



Molecular Rapid Diagnostic Test for Nosocomial Non-fermenting Gram-Negative Bacilli

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Abstract

Accurate diagnosis of nosocomial infections is crucial for appropriate antibiotic therapy, as well as avoiding unnecessary use of drugs. Mainly 3 gram-negative bacteria including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia* are involved in nosocomial infections. These bacteria are possibly misdiagnosed with together in traditional diagnostic methods. In this regard, a rapid molecular diagnostic kit was developed in Shiraz Burn Research Center. Using this diagnostic kit, the sensitivity and specificity analyses showed following results, respectively: *P. aeruginosa*: 100%, 96.8%; *A. baumannii*: 100%, 100%, and *S. maltophilia*: 96.7%, and 93.8%. Therefore, this multiplex polymerase chain reaction (PCR) method with 3 primer sequences could be a responsive technique in timely and appropriate detection of studied bacteria.

Keywords: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, Nosocomial infection

Background

In recent years, hospital-acquired infection or healthcare-associated infection (HAI) has been one of the major difficulties of healthcare professionals to tackle. HAI occurs during the hospitalization and usually is the result of an unsafe process in patient care (1). Although HAI often occurs during the hospitalization (usually 3 days after hospitalization), it may also appear after discharge from the hospital. Based on the latest reports of WHO, HAI represents the most frequent adverse event which is acquired during receiving care from healthcare providers, and no country or institute can claim to have solved it so far (2). One of the reasons for lack of control over HAI is frequent changes in the pattern of infections regarding their kind and resistance, which poses many challenges for health centers and patients. In general, several factors are involved in lack of infection control in patients. Since the first and most common method for infection

control in patients is prescribing antimicrobial agents, it is very important to identify the types of infection and their resistance patterns. 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: I) emergence of new infections, II) reemergence of resistant microorganisms, and III) difficulty of traditional methods in bacterial detections. For these reasons, in addition to infection prevention and control measures, proper policy making and timely implementation of infection detection and their proper standard resistance patterns can play an important role in infection control. Based on the WHO reports, approximately 15% of total hospitalized patients around the world (more in developing countries) 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

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viz Acinetobacter baumannii and *Stenotrophomonas maltophilia* have increasingly been recognized as emerging global opportunistic pathogens which have caused different kinds of HAI including bacteremia, pneumonia, endocarditis, and meningitis, as well as urinary tract, ocular, bone and joint, skin, soft tissue, and gastrointestinal infections (4,5). Considering some of the metabolic similarities between these bacteria, their identification and differentiation from each other can be problematic for microbiologists. In many cases, a misidentification may occur during isolation of these bacteria from clinical specimens (4,6). According to several reports around the world, these 3 infections are rapidly increasing, due to misdiagnosis of such group of infections (7, 8). For these reasons and some other problems such as being time-consuming and the cost of phenotypic traditional methods in the identification and differentiation of these 3 gram-negative bacteria, we developed a rapid molecular test based on simple polymerase chain reaction (PCR). In this method, 3 sequences of primers (specified in Table 1) were used. Primers were designed by Primer3 software (<http://bioinfo.ut.ee/primer3/>) using custom-designed primer sets. The designation was done based on the NCBI database sequences for 3 studied bacteria using the following specifications: *Pseudomonas aeruginosa*; GenBank ID: CP015117.1, *Acinetobacter baumannii*; GenBank ID: CP042841.1, and *Stenotrophomonas maltophilia*; GenBank ID: CP008838.1. In addition, the isolates of *Pseudomonas aeruginosa* (ATCC 25668), *Acinetobacter baumannii* (ATCC BAA-747), and *Stenotrophomonas maltophilia* (ATCC 13637) were chosen as positive controls. To confirm the applicability of the test, 30 isolates from each studied bacterium (total of 90 isolates) out of our collection bank were tested. These isolates were previously collected from different clinical samples in different hospitals affiliated to Shiraz University of Medical Sciences (Fars, Shiraz, Iran) and screened by gold standard microbiological tests. These isolates were previously confirmed by either API 20E test kit (BioMérieux, France) or a molecular method introduced in previous studies (9-12). All of the clinical isolates were tested by an introduced method for positive controls. In brief, designed multiplex PCR amplification was performed for the isolates in a 50- μ L

reaction volume containing 44 μ L of reaction mixture that contained 2 mM MgCl₂, 1X of PCR buffer, 0.25 mM each deoxynucleotide triphosphate, 0.4 pmol/ μ L of each primer, 2U of Taq DNA polymerase, and 2 μ L of each nucleic acid extract with the following setting: initial denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 1 minute, and a final extension at 72°C for 20 minutes. Final products were electrophoresed on 1.5% gel agarose. A 100 bp molecular weight marker was used for band detection. Specific bands with expected sizes (Table 1) were considered positive for test results. To evaluate the sensitivity of the test based on bacterial copy number, the mentioned test was performed in 3 categories. To this end, the test isolates were primarily prepared in 0.5 McFarland standard turbidity. Afterward, two more dilutions (1:10 and 1:100) were prepared from 0.5 McFarland dilutions. All of the dilutions were extracted and tested twice with the introduced method.

