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Molecular Rapid Diagnostic Method for Nosocomial Non-Fermenting Gram-Negative Bacilli

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Abstract

For appropriate antibiotic therapy and avoid unnecessary use of drugs, accurate diagnosis of Nosocomial Infections (NI) is crucial. There are three Gram-negative bacteria in NI's; *P. aeruginosa, A. baumannii* and *S. maltophilia*, which may misdiagnosed with together in traditional diagnostic methods. Due to this, a rapid molecular diagnosis kit has been designed in Shiraz Burn Research Center. According to the sensitivity and specificity percent analyses results, following results were obtained respectively: *P. aeruginosa*: 100%, 96.8%; *A. baumannii*: 100%, 100% and *S. maltophilia*: 96.7%, and 93.8%. Based on these results this multiplex-PCR kit with three primer sequences is responsible for timely and appropriate detection of studied bacteria.

Keywords: ***Pseudomonas aeruginosa*; *Acinetobacter baumannii*; *Stenotrophomonas maltophilia*; Nosocomial Infection**

Introduction

In recent years, hospital-acquired infection or Healthcare-Associated Infection (HAI) is one of the major difficulties for health care professionals to tackle. HAI is occurring during the hospitalization and usually is the result of an unsafe process for patient's care [1]. Although HAI often considered at the hospitalization time (usually 3 days after hospitalization) but this may also appear after discharge from the hospital. Based on the last WHO reports, HAI represents the most frequent adverse event during the health care provides, and no country or institute can claim to have solved it so far [2]. One of the reasons for lack of controlling the HAI is changing the pattern of infections in kind and their resistance, which poses many challenges for health centers and patients. In general, there are several factors in lack of infection control in patients. Since the first and most common method in patients infection control is prescribing antimicrobial agents, then it is very important to identify the type of infection and its resistance pattern. One of the problems facing the healthcare system is the lack of timely and accurate diagnosis of the infections. This condition can be mainly due to the following factors: I) emerging of new infections, II) reemerging of resistance microorganisms and III) the difficulty of traditional methods in bacterial detections. For these reasons, besides infection prevention and control measures proper policy making and timely implementation such as prevalent infection detection and their proper standard resistance pattern can play an important role in infection control. Based on WHO reports, approximately 15% of total hospitalized patients (more in developing countries) around the world suffer from HAI [3]. *Pseudomonas aeruginosa* is an opportunistic bacterium which is in the first line of Gram-negative Nosocomial infections in most centers. In recent years, two other Gram-negative bacteria; *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*, are increasingly recognized as an emerging global opportunistic pathogens causing different kind of HAI's such as bacteremia, pneumonia, endocarditis, and meningitis, as well as urinary tract, ocular, bone and joint, skin, and soft tissue, and gastrointestinal infections [4,5]. Due to some of the metabolic similarities between these bacteria, their identification and differentiation from each other can be problematic for microbiologists. In many cases, isolation of these bacteria from clinical specimens may be misidentified [4,6]. According to several reports around the world, these three infections are increasing rapidly, due to wrong or miss diagnosis of such group of infections [7,8]. For these reasons and some other problems such as Time-consuming and the cost of phenotypic traditional methods in identification and differentiation of these three Gram-Negative bacteria we designed a rapid molecular test based on simple Polymerase Chain Reaction (PCR). In this method, three sequences of primers with following specifications in Table 1 were used. Primers were designed by Primer3 software (http://bioinfo.ut.ee/primer3/) by using custom-designed primer sets. The designation was done based on the NCBI database sequences for three studied bacteria by following specifications: *Pseudomonas aeruginosa*; GenBank ID:

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Table 1: Novel three designed primers for detection of studied bacteria.

Primer Names	Sequences (5'->3')		Products size (bp)		
Ac-Ps-St-F	CGBATGAAGTTCAACCGTCG	Ta (C)	*P. aeruginosa*	*A. baumannii*	*S. maltophilia*
Ac-R	CTACAGCAGAGTCGGCC	57	241	691	307
Ps-St-R	CCATSGACAGGCGYTCCTT				

Table 2: Calculation of Accuracy, Sensitivity and Specificity predictive value.

Test results	True results (No., %)	False results (No., %)
Positive test results	True Positive (TP)	False Positive (FP)
	PA: 30, 100	PA: 1, 3.3
	AB: 30, 100	AB: 0, 0
	ST: 29, 96.7	ST: 2, 6.6
Negative test results	True Negative (TN)	False Negative (FN)
	PA: 30, 100	PA: 0, 0
	AB: 30, 100	AB: 0, 0
	ST: 30, 100	ST: 1, 3.3
$Accuracy = \frac{TP + TN}{TP + FP + FN + TN} \times 100$	$Sensitivity = \frac{TP}{TP + PN} \times 100$	$Specificity = \frac{TN}{FP + TN} \times 100$

