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Improvement Rapid Molecular Detection of *Pseudomonas aeruginosa* infected some Iraqi Patients and It's antimicrobial susceptibility.

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ABSTRACT

Pseudomonas aeruginosa is a one of an important opportunistic human pathogen and the greatest one of therapeutic challenges that represents the main cause of the morbidity and mortality in some patients. Fifty isolates of *P. aeruginosa* were collected between September 2015 and February 2016 from different hospitalization patients in Baghdad governorate. These samples were collected from different sites of infection represented by: diabetic foot ulcers, burns, ears, burns, keratitis, wounds and urine infections. All samples underwent to cultural tests, biochemical tests and ensured by molecular test using Real Time PCR as a rapid method using a commercial kit from Saccace then used ***gyrB*** as a specific gene in detection of this bacteria. These molecular tests gave the more accurate results in a short time comparatively with other biochemical and cultural tests . All isolated bacteria were tested for nine antimicrobial agents to detect their ability to resist the drugs. approximately(72%) of bacterial isolates were found highly resistence to Augmenten while most of them (94%) were found susceptible to Levofloxacine indicated that levofoxacine is the most effective antibiotic among the antimicrobail agents tested in this study were used in this study.

Keywords: *pseudomonas aeruginosa*, antimicrobial susceptibility, ***gyrB***, Real-Time PCR.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram negative; glucose non-fermenting, a strict aerobe, rod shape bacterium with grape-like odor which mostly associated with infections [1]. It represents the common nosocomial human pathogen that causes significant mortality and morbidity internationally. Patients may infect with *P. aeruginosa* when exposure to contaminated environment within the hospital, it ables to colonize in different environments and contacts with weakened populations lead to cause several types of community-acquired infections every year [2].

It causes many diseases such as diabetic foot ulcers [3], wounds, urinary tract, burns, otitis, bacteremia [4] and keratitis [5]. It is considered a major cause of morbidity and mortality in patients with cystic fibrosis[6].Wide use of broad-spectrum antibiotics against *P. aeruginosa* causes the high resistance to clinical drugs, that led to serious therapeutic problems Multi-drug resistant (MDR) of *P. aeruginosa* represents a global problem ,that resulting from the ability of these bacteria to develop its resistance to a different antibiotics, either by horizontal gene transfer or by mutations in chromosomal genes [7]. For example, mutations in the ***GyrB*** subunits of DNA gyrase represent one of the causative agents that play a major role in increasing the level of resistance to fluoroquinolone in *P. aeruginosa* and some other Gram-negative bacteria [8]. ***GyrB*** gene encodes the DNA gyrase subunit B which is protein consists of two domains: N-terminal domain that containing an ATP binding and hydrolysis site that is thought to play as a DNA clamp; and a C-terminal domain that is play a role in interactions with ***gyrA*** and DNA, and in strand passage. The two DNA gyrase subunits, ***GyrA*** and ***GyrB***, form a functional tetramer [9].

Present study conducted rapid molecular detection of *P. aeruginosa* using Real-Time PCR assay depending on the ***gyrB*** gene. Notably, this gene reported to be reliable PCR target to detect *P. aeruginosa* [2]. The results were compared with those obtained from a commercial biochemical and cultural identification tests as well as a specific commercial diagnostic RT-q PCR assays. This study was aimed to improve an development better way for rapid molecular detection of *Pseudomonas aeruginosa* and determine the most effective antimicrobials inhibition bacterial growth to reduce the efforts and costs of treatment.

MATERIALS AND METHODS

Samples collection

Fifty samples suspected ***P.aeruginosa*** were collected from patients with different diseases, using sterile swabs, which are diabetic foot ulcers, urin cultures, wounds, burns, keratitis, otitis for six months from September 2015 ,till February 2016, from hospitalization patient in four different Baghdad hospitals (Al-Kindi General Teaching Hospital, AlYarmuk General Teaching Hospital, AL-Kadimia Hospital, and AL-Shiakg Zaied Hospital).The study population targeted both gender from different ages groups .

Bacterial Isolation and cultivation

All collected samples were cultured on MacConkey agar, pseudomonas agar, citrimide agar, chromogenic agar, and blood agar incubated aerobically at 37°C for 24 hour [10].

