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Rapid Diagnosis of Invasive Fungal Infections Caused by *Candida* and *Aspergillus* Species in Patients Admitted to Intensive Care Unit of a Tertiary Care Hospital

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Abstract

Introduction: Invasive fungal infections (IFI) are common nosocomial infections in immunosuppressed individuals. Rapid diagnosis of IFIs is important to support the growing number of at-risk patients and standardize the treatment guidelines. The study aimed to assess the role of serum 1,3-β-D-Glucan (BDG) and galactomannan (GM) biomarkers and real-time PCR in the rapid diagnosis of IFIs.

Method: It was a cross-sectional and observational study. A total of 60 peripheral venous blood samples were collected from clinically suspected IFI patients from the intensive care unit (ICU) of Bangabandhu Sheikh Mujib Medical University (BSMMU). The study was conducted from December 2022 to August 2023.

Result: Out of 60 clinically suspected IFI patients, 12 (20%) were positive for fungus in blood culture, of which *Candida* species accounted for 11 (18.33%) and *Aspergillus* species accounted for 1 (1.67%). Using serum biomarkers (GM and BDG), 46.7% of patients were positive for the BDG assay, and 20.0% of patients were positive for the GM detection assay. Using real-time PCR, *Candida* species (*C. albicans, C. parapsilosis, C. glabrata, C. krusei*) were detected in 24 (40.0%) cases and *Aspergillus fumigatus* were detected in 14 (23.33%) cases. The agreement between real-time PCR and serum biomarkers was 81.67% and the kappa value was 0.639, which was considered good. The sensitivity of GM, BDG, and real-time PCR were 67.65%, 92.00%, and 92.00% respectively. The specificity of GM, BDG, and real-time PCR were 100.00%, 80.77%, and 58.34% respectively.

Conclusion: Serum GM and BDG biomarkers and PCR are promising and highly sensitive tests for rapidly diagnosing at-risk patients suspected of having invasive fungal infections.

Keywords: invasive fungal infections, serum biomarkers, real-time PCR, 1,3-β-D-Glucan, galactomannan

Introduction

Fungal pathogens cause at least 13 million infections and 1.5 million deaths worldwide each year, mainly in people with compromised immune function [1]. It is estimated that approximately 3 million people worldwide suffer from severe chronic fungal infections and nearly 1.9 million patients develop acute invasive fungal infection (IFI) each year [1]. In recent years, the prevalence of IFI has been increasing, mainly among hospitalized patients. The pathogens

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causing IFIs are mostly opportunistic, including *Candida*, *Cryptococcus*, and *Aspergillus* species [2]. The most common pathogenic fungi in immunocompromised patients are *Candida* and *Aspergillus* species [3].

The rate of candidemia varies significantly by country, and using a conservative 5 per 100,000 rate, a study estimated 8100 cases in Bangladesh each year [4]. Among other serious fungal infections, invasive aspergillosis (IA) is hardly recognized in Bangladesh. Among the estimated 34.5 million adults over the age of 40, there are an estimated 2,481,444 patients with COPD in Bangladesh (7.2%) [5]. Assuming that 13% of these patients are admitted to hospital each year and that 1.3% develop IA. Given the aforesaid data, invasive candidemia and aspergillosis constitute a serious burden in Bangladesh. Diagnosis of IFIs is conventionally done by microscopy and cultures which have limitations in cases of sensitivity, time to positivity (2-4 days) for *Candida* and *Aspergillus* species, and difficulty in collecting invasive specimens. The sensitivity of culture is only 2-8% in the case of *Aspergillus* species and 20-40% in the case of *Candida* species [6]. Novel diagnostic approaches based on non-culture-based methods have been developed that could enable rapid diagnosis and treatment of IFIs. The most promising techniques include the detection of fungal antigens (BDG and GM) in blood and real-time polymerase chain reaction (PCR).

