Detection of Exotoxin A gene in *Pseudomonas aeruginosa* from Clinical and Environmental samples

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Abstract

PCR was used to detect *Pseudomonas aeruginosa* from clinical and environmental samples by amplifying a 396-bp region of the exotoxin A (ETA) structural gene sequence. Specific primer amplified ETA – positive *P. aeruginosa* DNA, whereas other species of Pseudomonas and GC-rich bacteria did not yield any 396-bp fragment. The specificity of the assay was 1% (just one isolate from 100 isolates used in this study),this gene is not present in these isolates which means that these isolates do not have the ability to produce the Exo A toxin. With this PCR method, ETA – positive *P. aeruginosa* was detected only in one water sample in comparison with the use of the 16SrDNA gene which yielded 100% positive results of all tested isolates. This PCR method is rapid and more accurate than other diagnostic methods for the identification of *P. aeruginosa* strains, and it can be used to detect a low level of *P. aeruginosa* from different samples without using a selective medium or additional biochemical tests.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting both humans and animals. *P. aeruginosa* is an important cause of bacteremia in patients receiving organ transplants and is responsible for about 28% of most bacteremia episodes [1]. Pulmonary colonization with mucoid *P.aeruginosa* is also a major cause of morbidity and mortality in patients with cystic fibrosis [2].

Correa *et al.* 1991[3] have reported that vegetables were the main source of *P. aeruginosa* in hospitals even though 1% hypochloride solution is used for sanitizing the vegetables.

P. aeruginosa has a wide arsenal of virulence factors at its disposal. Among these virulence factors are a variety of secreted factors, such as proteases, phospholipases, and the exotoxin A.

P.aeruginosa strains also posses a type III secretion system that allows them to deliver toxins (effectors) directly into the cytoplasm of a host cell [4], Exotoxin A which causes tissue necrosis since it blocks protein synthesis [5].

P. aeruginosa strains producing relatively large amounts of exotoxin A (ETA) and proteases at the level of 107 cells per ml in drinking water of mice which can cause endogenous bactermia in few days [6;7].

Exotoxin A (ExoA, *toxA*) is a 66 kDA protein acts as a major virulence factor of *P. aeruginosa*, analogous in action to that of diphtheria toxin. ExoA is a highly virulent protein, exhibiting and LD50 of 2.5 mg/kg in mice, it has been shown that Δ*toxA* mutants are less virulent than wild type strains, and that vaccination against ExoA confers partial immunity to *P. aeruginosa* infection in animals [8].

Injection of purified ExoA results in leucopaenia, hepatic necrosis, hypotension and shock when injected into test animals. On a microscopic level, collagen is disrupted, proteoglycan ground substance is lost and widespread endothelial and epithelial cell death is observed [9].

P. aeruginosa produces two different ADP-ribosyltransferase toxins: ETA and exoenzyme S [10; 11; 8; 12]. Exoenzyme S causes significant tissue damage in lung, burn and wound infections [12]. The highly toxic ETA is produced by the majority of *P. aeruginosa* strains and can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor 2, similarly to diphtheria toxin [12].

ETA consists of two subunits; fragment A is catalytic, and fragment B is responsible for interaction with eucaryotic cell receptors. ETA is cytotoxic to numerous mammalian cells [13],

stimulates in vitro production of interleukin-1 in murine peritoneal macrophages [14] and induces murine cytotoxic T lymphocytes [15]. Gray *et al*. 1984 [16] have cloned and sequenced the ETA structural gene from *a P.aeruginosa* strain overexpressing ETA.

Because *P. aeruginosa* is medically important, various methods have been developed to rapidly and accurately identify *P.aeruginosa* species. Although conventional microbiological methods for identifying *P. aeruginosa* from environmental samples are reliable, they require several days to complete. PCR has the potential for identifying microbial species rapidly by amplification of gene sequences unique to a particular organism [17].

In this paper, PCR technique was used to detect *P. aeruginosa* strains in clinical and environmental samples by amplifying ETA structural gene.

Materials and Methods

Bacterial isolates and growth conditions

Fifty environmental isolates and fifty clinical isolates of *P. aeruginosa* were obtained from different sources (Table (1)). All isolates were maintained in MacConky broth (CDH, NewDelhi) containing 20% glycerol at -20°C.

Organisms were grown overnight at 37°C in MacConky broth or on citrimide agar (Microgen, New Delhi) plates. *P. aeruginosa* was isolated from water samples was isolated by plating on cetrimide agar and incubated at 37°C for 48 hrs.

Isolated colonies were identified by using the oxidase test, and using Api – 20 system [18]. A positive strain was obtained from the general health laboratory, Ministry of Health.

Table (1)
Clinical and Environmental isolates used to test the specificity of the ExoA gene in PCR assay to detect P. aeruginosa isolates.