Identification of Bacterial Isolates :

Pseudomonas aeruginosa isolates were identified by traditional biochemical tests including Oxidase, Catalase, Gram stain, growth on citrimide agar and **chromogenic** agar and by API 20EN biochemical kit (bioMérieux, France)[10]. Antimicrobial assay was performed using nine antibiotics showed in Table 1.using Kirby-Bauer's single disk diffusion technique [11].The detection of this bacteria was ensured by molecular identification.

Table (1) *gyrB* Oligonucleotides was used in this study

Oligonucleotides	Sequence (5'-3')	Reference
GyrB F-primer	CCTGACCATCCGTCGCCACAAC	[12]
GyrB R-primer	CGCAGCAGGATGCCGACGCC	[12]

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GyrB-TM	FAM-CGTGGTGGTAGACCTGTTCCCAG-BHQ	[6]

FAM = carboxyfluorescein; BHQ = black hole quencher

DNA Extraction:

DNA extraction was performed using Wizard genomic DNA purification kit (Promega ,USA) according to the manufacturing instructions.

Real Time PCR Assays

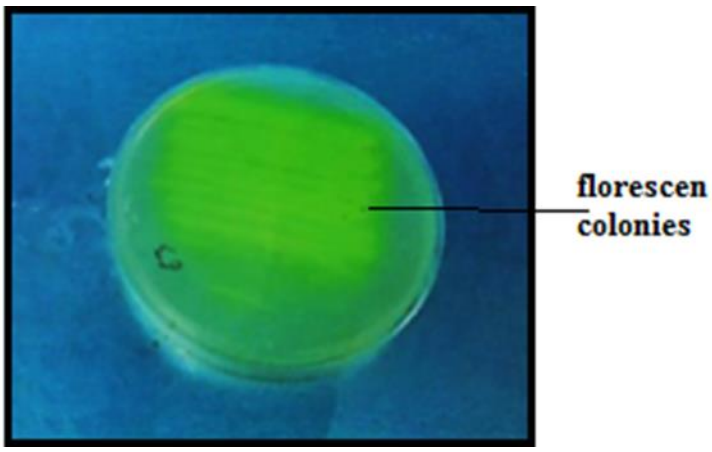
The quantitative PCR assay was performed with the Smart Cycler instrument (Cephied, USA). At first, using Real Time PCR Kit for detection of *P.aeruginosa* from Sacace (Italy), which is specific kit to detect this bacteria and the results was available in ~1hour and 4 minutes according to the manufacturing instructions .

The second test, using oligonucleotide primers and probes set that checked with GenBank to ensure their specificity to *P. aeruginosa* (showes in Table 2) for specific detection of a 220-bp fragment within the ***gyrB*** gene. The amplification mixture consisted of 12.5µl GoTaq Probe qPCR MasterMix (Promega), 2µl each primer, 1µleach probe, and 2µl of template DNA in a final volume of 25µl. Suspensions of negative and positive controls from the commercial kit were used as negative and positive controls, respectively in the detection of gyrB gene. Following an initial denaturation at 95°C for 900 seconds, the 45-cycle amplification profile consisted of secondary denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds. The final result was available in 1 hour and 6 minutes.

RESULTS AND DISCUSSION

The results of bacterial culture obtained from different patients 50 isolates were identified according to the following results of cultural characteristics, microscopic and biochemical test and molecular tests.

The bacterial colonies were feathered edges with a characteristics grapes oder, which appear pale on MacConky agar due to the in ability of these bacteria to ferment lactose and on blood agar *P.aeruginosa* produced clear zones around its colonies that represent beta hemolysis. Using Citrimide agar (Himedia, India) containing nalidixic acid is useful in isolation and identification this species from other species of *Pseudomonas* which appeared bright greenish figure1 [13]. Using pseudomonas Chromogenic agar (Conda, Spain) in identification of *P. aeruginosa* is very perfect [14] .depending on the manufacture company information that determine the color of *P.aeruginosa* colonies, this study represent one of rare studies that using this media in diagnosis this pathogen in Iraq. It is easily distinguishable due to the magenta colony color and the color of the medium that change from green to blue-green. The rest of bacteria are inhibited, and in case of growing, they grow as colorless colonies. figure2. Staining with gram stain, smears of these bacteria showed under compound light microscope at 40X, as a small single gram-negative rods, appeared singly or in groups figure3.



