ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Specific Genetically Detection of *Pseudomonas aeruginosa* by using *ecf*X gene.

Sana MH AL-Shimmary *, Sara H. Seddiq, and Asmaa M Salih Almohaidi.

Biology Department, College of science for women/University of Baghdad, Baghdad, Iraq.

ABSTRACT

Phenotypic identification of *Pseudomonas aeruginosa* from specimens of wounds and burns patients carries a high risk of misidentification. Rapid and sensitive molecular method for its detection in clinical samples is needed to guide therapeutic treatment and to control *P. aeruginosa* outbreaks. The present study was targeted *ecf*X as the specific gene, for the accurate and rapid identification of *P. aeruginosa* from wounds and burns using real time PCR technique. This technique was evaluated against 45 clinical isolates that were suspected as *P.aeruginosa*. The results were compared with those obtained using a commercial biochemical identification kit and several other *P. aeruginosa* PCR assays. The results showed that the real time PCR assay is highly suitable for routine identification of *P.aeruginosa* isolates from clinical samples of Iraqi patients. This reliable technique may offer a rapid tool that would help clinicians to initiate an appropriate treatment earlier. Further investigations are needed to assess the clinical benefit of this novel strategy as compared to phenotypic methods.

Keywords: *Pseudomonas aeruginosa*, real time PCR, *ecf*x gene

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium which an opportunistic human pathogen responsible for chronic and acute infections in both the community and health care settings. (Yetkin *et al*., 2006) It is found in soil, water, skin flora and most man-made environments throughout the world(Lavenir *et al*., 2007) .

Pseudomonas aeruginosa is a main cause of nosocomial infections that may be cause septicemia and death with burning patients (Manafi *et al*., 2009). Burns represent a suitable site for bacterial infections, so exposed tissues for a longer time cause invasive bacterial sepsis of patients (Albornoz *et al*., 2011). *P.aeruginosa* is the most commonly source of chronic or acute infection of burns and wounds in Iraq, Europe and United States (Al_Kaisse *et al*., 2015; Gallagher *et al*.,2007 & Guggenheim *et al*., 2009)

Different diagnostic assays have been used for this bacterium, including phenotypic tests (Jin *et al*., 2011), enzyme linked immune sorbent assay (ELISA) (Mauch *et al*., 2014) electrochemical techniques(Webster *et al*., 2014), and molecular methods including conventional PCR(Aghamollaei *et al*., 2015) and real-time PCR (Deschaght *et al*., 2011; Alshimmary, 2016). However, some of these methods are time-consuming, rather complex, require specialized expertise, costly instruments, so a simple, cheaper, and rapid detection method is needed. This pathogen is considered to be easily distinguishable using microbiology techniques such as biochemical and structural tests, but they need several days to get the results (Procop, 2007). Rapid detection of pathogens which cause hospital infections is very important for rapid and accurate treatment decision of patients. Real time PCR has the possibility for rapid identifying microbial species by amplification of sequences singular to a particular organism (Cattior *et al*, 2010). Different molecular targets have been used such as , *opr*I, *opr*L, 16 S rRNA, *tox*A, *alg*D, *gyr*B, and *ecf*X genes (Lavenir *et al*. 2007; Qin *et al*. 2003; Jaffe *et al*. 2001). Since false negative results (with *tox*A and *alg*D genes) as well as false-positive results (with *opr*I and 16 S rRNA genes) have been reported, the *ecf*X gene is a best target for specific identification of *P.aeruginosa* isolates (Anuj *et al*., 2009; Laviner *et al*., 2007). The *ecfX* gene encodes an ecf sigma factor (extracytoplasmic function sigma factor), which is involved in haem uptake and virulence, so the *ecf* factors, involved in organizing the systems of iron uptake, are classified as the iron-starvation class (Leoni *et al*., 2000). These characters are represented by controlling the transcription of the gene which is responsible of producing pyoverdin siderophore (Imperi *et al*., 2013). Pyoverdin is used to solubilize ferric iron in acute infections and plays a role in forming QS during chronic infection (Nguyen *et al*., 2014). The *ecfX* gene is a suitable target for specific identification and detection of *P. aeruginosa* isolates, many studies showed that *ecfX* PCR test gives a highly reliable, and PCR products for all *P. aeruginosa* strains tested and not amplifying DNA for other species (lavenir *et al*., 2007; Cattoir *et al*., 2010).

