder in sterile cups from mastitic and apparently healthy cows, irrespective of age and season from different local cow`s farms at different districts of Wasit governorate.

Culturing and Identification

The milk samples were incubated at 37 °C overnight. Then, cultured on nutrient agar, LB agar and MacConkey's agar (HiMedia/India); incubated aerobically at 37 °C for 24-48 hours. Suspected colonies were picked up and streaked on Cetrimide agar**;** and was identified by biochemical tests, including: oxidase test (Fluka/Switzerland), API 20NE kit (BioMerirux/ UK) (12) and APiGN24 (Diagnostic.SK/ Slovakia).

Antimicrobial Susceptibility

P. aeruginosa isolates was determined by the disk diffusion test (DDT) according to (13), and the antibiotic discs used were ciprofloxacin 10 mcg, gentamicin 10 mcg, tetracycline 10 mcg, cephalothin 30 mcg and nalidixic acid 30 mcg (Bioanalyse/UK).

Sensitive and resistant isolates were detected depending on the recommendations made by (14).

Total Genomic DNA

The DNA was extracted from the selected bacterial isolates by using bacterial genomic DNA Extraction Kit Quick Protocol System supplied by Tonk Bio.

Amplification of Antibiotic Resistance Gene

The amplification of antibiotic resistance gene was achieved by using specific primers (15) as indicated in Table 1.

Lyophilized primers were dissolved in nuclease free distilled water to give a final concentration of 100 pmol/µl as a stock DNA solution.

Table 1. Specific primers for a resistant gene in *P. aeruginosa* isolates

Gene name	Primer sequence	Predicated amplification size (bp)
aac(3)-Ib	**F:** GCGGAACAGCAATAGGTGG **R:** CCACCTATTGCTGTTCCGC	**530**

Polymerase Chain Reaction (PCR)

DNA extracted from bacterial isolates was used for amplification of the resistance gene and trans-posable elements by using specific primers in a thermal cycler. PCR was carried out according to the amplification program (initial step: 95 °C/5 min/1 cycle, denaturation step: 94 °C/1 min/30 cycles, annealing step: 55 °C/2 min/30 cycles, extension: 72 °C/6 min/30 cycles and final extension: 72 °C/5 min/1 cycle).

PCR carried out in a total volume of 20 µl; the reaction components included: 10 µl of Master Mix: Taq DNA Polymerase, dNTPs, $MgCl_2$, reaction buffer; 0.5 µl of each forward and reverse primers; 2 µl of DNA template and 7 µl of D.W. Then, the amplified products were analyzed on agarose gel (2%) in presence of 100 bp DNA ladder marker (Promega, UK).

Results and Discussion

All of the six isolates grew faster on LB agar at 37 °C and appeared as convex, smooth, non-lactose fermenting colonies with regular margin and pale color. On MacConkey agar and nutrient agar, these bacteria appeared smooth at fresh isolation, converted to mucoid spreading growth due to bacterial swarming, with conversion of almost dish to the greenish color or without greenish pigment production in some isolates; some isolates produced water-soluble greenish pigment on nutrient broth (Figure 1 A, B).

P. aeruginosa isolates differ from other species of *Pseudomonas* by growth in selective medium (Cetrimide agar (Figure 1 C). The bacterial colonies on Cetrimide agar were seen as convex, smooth at fresh isolation, and then converted to mucoid distinguished in their color and spreading growth.