Figure(1) *P.aeruginosa* growth on Citrimide agar and examined under UV to see the florescence of its colonies

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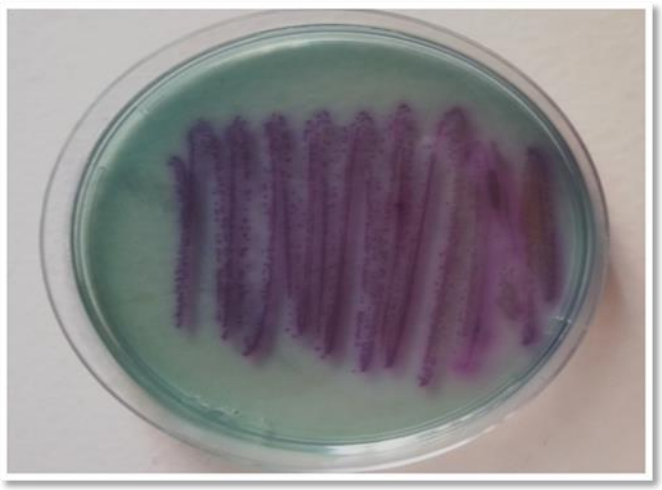

Figure (2) *P. aeruginosa* growth on Chromogenic agar

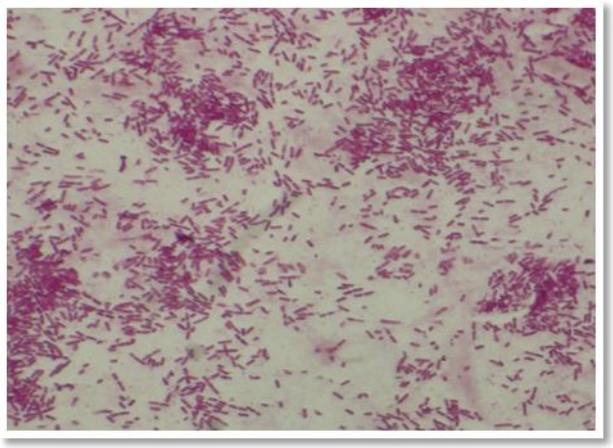

Figure (3) *P. aeruginosa* stained with Gram stain

The results of the biochemical tests for *P.aeruginosa* isolates show in Table (2). Oxidase reaction gives appositive results in 2-3second, but the mucoid strains of this bacteria gave positive after ~20 seconds. All isolates showed positive results for catalase test (production of gaseous bubbles represents appositive results). API 20EN test recorded positive results for all types of isolates with just 5(33.3%) isolates from diabetic foot ulcers.

Table (2) the results of biochemical tests for *P. aeruginosa* isolates

Types of isolates	Numbers of isolates	Oxidase test positive/total	Catalase test positive/total	API 20 EN positive/total
Diabetic foot ulcers	15	15/15(100%)	15/15(100%)	5/15(33.3%)
otitis	8	8/8(100%)	8/8(100%)	8/8(100%)
keratitis	7	7/7(100%)	7/7(100%)	7/7(100%)
Urine	10	10/10(100%)	10/10(100%)	10/10(100%)
Wounds	6	6/6(100%)	6/6(100%)	6/6(100%)
Burns	4	4/4(100%)	4/4(100%)	4/4(100%)
Total	50	50/50(100%)	50/50(100%)	40/50(80%)

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The data for the real time PCR assays show in table **(3),** all isolates from different sources were tested by commercial kit and also retested by specific ***gyrB*** gene. The present results showed that the 98%(49 isolates) were identified by the diagnostic commercial kit which were (100%) positive results for (diabetic foot ulcers, urine, otitis, burns and keratitis) isolates , while just one isolate from wounds was misidentified in this kit, so the positive results for the wounds isolates were (83.3%).