CP015117.1, *Acinetobacter baumannii*; GenBank ID: CP042841.1, and *Stenotrophomonas maltophilia*; GenBank ID: CP008838.1. For control positive, following isolates were chosen; *Pseudomonas aeruginosa* (ATCC 25668), *Acinetobacter baumannii* (ATCC BAA-747) and *Stenotrophomonas maltophilia* (ATCC 13637). For following of the confirmation and applicability of the test, 30 isolates from each studied bacteria (total of 90 isolates) from our collection bank were tested. These isolates have been previously were collected from different clinical samples in different hospitals affiliated with Shiraz University of Medical Sciences (Fars, Shiraz, Iran) and screened by gold standard microbiological tests. Either these isolated were confirmed previously either by API 20E test kit (BioMrieux, France), and molecular method with introduced methods in previous studies [9-12]. All of the clinical isolates were tested either by introduced method for control positives. In brief, designed multiplex-PCR amplification for isolates was performed in 50 µl reaction volumes containing 44 µl of reaction mixture containing 2 mM $MgCl_2$, 1X of PCR buffer, 0.25 mM each deoxy nucleotide triphosphate, 0.4 pmol/µl of each primers, 2 U of Taq DNA polymerase and 2 µl of each nucleic acid extracts with the following setting: 1 cycle at 94°C for 10 min denaturation followed by 35 cycles at 94°C for 45 sec, annealing temperature 57°C for 45 sec and 72°C for 1 min and a final extension at 72°C for 20 min. Final products were electrophoresis on 1.5% agarose gel. A 100 bp molecular weight marker was used for band detection. Specific bands with expected sizes (Table 1) have been considered positive for test results. Although the primer blast evaluations for designed primers showed no probable nonspecific band for other microorganisms, but still for probable false positives some other clinical isolates (total of 30 isolates) of Gram-negative bacteria such as *E. coli*, *Klebsiella* sp., *Burkholderia* sp. and *Shigella* were tested either. For accuracy, sensitivity and specificity of designed test, following analysis in Table 2 was performed [13]. Based on these analyses the accuracy, sensitivity and specificity percent for studied bacteria were as following respectively: *P. aeruginosa*: 98.4, 100, 96.8; *A. baumannii*: 100, 100, 100 and *S. maltophilia*: 95.2, 96.7, and 93.8.

According to these results it can be deducted that designed primers for the studies bacteria are responsible for isolation and detection of pointed bacteria from clinical samples. Although there are many techniques such as phenotypic microbiological tests and molecular tests based on different primers, these methods are very time consuming and costly. In this new designing we tried to reduce the microbiological tests processes except colony purification. Either for reducing the costs and steps in molecular technique, a multiplex-PCR with three primers and high specificity and sensitivity has been designed. This test can be used as an alternative for diagnosis of three common and emerging infections (*P. aeruginosa, A. baumannii and S. maltophilia*) with conventional diagnostic methods. Using this test can reduce the detection time from at least 4 to 5 days to a maximum of two days. This alternative can be very helpful in timely diagnosis and treatment of patients and to accurately distinguish these three infections in hospitalized patients. To improve the methods of diagnosis of infectious diseases and apply new techniques in this regard it is very important to know, lack of perfect diagnosis and accurate differentiation of new emerged infection with previous probable infection can be problematic in control infection in hospital settings. This bunch of errors may results in prolonged hospital stays, long-term disability, and increased resistance of microbes to antimicrobials, additional costs for healthcare systems, high costs for patients and their family, and increased unnecessary mortality rate.

Some of the important policies which should be following up seriously are evaluating prevalent infections periodically in terms of type and resistance pattern. According to some experiences, it is recommended this kind of evaluation perform for inpatient treatment centers, separately and base on wards [14]. In following, the output of these evaluations should be made available to clinicians monthly to make empirical treatment based on that. In a previous study by the same team in southwest of Iran burn center (Amir-Al-Momenin burn hospital, affiliated with SUMS) it has been resulted that *Acinetobacter* sp. have been emerged and have miss diagnosed with *Pseudomonas* sp. is many collected samples. Nowadays, PCR method is a significant technique in the diagnosis of specific pathogens which are difficult or time-consuming in detection by phenotypic procedures for clinical aspects [15].

However, according to different studies results application of PCR to clinical specimens has many potential pitfalls due to the

susceptibility of PCR to inhibitors, contamination, and experimental conditions [16-18]. Given that it has been shown that the sensitivity and specificity of a PCR method depend on many factors such as: Target genes, primer sequences, PCR technique, and DNA extraction methods, in the recent study the designation have been improved based on recognizing these problems. One of the problems with the application of molecular methods in clinical practices is the detection of infection in different samples such as wound, blood, urine, sputum, and others. This difference in regard to the nature of the content and amount available samples, careful design of the molecular is essential. One of the important points in designing the molecular methods is the application of appropriate DNA extraction method based on the sample nature [15,19]. Based on these, the recommendation for the recent study is culturing the samples (from any kind) on general or specific enriched bacterial media for purification purposes. In following, samples management, the extraction technique and the molecular sensitivity and specificity of method will be organized perfectly in the hospital laboratory settings. In conclusion, for appropriate antibiotic therapy of infections and avoid unnecessary antibiotic use, accurate diagnosis of bacterial infection is crucial. Although the history and examination of patients to find disease definitions seem to fits with clinical aspects more than one diagnostic modality is usually required for confirmation either to confirm or exclude a diagnosis.

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