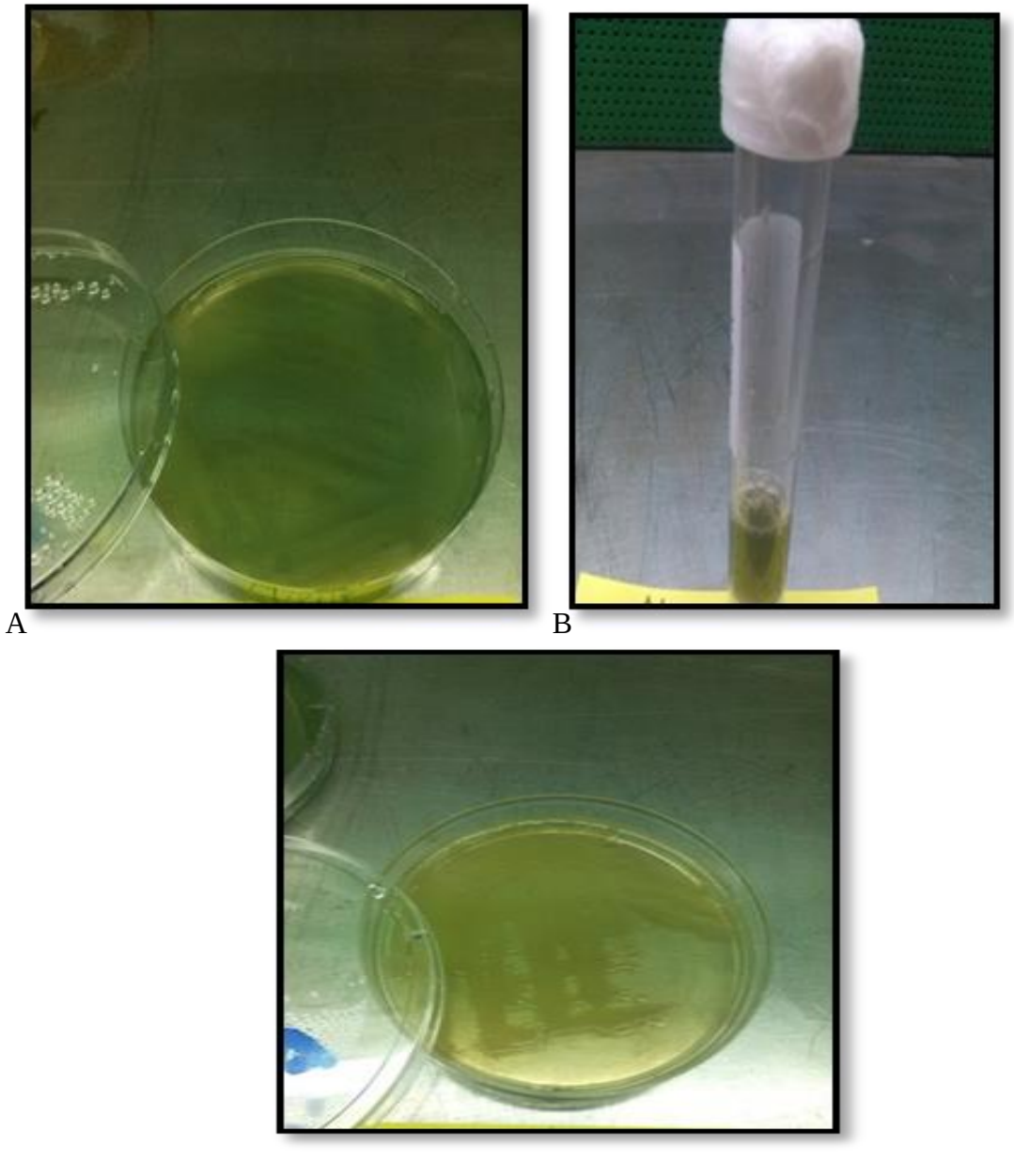

Figure 1. Growth of *P. aeruginosa* on: A. Nutrient agar, B. Nutrient broth, C. Cetrimide