GM is a carbohydrate component of the *Aspergillus* cell wall that is released by all *Aspergillus* species during growth in the tissue. GM is escaped into the blood and other body fluids even in the early steps of *Aspergillus* invasion. Circulating GM could be sensed in the serum before diagnosis by clinical and radiological examination in about 65% of patients [3]. BDG is a cell wall component of many fungi and can be detected in IFIs due to *Aspergillus* species, *Candida* species, *Pneumocystis jirovecii, Fusarium* species, *Trichosporon* species, and *Saccharomyces* species, but absent in *mucormycetes* and *cryptococcus*. However, unlike GM, which is specific to *Aspergillus*, BDG is found in several fungal species. Therefore it can be considered a pan-fungal marker and a predictor of systemic fungal infection when demonstrable in blood or other normally sterile body fluids [7]. Pathogen detection using real-time PCR is undoubtedly the most powerful tools for rapid detection of human pathogens. The potential value of nucleic acid-based methods for the detection and identification of fungus in immunosuppressed patients is undeniable. IFIs present a major challenge in the treatment of immune-compromised patients. Early diagnosis is required to improve survival from these infections. As numerous articles show, conventional microbiological, histological, and radiological techniques still form the basis of diagnosis but are insensitive and have little impact on clinical decision-making. There is an urgent need to develop new effective diagnostic methods. These tests must be rapid and highly sensitive.

Materials and Methods

Study design

This is an observational and cross-sectional study conducted in a tertiary care hospital in a time span of 9 months, from December 2022 to August 2023.

Study sites

Peripheral venous blood was collected from the patients admitted to the intensive care unit of Bangabandhu Sheikh Mujib Medical University (BSMMU). Laboratory work was performed in the Department of Microbiology & Immunology, (BSMMU).

Participants

Clinically suspected patients of IFIs were admitted to the intensive care unit of BSMMU.

Inclusion criteria

Clinically suspected patients of IFIs with any of the following factors were included in this study.

The factors are:

Neutropenia (<0.5x 109 neutrophils/L).

Use of corticosteroid for a prolonged time (minimum dose of 0.3 mg/kg/day of prednisone equivalent, for >3 weeks).

Fever refractory to at least 3 days of appropriate antibiotics/fever relapsing after a period of defervescence of at least 48 hours while still receiving antibiotics.

Exclusion criteria

Participants were not be enrolled if they

Had any bacterial/parasitic infections.

Were receiving immunoglobins and albumin.

Were on the regimen for anti-fungal therapy.

Data collection

Relevant data were collected from patients or their attendants or the clinical history records and investigations of the patients in a predesigned data collection sheet. Results obtained from laboratory methods were recorded in a separate data collection sheet.

Among the 60 patients', X-ray was done in all the patients. HRCT chest was done where clinically indicated.

Procedures in the laboratory

Peripheral venous blood was collected under aseptic conditions using a sterile disposable syringe tagged with a butterfly needle after preparing the patient's skin with, at first

70% alcohol and hereafter with 1% tincture iodine [8]. A total of 16 ml of blood was collected and divided into three parts. The first part is for blood culture which consists of 10ml. According to the procedure, this part was inoculated into an automated blood culture bottle. The second part consists of 3ml of blood, which was taken in a sterile EDTA-coated vials for DNA extraction and shaken gently for proper mixing. Another 3 ml of blood was taken into a sterile test tube without anticoagulant for serum separation.

Blood culture and identification of yielding fungi

Inoculation of 10 ml of blood into the BD-BACTEC™ Plus Aerobic/F blood culture bottle after disinfection of the head of the bottle with 70% alcohol [9]. Then the blood culture bottle was inserted into the BD-BACTEC™ FX40 (Becton, Dickinson and Company, Sparks, MD 21152, USA) blood culture machine for incubation at 37^0 C temperature for 1-5 days according to manufacturer's instruction [10]. After showing positive indicators on the machine; with all necessary aseptic precautions, isolation of microorganisms was done by sub-culturing a small amount of liquid media on Sabouraud dextrose agar (SDA) media, blood agar media, and MacConkey agar media and were incubated at 37^0C for 24 hours aerobically, up to 4-5 days for SDA media, as maximum time required for *Candida* and *Aspergillus* species to grow in SDA media is 4-5 days [11]. A smear was also made from growth on subculture media and Gram stain preparations were performed. Culture plates with growth of organism other than *Candida* and *Aspergillus* species were excluded. All the bottles and the culture media were discarded after 7 days according to the proper safety procedure [12]. Identification of *Candida* spp. and *Aspergillus* spp. by colony morphology, wet film, Gram staining, and microscopy was done.