Table (3) summarizes the data of real time PCR assays for commercial kit comparing with the specific gene

Types of isolates	Results of detection by the commercial kit	Results of *gyrB* gene detection
Diabetic foot ulcers	15/15(100%)	11/15(73.3%)
Ear swab	8/8(100%)	8/8(100%)
Eye swab	7/7(100%)	7/7(100%)
Urine	10/10(100%)	10/10(100%)
Wounds	5/6(83.3%)	6/6(100%)
Burns	4/4(100%)	4/4(100%)
Total	49/50(98%)	46/50(92%)

The same table recorded the results of ***gyrB*** gene test ,the positive results were 92% (46 isolates) which are (100%) positive results for the isolates from (urine, otitis, burns, wounds and keratitis),while four isolates from diabetic foot ulcers were misidentified in this test, so the positive results for these isolates were (73.3%).

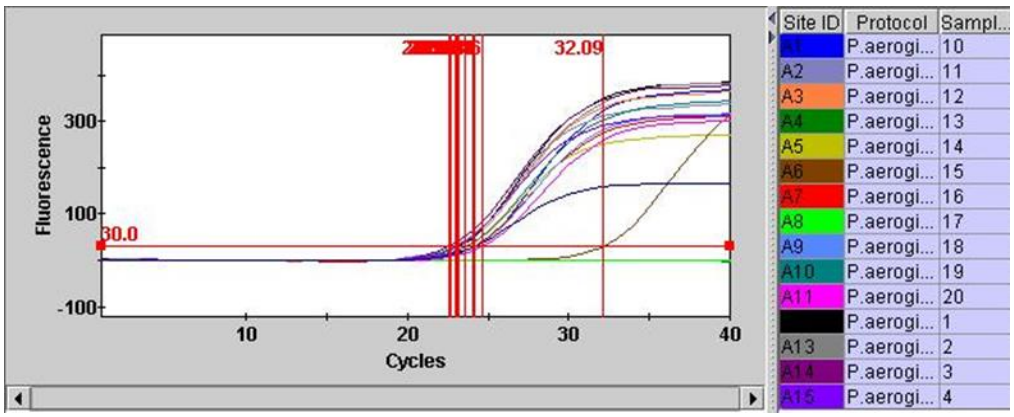

Figure (4) shows the results of some isolates in real time PCR technique using the specific detection of *P. aeruginosa* commercial kit using CY3dye in the detection

Note: the last curve arise for isolate from diabetic foot ulcers referred that isolate contains less copies of target gene so it needs more than 32cycles to arise

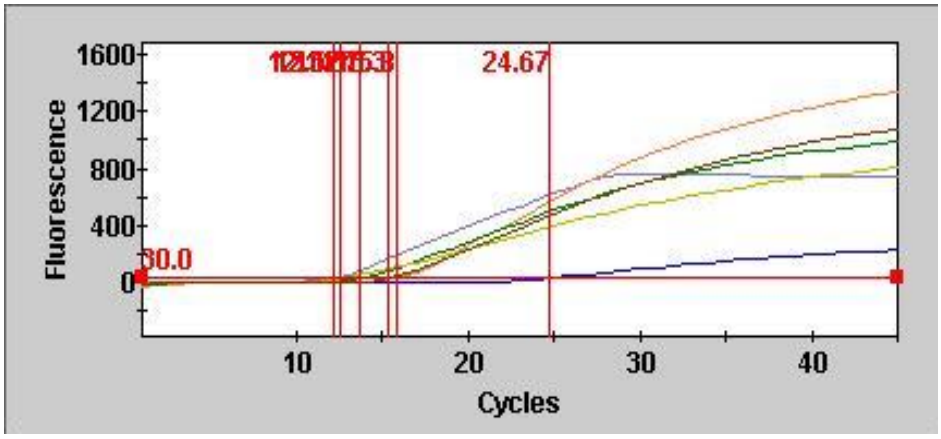

Figure (5) Shows the results of some isolates in real time PCR technique depending on *gyrB* gene using FAM dye in the detection and the last curve arise for isolate from diabetic foot ulcers

Detection of this bacteria by the commercial kit depending on the curves of CY3dye(Cyanine type3dye) as an evidence of amplification (CT values ranged from 12 to 32 cycles in this test)as in figure.4,which shows different types of melting peaks which represent the amplified DNA products for commercial kit as distinct melting peaks with their specific melting temperature. Amplification of detection of *P. aeruginosa* depending on ***gyrB*** gene illustrates in Figure.5, the amplification of ***gyrB*** gene can be determined by serving the fluorescence emission curves, the fluorescence curves which exceed the threshold line represent the amplified ***gyrB*** gene of *P. aeruginosa* and the evidence of amplification was the curves of FAM dye using 45 cycles (CT values ranged from12 to ~25cycles).