Basically, oxidase test was used to confirm presence of *pseudomonas* bacteria, all bacterial isolates were positive for oxidase test, which indicated by appearance of deep purple-blue color.
Isolates of *P. aeruginosa* were identified from other *Pseudomonas* spp by growth at 42 °C and pyocyanin production, in which only *P. aeruginosa* has this ability. *P. aeruginosa* isolates were further identified by APiGN24 and Api-20E tests, these tests contain a set of biochemical reactions.
By using APiGN24, the percentage of *P. aeruginosa* identification was 99% recorded as excellent results. Positive results in Api-20E tests showed the ability of all isolates for citrate utilization, gel liquefaction, and oxidase production, while were negative to indole production and Voges-Proskauer tests. The antibiotic susceptibility of mastitic *P. aeruginosa* isolates is shown in Table 2, where most isolates showed multidrug resistance to 5 types of antibiotic discs used. The rate of resistance ranged from 33.3% - 100%.
In Quinolone group, 100% of the isolates were resistant to nalidixic acid, and in aminoglycosides group 50% of the isolates were resistant to gentamicin. While in β-lactams, all isolates were resistant to tetracycline (100%). In Cephalosporin group, only two isolates were resistant to cephalothin, and in fluoroquinolone group, 16.6% of the isolates were resistant to ciprofloxacin.

Table 2. Rate of antibiogram of mastitic isolates of *P. aeruginosa*

Antibiotic types	No. of sensitive isolates	percentage of sensitive isolates	No. of resistance isolates	percentage of resistance isolates
Nalidixic acid	**0**	**0%**	**6**	**100%**
Gentamicin	**3**	**50%**	**3**	**50%**
Tetracycline	**0**	**0%**	**6**	**100%**
Cephalothin	**4**	**66.6%**	**2**	**33.3%**
Ciprofloxacin	**5**	**83.3%**	**1**	**16.6%**

The results revealed DNA bands representing a chromosomal DNA after electrophoresis of extracted DNA on agarose gel. PCR detection for aminoglycoside 3, N-acetyltransferase *(aac(3)-Ib*) gene has been done for positive *P. aeruginosa* isolates taken from mastitis milk of cows. Six isolates out of 100 milk samples showed their resistance and sensitivity against gentamicin in antibiotic sensitivity test. The result in Fig. 2 showed that 50% of the bacterial isolates had the *aac(3)-Ib* gene, which showed a molecular size of 530 bp after electrophoresis on 2% agarose gel.

Figure 2. Agarose gel electrophoresis of PCR assay shows the positive aminoglycoside antibiotic resistance genes in some *P. aeruginosa* isolates. Lane (L) DNA marker (1000-100 bp), Lane (1,2, and 3) positive for *AAC-3- Ib* gene at 530 bp, Lane (4-6) *AAC-3-Ib* negative isolates

Mastitis is probably the most important health disorder on dairy farms. This is reflected in relatively high incidence of clinical mastitis and on many farms a high prevalence of subclinical mastitis.

In case of *Pseudomonas*, mastitis is only sporadic, but occasionally it may be a serious herd problem, and udder infection is usually regarded as an opportunist, being relatively non-invasive and producing disease more often after injury of debilitating conditions, or secondary to other infectious agents. Also, the use of common or non-sterile teat cannulas for intramammary administration of antibiotics have been involved in the introduction and spread of *Pseudomonas* mastitis (16). The milk samples collected from cattle in the present study revealed presence of *P. aeruginosa* in 6% of the cases. Such isolation of *P. aeruginosa* was recorded recently by other workers such as (17) in which 30 milk samples were taken from milk of cattle infected with mastitis from different fields in Al-Diwanyia province (18) recorded that conta-mination of raw cow milk and soft cheese samples with *P. aeruginosa* in Baghdad was (76.7%); (19) isolated 10%; (20) isolated 3.0%; (21) isolated 6.9% and (22) reported 3.6% isolation in mastitic cows. *P. aeruginosa* isolates showed characteristic features associated with blue-green fluorescence production (23, 24, 25); (26) in Gujarat isolated 3.6%; (27) isolated 9.4% and (28) reported that *P. aeruginosa* was associated with bovine subclinical mastitis cases.

The fact that aminoglycosides used in veterinary treatment as antipseudomonal vision to these medications let us worry more than the past, since these aminoglycoside resistance qualities are generally situated on portable hereditary elements. There is a developing worry about the spread of resistance genes and be scattered among other microscopic organisms (29, 30).