Sample Sources	*No. of P. aeruginosa isolates*
Environmental samples	
• Dijla, Al-Furat and Diyala Rivers	29
• Wells	21
Clinical samples	
• Exudates from wounds of the burn units patients	23
• Seputum from CF patients	14
• Urine from UTI patients	13

Bacterial DNA preparation

P. aeruginosa (from clinical samples) was grown overnight in MacConky broth at 37°C. Cells were harvested from 1-ml bacterial cultures, washed once with TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0, and resuspended in 10 ml of sterile distilled water.

Also cell recovery from water samples by filtration of *P. aeruginosa* isolates was grown overnight in MacConky broth at 37°C and collected by filtration through 45-mm-diameter Fluoropore filters under vacuum [19].

Isolation of genomic DNA

Genomic DNA was isolated from bacteria using DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

Genomic DNA Mini kit system can provide a rapid method to purify chromosome and plasmid DNA, the procedure followed the manufacture company instruction.

Primer selection

The primers used in this study are given in Table (2). The primers (ETAl and ETA2) specific to *P. aeruginosa* were chosen from the published sequence [16] of the ETA operon and amplify a 396-bp region of the structural gene.

Amplification

The amplification reaction was performed by using a Verti 96 wells DNA thermal cycler (AB: applied Biosystem Company, USA) and GeneAid kit with Taq DNA polymerase enzyme in 0.5-ml microcentrifuge tubes. The

reaction mixture (25-µl total volume) consisted of 6.5 µl of sterile water, 12.5 µl of 10x PCR Master Mix buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 4 u1 of deoxyribonucleo-side triphosphates; 2.5 mM each dATP, dTTP, dGTP, and dCTP, and 0.25 µl (0.5 U/Iul) of Taq DNA polymerase), 0.5 µl of each primer (stock concentration, 100 µM), 5 µl of template. The samples were subjected to 30 cycles of amplification [17].

Preincubation was at 95°C for 2 min. 30 PCR cycles were run under the following conditions: denaturation at 94°C for 1 min, primer annealing at 68°C for 1 min, and DNA extension at 72°C for 1 min in each cycle. After the last cycle, the PCR tubes were incubated for 7 min at 72°C. Five microliters of the reaction mixture was analyzed by standard gel electrophoresis (1.5 % µg/ml agarose; 5 V/cm), and the reaction products were visualized by staining with ethidium bromide (0.5 µg/ml in the running buffer).

A reagent blank contained all components of the reaction mixture except template DNA, for which sterile distilled water was substituted. This step was included in every PCR procedure.

Table (2)
Sequences of oligonucleotide primers.

a	*b*	*c*	*d*
Region	***Size bp***	***Primer Sequence***	***Tm (°C)***
1001-1024	396	ETAl 5'-GACAACGCCCTCAGCATCACCAGC-3'	73
1373-1396	396	ETA2 5'-CGCTGGCCCATTCGCTCCAGCGCT-3'	75

a. Positions of the first and last nucleic acids of the primer in the targeted fragment. The structural gene extends from position 959 to 2662.
b. Number of nucleotides in the amplified fragment.
c. ETAl-ETA2 is a primer pair.
d. The melting temperature (Tm) was calculated by using the following formula:

Tm=[4°C (G+C content of oligonucleotide) + 2°C (A +T content of oligonucleotide)] – 5C.

Results and Discussion

Morphological properties and biochemical tests for the isolates agreed with those for *P. aeruginosa* [1]. All isolates gave positive results for the smears taken as a gram negative bacteria, the oxidase test was positive, and tests of the Api-20 system gave positive results which were in accordance with the properties mentioned by Brook [1].

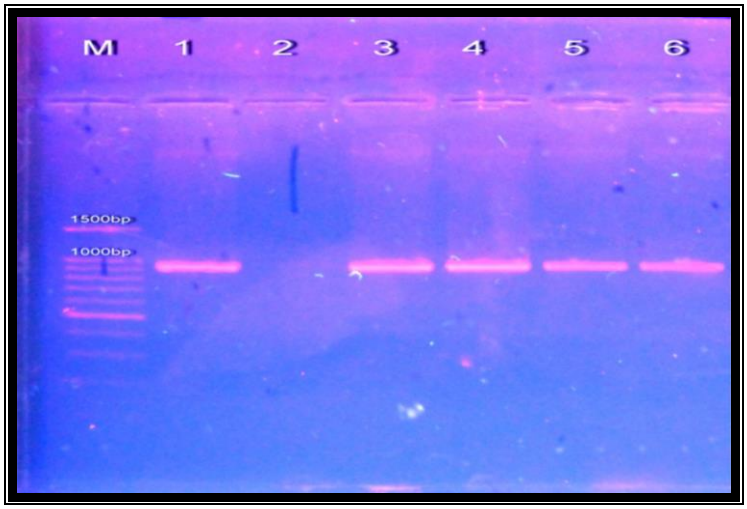

Fig.(1) Amplification of the 16SrDNA gene to ensure that these isolates are P. aeruginosa. lane 1;positive control, lane 2; negative control, lanes 3,4,5 & 6 ; positive results with 996bp gene product.