Though the primer blast for designed primers showed no nonspecific band for other microorganisms, for preventing probable false positive results, some other clinical isolates (total of 30 isolates) of gram-negative bacteria such as *E. coli*, *Klebsiella* species, *Burkholderia* species, and *Shigella* were also tested. The accuracy, sensitivity, and specificity of the designed test were evaluated by an analysis based on Table 2 calculations (13). Based on this analysis, the accuracy, sensitivity and specificity of the studied bacteria were as follows, 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 deduced that designed primers for the studied bacteria are responsible for the 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 design, we tried to reduce the microbiological test processes except for colony purification. Therefore, for reducing the costs and steps in molecular techniques, a multiplex PCR with 3 primers and high specificity and sensitivity was designed. This test can be used as an alternative for the diagnosis

Table 1. Novel 3 Designed Primers for Detection of Studied Bacteria

| Primer Names | Sequences (5'->3') | Temperature annealing (°C) | Product Size (bp) | | |
|-------------------|----------------------|----------------------------|----------------------|---------------------|-----------------------|
| | | | <i>P. aeruginosa</i> | <i>A. baumannii</i> | <i>S. maltophilia</i> |
| <i>Ac-Ps-St-F</i> | CGBATGAAGTTCAACCGTCG | 57 | 241 | 691 | 307 |
| <i>Ac-R</i> | CTACAGCAGAGTCGGCC | | | | |
| <i>Ps-St-R</i> | CCATSGACAGGCGYTCCTT | | | | |

Note. B: C or G or T, S: G or C, Y: C or T.

Table 2. Calculation of Accuracy, Sensitivity, and Specificity Predictive Values

| Test Results | True Results (No., %) | False Results (No., %) |
|-----------------------|-----------------------|------------------------|
| Positive test results | True positive | False positive |
| | 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 | False negative |
| | PA: 30, 100 | PA: 0, 0 |
| | AB: 30, 100 | AB: 0, 0 |
| | ST: 30, 100 | ST: 1, 3.3 |

$$\text{Accuracy} = \frac{TP + TN}{TP + FP + FN + TN} \times 100 \quad \text{Sensitivity} = \frac{TP}{TP + FN} \times 100 \quad \text{Specificity} = \frac{TN}{FP + TN} \times 100$$

Note. PA: *P. aeruginosa*, AB: *A. baumannii*, ST: *S. maltophilia*, TP: True positive, TN: True negative, FP: False positive, FN: False negative.

of 3 common and emerging infections (*P. aeruginosa*, *A. baumannii*, and *S. maltophilia*) to conventional diagnostic methods. Using this test can reduce the detection time from at least 4-5 days to a maximum of two days. This alternative test can also be very helpful in timely diagnosis and treatment of patients, as well as accurately distinguishing these 3 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 that lack of perfect diagnosis and accurate differentiation of new emerged infections from previous probable infections can be problematic in controlling infection in hospital settings. This bunch of errors may result 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 families, and increased unnecessary mortality rate.

One of the important policies which should be followed seriously is evaluating prevalent infections periodically in terms of type and resistance pattern. According to some experiences, it is recommended that this kind of evaluation be performed for inpatient treatment centers, separately and based on wards (14). Thereafter, the output of these evaluations should be made available to clinicians monthly in order to make empirical treatments based on those evaluations. In a previous study conducted in southwest of Iran, Shiraz Burn Center (Amir-Al-Momenin Burn Hospital, affiliated to SUMS), it was shown that the emerged *Acinetobacter* species was misdiagnosed with *Pseudomonas* species which is the mostly collected sample. Nowadays, PCR method is a significant technique in the diagnosis of specific pathogens which are difficult or time-consuming to be detected by phenotypic procedures considering clinical aspects (15). However, according to different study results, application of PCR for clinical specimens has many potential pitfalls due to

the susceptibility of PCR to inhibitors, contamination, and due to 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 current study the designation was improved considering these problems. Moreover, one of the problems with the application of molecular methods in clinical practices is the detection of infection in different samples such as wounds, blood, urine, sputum, and others. Considering differences in the nature of the content and the amount of available samples, careful design of the molecular technique is essential. One of the important points in designing the molecular methods is the application of an appropriate DNA extraction method based on the sample nature (15,19).

Considering the abovementioned, this study recommends culturing the samples (from any kind) on general or specific enriched bacterial media for purification purposes. In the next step, samples management, the extraction technique, and the molecular sensitivity and specificity of the method would be organized perfectly in the hospital laboratory settings.

Conclusion

Generally, accurate diagnosis of bacterial infection is a crucial factor for appropriate antibiotic therapy of infections and avoiding unnecessary antibiotic use. Although the history of patients and their clinical examination seem to fit to find their infection diseases, with clinical aspects, laboratory confirmation tests are needed for diagnostic modality and usually required either to confirm or exclude a diagnosis.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

None.

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