The two Real Time PCR assays gave positive results of *P.aeruginosa* in 45 isolates, while the different results (negative results) between them was in 5 isolates, at the same time one isolate from diabetic foot ulcers delayed until 32.09comparing with the other isolates by using commercial kit, the last curve arise for isolate from diabetic foot ulcers referred that isolate contains less copies of target gene so it needs more than 32cycles to arise, while another isolates from diabetic foot ulcers delayed until 24.67 comparing with the other isolates in the ***gyrB*** detection as noticed in figure5 because this isolates has less copies of ***gyrB*** gene therefore it needs more than 24 cycles to arise. The negative samples were not amplified and the fluorescence curve still remained below the threshold line, so these different results may be caused by the variations of sequences in probe or primer **target**.

Table (4) illustrares the results of sensitivity of *P.aeruginosa* against nine antimicrobial agents according toCLSI [15]. The results showed that the most isolates were sensitive to Levofloxacin (94%), Ticarcillin-clavulanic acid (88%), Gentamicin(82%) and (80%) to both Pipracillin and Imipenem, while the isolates appeared resistant to another antimicrobials represented by (72%) to Augmentin, (34%) to Ceftazidime, (38%) to Colistin, and (28%) to ciprofloxacin. These results disagreed with the other study in India that showed (100%) resistant to Pipracillin, (42.8%) resistant to Gentamicin and (57.1%) resistant to Levofloxacin [16], present results disagreed with other study in Pakistan [3] that showed a high resistant of *P.aeruginosa* against Augmentin (97.6%) at the same time showed a lower resistant to Imipenem (10.4%) that close to our study.

Table (4) types of antimicrobial agents and the results of susceptibility tests of *P. aeruginosa* using Kirby-Bauer's single disk diffusion technique

Antibiotics	Diameter of inhibition zone (mm)			Number of bacterial isolates and % of the results		
	R	I	S	R	I	S
Imipenem10µg	≤15	16-18	≥19	3(6%)	7(14%)	40(80%)
Ciprofloxacin5µg	≤15	16-20	≥21	14(28%)	4(8%)	32(64%)
Levofloxacin5µg	≤13	14-16	≥17	2(4%)	1(2%)	47(94%)
Ticarcillin75µg+Clavulanic acid 10µg	≤15	16-23	≥24	4(8%)	2(4%)	44(88%)
Gentamicin10µg	≤12	13-14	≥15	9(18%)	_	41(82%)
Colistin sulphate 25µg	≤10	-	≥11	19(38%)	_	31(62%)
Ceftazidime 3µg	≤17	18-20	≥21	17(34%)	4(8%)	29(58%)
Pipracillin 100µg	≤14	15-20	≥21	7(14%)	3(6%)	40(80%)
Augmentin30 µg	≤13	14-17	≥18	36(72%)	3(6%)	11(22%)

This report agrees with ALKaabi (2013) in the low resistant to Imipenem which is (30.76%) ,but disagreed with them in the sensitivity to Levofloxacin[17].This study rather agreed with AL-Taei (2012) that represent the best antimicrobial against this bacteria is quinolones like Ciprofloxacin [18], but the common use of this antibiotic led to increase progression the resistance in many bacterial pathogens. The outer membrane of this pathogen appears a little permeability to the small hydrophobic molecules, which may cause to form the essential resistance of *P. aeruginosa* against quinolones, the outer membrane is less permeable (10- to 100 fold) to antibiotics than of *E. coli* [19]. Mutations in the DNA gyrase subunits may play a major role in forming a high rate of resistance to fluoroquinolone in *P. aeruginosa* and other Gram-negative bacteria [8].