To determine the presence of *P. aeruginosa* in water samples, ETAl and ETA2 primers were used. Preliminary experiments were made to determine the optimum PCR amplification conditions utilized genomic DNA from a *P.aeruginosa* ETA gene positive isolate as the source of template DNA. All the 100 isolates used in this study gave positive results for the prescence of the 16SrDNA gene.

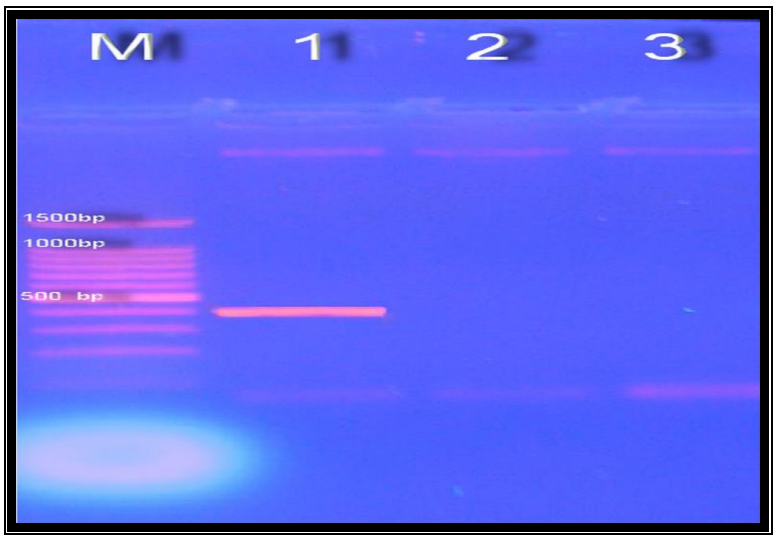

Fig.(2) Agarose gel electrophoresis of 396-bp ETA amplified DNA from environmental sample. Lane1, 396-bp amplified product from environmental sample; lane2 positive control, no product; lane 3 negative control.

Primer annealing temperatures close to the theoretical primer melting points allowed amplification of a single 396-bp product in one isolate only (Fig. (2), lane 1).

The amplified products (5 µl) were analyzed on a 1.5% agarose gel (Fig. 2). These results showed a 396-bp PCR-positive DNA for one sample only in lane 1 (Fig.(2)).

One percent of the 100 strains of *P. aeruginosa* was positive for the ETA gene tested by PCR in comparison with morphological and biochemical characteristics and the amplification results of the 16SrDNA gene product of *P. aeruginosa*.

These results indicate that sequences in these isolates which are upstream or downstream of the ETA structural gene are absent, or are rearranged from isolate to isolate, or the gene may not be found.

Interestingly, Engel(2003) found that sequences upstream of ETA genes are rearranged between strains [8].

In conclusion, these results indicated the non-specificity of PCR technique to detect *P. aeruginosa* isolates which have ETA gene.

Also we have presented data suggest that PCR can be used to provide rapid and specific detection of small numbers of *P. aeruginosa* in environmental samples against a background of other bacterial species.

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الخلاصة

استخدمت تقنية الـ PCR للتحري عن بكتريا الـ *Pseudomonas aeruginosa* مِن العيناتِ السريريةِ والبيئيةِ بتَضْخيم منطقة حجمها 396bp من تسلسل السلسلة الهيكليةِ للجين exotoxin A (ETA) . يقوم بادئ متخصص بتضخيم الجين في البكتريا الموجبة لوجود الجين فيها، بينما الانواع الاخرى لجنس السيدوموناس وغيرها من البكتريا الغنية بمحتوى القواعد GC لاتعطي أي ناتج للتضخيم . يبلغ التخصص لهذه الطريقة ١ % فقط (فقط عزلة واحدة من مئة عزلة استخدمت في هذه الدراسة)، امكانية عدم وجود الجين في السلالات قيد التحري والذي يعني انها غير قادرة على انتاج هذا النوع من السموم (Exo A toxin) . مع طريقة التضخيم هذه،تم فقط الكشف عن وجود عزلة واحدة موجبة لوجود الجين هذا فيها في عينة ماء واحدة فقط بالمقارنة مع تم استحصاله من نتائج لتضخيم الجين 16SrDNA gene الخاص بهذه البكتريا والذي اعطى نتائج ايجابية بنسبة ١٠٠% . تعتبر تقنية الـ PCR طريقة سريعة واكثر دقة من طرق التشخيص الاخرى للتحري عن بكتريا الـ *Pseudomonas aeruginosa*، وهي ممكن ان يستفاد منها حتى في كشف الكميات القليلة جدا من البكتريا في النماذج المختلفة من دون الحاجة لاستخدام اوساط اختيارية للنوع المعين او دون اللجوء لاجراء الاختبارات البيوكيميائية التقليدية.