The rate of resistance against 5 types of antibiotic used in the present study ranged between 16.6-100% for mastitic isolates, in which 100% of the isolates were resistant to nalidixic and tetracycline, 50% were resistant to gentamicin, 33.3% were resistant to cephalothin and 16.6% were resistant to ciprofloxacin. This research focused on aminoglycosides resistant isolates particularly gentamicin resistant isolates; 50% of mastitic isolates had the amplified products of *aac(3)-Ib* gene with a molecular size of 530 bp after electrophoresis. The highest resistance rates to both carbapenems and aminoglycosides were reported in some European countries (28, 29).

Also, (30) in 2016, demonstrated that the percentage of aminoglycoside modifying enzymes genes in bovine mastitic *P. aeruginosa* was 91% and 18.1% for *aac3-Ib* gene. (31) detected the *aac3-Ib* gene in 8.3% of mastitic cattle.

These enzymes are categorized into the three families, based upon the chemical modification they mediate: (i) amino-glycoside phosphoryl transferase enzymes that phosphorylate the drug molecule, (ii) amino-glycoside acetyltransferase enzymes, which acetylate the drug molecule such as *aac-3* gene types, and (iii) aminoglycoside nucleotidyltransferase enzymes that adenylate the drug molecule. Although the range of aminoglycosides inactivated by specific enzymes within this family can differ, the ability of *P. aeruginosa* to carry the genes for multiple aminoglycoside-inactivating enzymes provides individual strains with the potential to develop resistance to all aminoglycosides.

Making complete scan about all resistance genes that provide bacterial resistance against all chemical substances in circular and liner genome and studying all mechanisms that bacteria do to resist the antibiotics generally and aminoglcosides specially (32).

Therefore, continuous isolation of bacteria and detection of genes types other than those used in the present study are more important to acknowledge the development of *P. aeruginosa* especially that isolated from mastitic cattle and for detection of effective treatments which prevent the improper use of antibiotic.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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الحساسية للمضادات الحياتية والتحديد الجزيئي لجرثومة الزوائف الزنجارية المعزولة من الابقار المصابة بالتهاب الضرع

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الخلاصة

هدفت هذه الدراسة إلى عزل جرثومة الزائفة الزنجارية من حليب الأبقار المصابة بالتهاب الضرع وذلك لتوصيف قابليتها المضادة ضد بعض المضادات الحيوية، ولتحديد موروث أمينوغليكوزيد أسيتيل ترانسفيراز (aac-3-Ib). ولذلك تم جمع ما مجموعه 100 عينة من حليب الأبقار بشكل عشوائي من مختلف مزارع الأبقار المحلية في مناطق محافظة واسط ، العراق. تم الحصول على ستة عزلات من نوع *P. aeruginosa* باستخدام طريقة استنبات البكتريا وتم تحديدها بواسطة مؤشر الملف التحليلي (API-20E). تم اختبار فحص الحساسية للمضادات الحياتية باستعمال طريقة نشر الاقراص وكانت اعلى نسبة مقاومة من بين خمسة انواع من المضادات الحياتية ل (نااليدكس اسد و تتراسايكلين) 100% يتبعها الجنتامايسين 50% بينما كانت اقل نسبة مقاومة ل سبروفلوكساسين والسيفالوثين (16.6% و 33.3%) على التوالي. واظهرت نتائج تقنية سلسلة التفاعل البلمرة التي اجريت لجميع عزلات الزائفة الزنجارية ان هناك3 عزلات مقاومة لمضاد الجنتامايسين ضمن عائلة الامينوكلايكات كونها تمتلك جين المقاومة الدوائية (ا ي اي سي 3- اب) ذات الوزن الجزيئي 530 زوج قاعدي. من نتائج الدراسة الحالية، استنتجنا إلى أن *P. aeruginosa* المعزولة من الأبقار الضارية قد طورت مقاومة ضد الأمينوغليكوزيدات من خلال وجود الموروث aac (3) -Ib، وان سيبروفلوكساسين و السيفالوثين يمكن ان يؤخذ كخيار جيد للعلاج.

الكلمات المفتاحية: المضادات الحياتية، الحساسية، الزائفة الزنجارية، الأبقار، التهاب الضرع