Aim of study

Rapid detection of *P.aeruginosa* by using real time PCR assay with *ecf*X gene which were isolated from Medical sources

MATERIALS AND METHODS

Bacterial Collection and Isolation

The 45 isolates of *Pseudomonas aeruginosa* were collected in six mounths starting in December 2016 till May 2017. The clinical samples were collected from wounds and burns patients in Al-Yarmouk General Teaching Hospital in Baghdad. The study population included both gender with different ages.

All collected samples were cultured on MacConkey agar, Blood agar and nutrient agar, then incubated aerobically at 37°C for 24 hours (Macfaddin, 2000). The next day the single colony test for biochemical tests represented by Oxidase, Catalase and API 20NE tests depending on manufacture company information.

ISSN: 0975-8585

Extraction of Bacterial DNA

The bacterial DNA was extracted using Wizard genomic DNA purification kit (Promega ,USA) according to the manufacture protocol. The extracted DNA [shown in fig.(1)] solution was stored at 20- C˚.

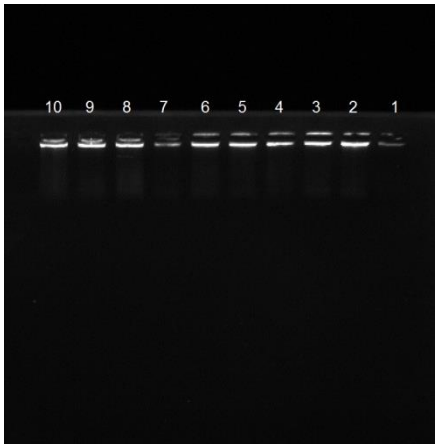

Fig.1: agarose gel electrophoresis of genomic DNA using 0.8% agarose.

Real Time PCR Assays

The quantitative PCR assay was performed with the mic PCR version 2.0.0. In the test, using oligonucleotide primers and probe set that checked with GenBank to ensure their specificity to *P.aeruginosa* [shown in Table (1)] for specific detection of a 152-bp fragment within the *ecf*X gene. The amplification mixture consisted of 12.5µl GoTaq Probe qPCR MasterMix (Promega), 2µl each primer, 1µl each probe, and 2µl of template DNA in a final volume of 25µl. Following a hold step including 1) at 50 C for 2 min. 2) 95 C for10 min. 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 60 seconds. The final result was available in 1 hour and 6 minutes.

Table 1: *ecf*X Oligonucleotides was used in this study

Oligonucleotides	Sequence (5'-3')	Reference
*ecf*X F-primer	TTCCATGGCGAGTTGCT	(Cattoir *et al*,2010)
*ecf*X R-primer	CGGGCGATCTGGAAAAGAA	(Cattoir *et al*,2010)
*ecf*X prob	FAM-GCTGAAATGGCCGGGC-BHQ	(Cattoir *et al*,2010)

FAM = carboxyfluorescein; BHQ = black hole quencher

RESULTS AND DISCUSSION

P.aeruginosa is considered as a ubiquitous microorganism which colonizes in a wide spectrum of habitats. It is extremely detected in many environments and the danger to human health related with its has been estimated (Mena and Gerba 2009).

All isolates of *P.aeruginosa* which gave positive results in MacConky agar and blood agar, Oxidase and Catalase and API20 NE tests [shown in table (2)], undergo to DNA extraction to get a pure bacterial DNA. Molecular identification of *P. aeruginosa* isolates that were recovered from burns and wounds samples represented by using real time PCR technique which target *ecf*X gene. The positive results represented by curves that cross threshold line. The CT values ranged from 25 to~35cycles [shown in fig.2].

