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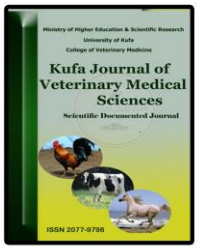

Rapid Identification of *Pseudomonas aeruginosa* by Using Real Time PCR

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Abstract

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium which has emerged as one of the most problematic nosocomial pathogens.To characterizes *P. aeruginosa* strains that are widespread in patients in Iraq, 90 clinical samples were collected from wounds, burn, ear infection and urinary tract infection taken from three general hospitals of different areas of the region in Baghdad . Methods for isolation and identifying *P. aeruginosa* based upon culture methods coupled with biochemical tests, were used in this study. The results show that, the selective medium (cetrimide agar) at 42˚C aerobically had highest recovery in the isolation of *P. aeruginosa* strains, they were produced greenish-yellow or blue pigment colonies, catalase and oxidase was positive whereas negative for methyl red, and indole.; however, some of these methods are time consuming and may not be very accurate whereas API 20E is rapid method which performs at least 20 different biochemical tests at once, however it proved difficult to obtain additional information concerning the relationship between these strains. Molecular study for identifying *p.aeruginosa* include DNA extraction , than Real time assay by using powerchek *P. aeruginosa* Real time PCR Kit with probe, the results showed that RT-PCR has found to be rapid and more sensitive and specific in identification of *P. aeruginosa*, however Real time PCR Kit with probe, specific marker is recommended

Key :- *RT-PCR* **,** *Pseudomonas aeruginosa ,opal gene*

Introduction

Pseudomonas aeruginosa is a rod-shaped gram-negative obligatorily aerobic bacterium belonging to the family of Pseudomonadaceae. *Pseudomonas aeruginosa* is capable of growing in a wide variety of niches with a preference for moist environments. In addition, *P. aeruginosa* is one of the three most abundant bacterial species causing nosocomial infections in intensive care units (Spencer, 1996). *P. aeruginosa* has emerged as one of the most problematic nosocomial pathogens, it is considered an opportunistic pathogen that causes infection in immune depressed subjects (Brooks *et al.,* 2007).It is the leading cause of wound infections, urinary tract, surgical wound and ear infection (Todar, 2008). *P. aeruginosa* infections are nosocomial in nature,hospital reservoirs of growth are many and include respiratory

equipment, solutions, medicines, disinfectants, sinks,mops, food mixers and vegetables (Muscarella, 2004; Trautmann *et al*., 2005). A rapid and accurate system for the identification of *Pseudomonas* is important to isolate patients and prevent further spreading of the diseases. For the genetic identification and characterization of pseudomonas species via PCR-based methods, various targets have been reported, such as 16S rRNA (Relman *et al*. 1992), *toxA* (Khan and Cerniglia 1994), *oprI,* (De Vos *et al*. 1997), *algD* (da Silva Filho *et al*. 2004) . The 16S ribosomal RNA (rRNA) gene is most commonly used, but it is not feasible to develop highly specific primer and probe sets using this gene because of the high similarities of the 16S rRNA gene sequences (Moore *et al*. 1996; Yamamoto *et al*. 2000). real-time PCR assayas a highly sensitive and specific assay when tested with *P. aeruginosa* strains and species of pseudomonads closely related to *P. aeruginosa* (Lavenir *et al*. 2007). Spcies specific PCR targeting the outer membrane lipoprotein gene *oprL*, was developed (Lim *et al*,1997).It was used for the direct detection and identification of *P. aeruginosa* in clinical samples(Nde *et al.,* 2008). Positive PCR results were obtained using primer specific for *oprL* gene for 150 strains *of P. aeruginosa* isolates, including strains of clinical and environmental origin (Jaffe *et al*., 2001).The aims of this study are: Isolation and identification of *P. aeruginosa* from clinical sample, using phenotypic test, and polymerase chain reaction using species specific primers

Materials and Methods

Sampling

Between August and December 2014, 90 samples were taken from three general hospitals of Baghdad. The swab samples were taken from patients with infected wounds including, burn, wound and ear infections, and The clinical swaps were collected from surface of burn's patients , flooring of burns unit , tools of burns unit, handles laundries in the water cycle in burn unit ,wound ,inflamed ear and urine samples from people with inflamed of the urinary tract .

Phenotypic identification of *P. aeruginosa*

According to Chessbrough (1991), the swabs were enriched in brain-heart infusion broth, plated onto MacConkey agar. A single colony was selected and inoculated on the selective medium (cetrimide agar). Then phenotyping characteristics *of P aeruginosa* was described after Gram staining, including pigments production after incubation at 37ºC,and the biochemical test done according to MacFaddin, (2000) which include:,motility, Indole production test. Methyl red test. **,**Citrate utilization test **,**Oxidase test and the catalase test , then API 20E System used for biochemical test which consists of 20 microtubes, containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents and the identification is obtained by referring to the analytical profile index.