Although classical scenario for identification of *P. aeruginosa* from clinical isolates is usually achieved by modest phenotypic tests, but precise identification of *P. aeruginosa* strains were difficult in some chronic infection at the early stage of disease after onset of infection because strains undergo a series of phenotypic changes during adaptation to the infection[20] besides it takes several days around(4-5days). Present study focused on using a molecular tools for identification comparing with the microbiology laboratories, like API20EN test, identified isolates as *P. aeruginosa*, they were perfect on almost 98% of cases, that was agreed with another study in Australia[21],but unfortunately when the laboratories made mistakes, the patients may receive unnecessarily and prolonged antimicrobial therapy that may expose them to infect with the clonal complexes of dominant *P. aeruginosa* from another patients accidentally[22]. Growth on citrimide agar and chromogenic agar help in differentiation this species from other gram negative rods. Oxidase testing and catalase testing may reduce misidentification of *P. aeruginosa*. Using API 20EN systems exhibit results ranged from excellent to acceptable results and the false results at genus level less than at species level [23],at the same time all of phenotypic test have problems, e.g., (i) not all strains within species may give a particular characteristic, (ii) the one strain may exhibit different results when repeated testing[24], and (iii) limited the identical databases[23] .For all previous reasons in present study, we try to use a genotypic identification assays which may be an alternative or complement to phenotypic and biochemical detection procedures [4].

In this study, two real time PCR provided conclusive results (concurrent positive and negative results to the specific detection of *P.aeruginosa* kit from (sacace and ***gyrB*** targets) for 50 isolates. One laboratory are usually depend on oxidase testing, morphology of colonies, and growth on MacConkey agar and in some hospital ensure by API 20NE analysis during the study period, that do not improve the true identification or misidentification rates, besides it tacks along time (several days) that may lead to determine unsuitable drug which may be caused to appear multi drug resistance in nosocomial pathogens.

The first focus was to check isolates identified by clinical microbiology laboratories as *P. aeruginosa*. Isolates reported as genus and species other than *P. aeruginosa* were not presented and were out the scope of this study. Second, all isolates that show positive in the two real time PCR results were identified as *P.aeruginosa*. Finally, all *P.aeruginosa* tested to different antimicrobials to define its Susceptibility against them. As a knowledge there are some antibiotic mechanisms activity that prevents the growth and colonization of the pathogenic bacteria such as: (i) interference with cell wall synthesis like Beta-lactam antibiotics such as Imipenem and Ceftazidime prevent the formation of peptidoglycan layer [25], (ii) impeding the nucleic acid synthesis like Levofloxacin and Ciprofloxacin, which inhibit DNA synthesis during replication by interference of DNA gyrase (***gyrA***, ***gyrB***), type II topoisomerase and type IV topoisomerase[26], (iii) disorganizing of the cell membrane, it is hypothesized that polymyxins like colistin increase bacterial membrane permeability causing infiltration of bacterial content [27], (iv) inhibition of protein synthesis like Aminoglycosides (e.g.gentamicin) which inhibit initiation of protein synthesis by binding to the 30S ribosomal subunit[28],(v) inhibition of a metabolic pathway[26] . The term "multidrug-resistant pathogen" might be called when it is resistant to more than one drug [29]. Bacterial strains become resistant by mechanical and genetic ways. There are many different resistant mechanisms including: (i) Antibiotic inactivation By hydrolysis in the case of extended spectrum b-lactamases (ESBLs) [30]. (ii) Antibiotic inactivation by group transfer like aminoglycosides [31]. (iii) Antibiotic resistance via target modification [32]. The genetics of antibiotic resistance include. (i)Antibiotic resistance via mutations of gene that encode the target of antibiotic like resistance to fluoroquinolones [33] or by mutations of the efflux systems reduced expression of the OprD porin of *P. aeruginosa* reduces the permeability of the cell wall to imipenem [34], (ii) by horizontal gene transfer many strains of bacteria become resistant to some antibiotics like (ESBL) against cephalosporins[35].

CONCLUSION

The difference in the number of positive isolates when diagnosed by chemical API20 test and Real Time PCR technique may be due to the highly specificity and greater sensitivity of the Real Time PCR than chemical tests so the rapid and **accuracy in** detection of pathogen may help in a precise treatment that may prevent a rise of resistant strains of *P.aeruginosa*. Finally present data shows that diabetic foot ulcers isolates need more specific studies at the same time the molecular method by using Real Time PCR looks more perfect for rapid diagnosis of this pathogen.

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