ISSN: 0975-8585

Table 2: summarizes the data of cultural and biochemical technique

Types of isolates	MacConkey agar	Blood agar	Oxidase test	Catalase test	API20NE test
27 isolates from burns	+	+	+	+	+
18 isolates from wounds	+	+	+	+	+

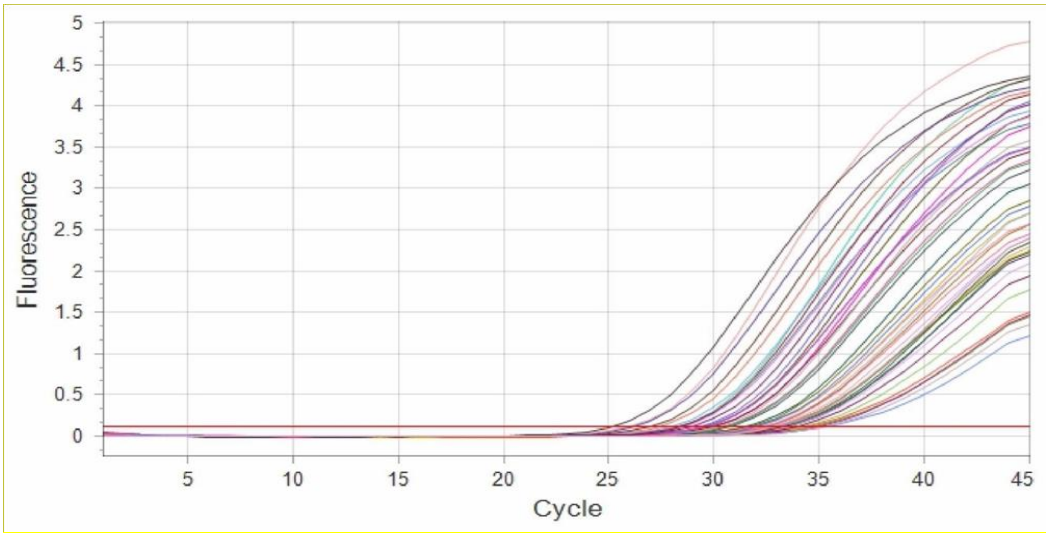

Fig.2: Shows the results of *P. aeruginosa* isolates in real time PCR technique depending on *ecf*X gene

Exact identification of *P.aeruginosa* in the laboratory remains an essential component of patient treatment and controlling the nosocomial infections.

Unfortunately, there is no single test that using to identify *P. aeruginosa*. The limitations of biochemical and phenotypic tests which are documented by many reports (Qin *et al*. 2003; Anuj *et al*. 2009; Alshimmary *et al*., 2016) which they showed *P. aeruginosa* isolate was misidentified by using the API 20NE test. For previous finding, the demanding needs for accurate and specific test, so that Molecular diagnostic by targeting the *ecf*X gene with real time PCR assay may be more suitable for the identification of *P. aeruginosa*.

Real time PCR is considered more sensitivity, accurate and faster methods than the routine biochemical and cultural techniques for identification of bacteria and investigated a suitable for routine identification purposes (Ferreira *et al*., 2011). One study in Indonesia by Hatta *et al*., (2007) recorded a sensitivity of PCR is 94.5%, so they were concluded that the PCR method was much better to others tests giving very high sensitivity and specificity. Molecular techniques need more careful processing of samples to get a good quality of DNA for test, sheared or degraded DNA that used for the PCR (Maddox and Fales, 1991). The main advantage of PCR technique than classical assays is providing results in few hours while routine biochemical and cultural tests need several days (Jamil *et al*., 2007). In fact, because of the rapid and accurate diagnosis, hospital entry of the patient can be averted, reducing suffering of patients, save working days and unnecessary cost on misdirected and unrelated treatment which probably many times higher than the expenditure of PCR (Hatta *et al*., 2007). The molecular tests may be used to detect the resistance genes like emerging carbapenemase genes(Potron *et al*., 2015) In fact, the choice of treatment by antibiotic has to consider local and national epidemiology because multidrug resistant *P. aeruginosa* isolates are progressively reported worldwide (Rahim *et al*., 2016), therefore, in order to decrease the risk of unsuitable treatment, the use of set antimicrobial therapy, until antimicrobial sensitivity results become known, may be superior in many situations(Bhawsar and Singh, 2017). This is confirming by the fact that the *P. aeruginosa ecf*X gene was described as being reliable PCR targets for detection of this bacterium previously (Anuj *et al*, 2009; Lavenir *et al*. 2007; Qin *et al*. 2003) and that no false-negative results or cross-reactions were noticed using the *ecf*X gene

target in these studies. The final conclusion from present work is using molecular diagnostic by specific genes and *ecf*X looks an optimum gene.

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