DNA EXTRACTION

Genomic DNA was prepared for forty nine samples chosen according to Cardinal *et al.,* (1997) method with some modification as follow: Ten ml overnight cultures were prepared in broth media from fresh single colony. Cells were harvested in a centrifuge for 5 min at 6000 rpm. Then the cell pellets were re-suspended in 1ml of

sterile water. The resuspended cells were re-centrifugated at 12,500Xg for 15min. The pelleted cells were then used for DNA extraction as followed by manufacturer instructions for geneaid DNA miniprep kit.

Real time assay by using powerchek *P. aeruginosa* Real time PCR Kit .

The RT-PCR assay working with the fluorescent labelled hydrolysis probes provided from kogenebiotech (germany) .This Kit is intended for 50 tests, including
Primer /probe mix *p.aeruginosa* ,2XReal-Time Pcr Master Mix.

RT-PCR Reaction Mixture

Composition	volume
Probe	4µl
2xreal time pcr master Mix	10µl
Template DNA	4µl
Free dnase water	Adjusted to 20µl
Total	20µl

Results and Discussion

Isolation and Identification of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa get positive result in clinical samples included burn's patients (*n*=20)(66.6%), flooring and tools of burns unit (*n*= 6)(42.8%), handles laundries in water cycle (*n*=11)(84.6%), urine samples from people with inflammation of the urinary tract (*n*= 4)(36.6%), wounds (*n*= 3)(30.0 %) and ear inflamed (*n*= 5)(41.6%).

Types of clinical samples, number of clinical samples for each type, and the number isolates were belonged to *P. aeruginosa* in each type were illustrated by the table (1).

Table (1): clinical samples number, positive isolate for *Pseudomonas aeruginosa* and percentage of positive isolates

Types of clinical samples	No	Positive	Percentage
Burn's patients	30	20	66.6 %
Flooring and Tools of burns unit	14	06	42.8 %
Handles laundries in in water cycle	13	11	84.6 %
Urine samples from people with urinary tract infection	11	04	36.6 %
Wounds	10	03	30.0 %
Ear infection	12	05	41.6 %
Total	90	49	54.4 %

As shown above, most *P. aeruginosa* isolates collected from burns patient, so this bacterium was considered the major agents of nosocomial infections in burn unit and the major cause of mortality and morbidity in These results agree with results of Al-Ammary(2013),fromBurn's patients = 66.6%,while these results are a little more of Jabbar(2014) from burn's patients = 60.0%. This closeness in the results may be due to the samples taken from the same place which dealt with the same ospitals.hospitalized patients (Saderi*et al*., 2010).

Bacterial Diagnosis

Pseudomonas aeruginosa was G-ve rods, motile and colonies on MacConkey agar appeared as small pale colonies due to lactose non-fermenting. This bacterium grew on Cetrimide agar as a selective medium,

and produced pigment capable to grew at 42°C and also at 4°C. It gave positive result for oxidase, catalase and Simmon's citrate, did not ferment glucose on Triple Sugar Iron medium (alkaline for both slant and butt) and did not produce H2S. Indole test for this bacterium was negative. Most isolates produce fluorescent greenish blue growth and pigment diffuses into medium called pyocyanin (Forbes *et al*.,2007). Ryan and Ray (2004) mentioned that the combination of oxidase positive colonies characteristicpyocyanin production and the ability to grow at 42°C as described by (Jawetz*et al*., 2001).

Molecular Methods for Identification of *Pseudomonas aeruginosa*

DNA extraction

The result showed that, the full amount of DNA obtained(Figure: 1) using this protocol was very efficient method for DNA extraction from *P. aeruginosa*, since good yields of genomic DNA were obtained .

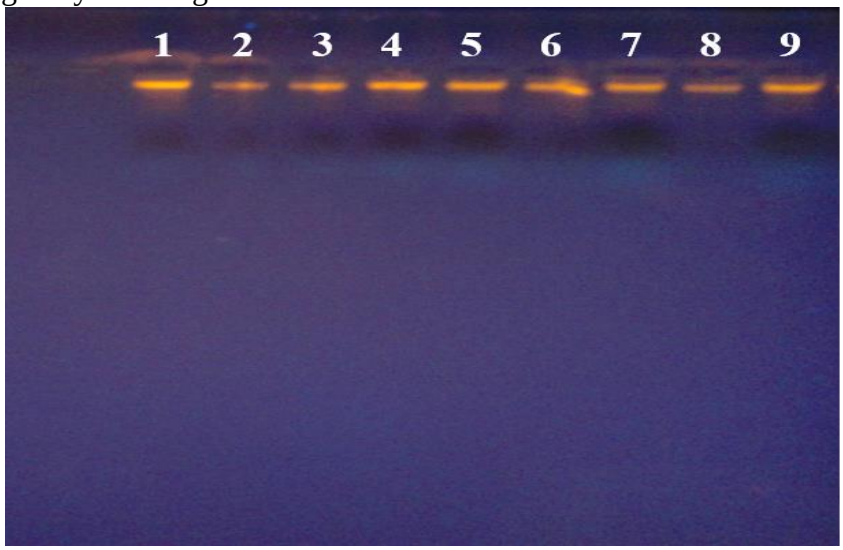


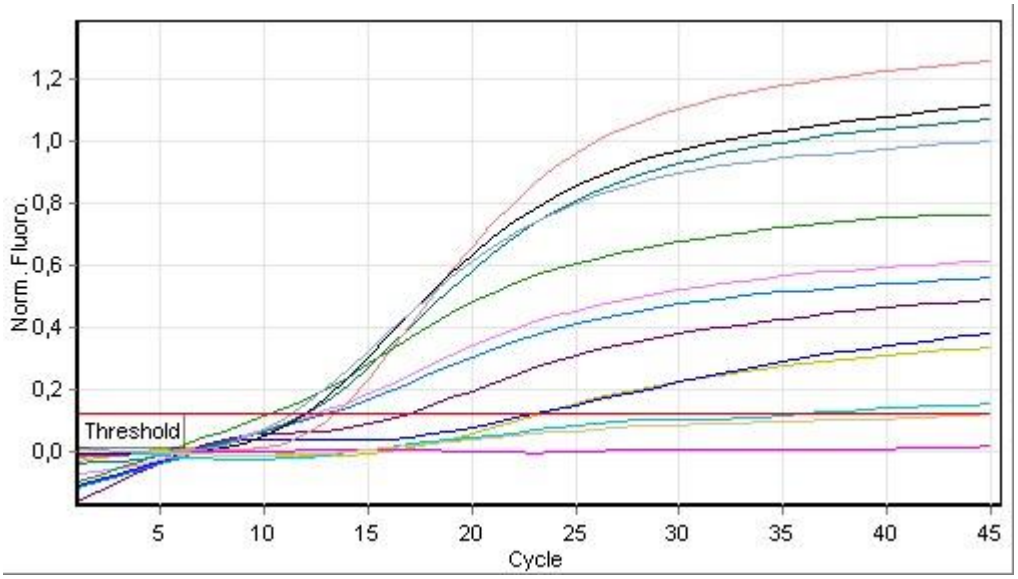
Figure (1): Genomic DNA bands visualized under UV after staining with ethidium bromide on 1% agarose gel at 1 volt/cm^2 for 30 min.

Real time PCR assay

real-time PCR detection with good sensitivity that can rapidly detect the target bacteria. *Pseudomonas aeruginosa* Detection Kit uses proven real-time PCR technology designed to provide: Highly selective identification of *P. aeruginosa* in a wide variety of samples , Verified performance , Ready-to-use convenience , Reduced risk of contamination ,(Khodabakhshi, *et al*., 2014**)** We use power chek *psedomonase aeruginosa* real-time PCR kit protocol to detected specific sequence of pathogen specific *oprl* gene for pseudomonase aeruginosa so this kit provides real time pcr master mix with enzyme components and the specific primer/ probe labeled with the fluorescent dyes.The target sequences are detected through the FAM channels for super-fast testing by Real-time PCR assay . The fluorescence curves are analyzed on FAM fluorescence detection to predict the presence or absence of *pseudomonas aeruginosa* specific gene in samples by analyzing the real time PCR results.

The evaluation power chek *psedomonase aeruginosa* real-time PCR kit By using qPCR all *p.aeruginosa* isolates were identified correctly fig2.So this study targeted the oprL gene, previously shown to be a more sensitive gene locus than the exotoxin A locus and evaluated

whether qPCR can improve early detection of *P. aeruginosa* in samples from patients, not yet infected with this organism.(Musafer,2013**)** In the past decade,several PCR formats and other molecula rmethods for the detection of *P. aeruginosa* have been developed. Some studies found a higher sensitivity of PCR in comparison with cultureand/or biochemical tests for the detection of *P.aeruginosa* from samples of patients by using real time PCR (Hassan,2012 pdf 7;Heyrman2007 pdf6)



Figure(2) RT-PCR detection of P.aeruginosa by using *oprl* probes

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