Galactomannan assay

Properly stored serum from patients of clinically suspected invasive fungal infections was used for determination of Galactomannan (GM) by using sandwich ELISA according to the manufacturer's instruction (Genobio Pharmaceutical Co., Ltd, Tianjin). After measuring the absorbance values for each control and samples, the mean cut-off control value was calculated and then sample index (SI) was calculated. SI value >0.5, were considered as positive.

1,3-β-D-Glucan assay

Properly stored serum from patients of clinically suspected invasive fungal infections was used for determination of BDG by using the Kinetic ELISA method according to manufacturer's instruction (Genobio Pharmaceutical Co., Ltd, Tianjin). The results were expressed in pg/mL of sample and ranged from non-detectable (<31.25 pg/mL) to >500 pg/mL and read from the standard curve. BDG values <60 pg/mL were interpreted as negative results. Values ≥80 pg/mL were interpreted as positive. Values from 60 to 79 pg/mL were considered possible fungal infections.

DNA extraction

DNA extraction from peripheral venous blood was done according to the manufacturer's instructions (Qiagen QIAamp DNA Mini extraction kit). The volume of the clinical sample for DNA extraction was 200µl. Extracted DNA stored at -20°C until use. The concentration of DNA was measured by spectrophotometric assay performed using a Nanodrop 200 spectrophotometer (Thermo Fisher scientific, Waltham, MA, USA) according to the manufacturer's instruction.

Detection of *Candida* species and *Aspergillus* species by real-time PCR

Molecular detection of *Aspergillus* species (*Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus terreus*) And *Candida* species (*Candida albicans*, *Candida parapsilosis*, *Candida glabrata* and *Candida krusei*) was done using an *Aspergillus* differentiation kit (VIASURE) and *Candida* spp. Detection kit v2 (Anatolia Geneworks, Bosphore, Turkey) according to the manufacturer's instructions respectively. The real-time PCR instrument was CFX Opus 96 Touch™ real-time detection system (Bio-Rad). PCR was considered as positive when for a single sample, the CT value was ≤40 for *Aspergillus* species detection and ≤37 for *Candida* species detection.

Ethical approval

This study was ethically approved by the Institutional Review Board (IRB), BSMMU on 12/12/2022.

Statistical analysis

Data analysis was done using SPSS software package version-27 (Strata Corporation, College station, Texas). P value was calculated by Chi-square test to explore the association of categorical data. P value <0.05 was considered statistically significant. Median were calculated for asymmetric quantitative data and mean with 95% confidence interval and standard deviation. Agreement between the three methods was analyzed using Kappa statistic. EORTC/MSG guideline 2020 [13] was as reference gold standard to calculate true positive and true negative cases. Proven and probable cases were considered as true positive and no IFIs cases were considered as true negative.

Results

In this study, a total of 60 blood samples were collected from clinically suspected patients of invasive fungal infections were collected. Automated blood culture, measurement of serum biomarkers (BDG and GM), and real-time PCR were performed in all 60 blood samples. Out of 60 clinically suspected invasive fungal infections patients' blood samples, 12 (20%) were positive for fungus, 17 (28.3%) were positive

for bacteria and 31 (51.7%) yielded no growth, shown in a pie chart Figure 1. In Table 1, elaboration of the laboratory test results of patients with clinical suspicion of IFIs are shown. Out of 60 clinically suspected IFIs patients', 12 (20%) were positive for fungus in automated blood culture, of which *Candida* species accounted for 11 (18.33%) and *Aspergillus* species accounted for 1 (1.67%). Using serum biomarkers, 46.7% of patients enrolled were positive for a BDG detection assay, and 20.0% of patients enrolled were positive for a GM detection assay. Using real-time PCR, *Candida* species were detected in 24 (40.0%) of cases and *Aspergillus* species were detected in 14 (23.33%) of cases. In the present study thirty-eight (63.33%) cases were positive by real-time PCR assay. Among them, 24 (40.00%) were *Candida* species and 14 (23.22%) were *Aspergillus* species. All the Aspergillus were *Aspergillus fumigatus*. Among the identified *Candida* species 8 (21.60%) were *Candida albicans*, 9 (23.30%) were *Candida parapsilosis*, 6 (15.78%) were *Candida glabrata* and 1 (2.70%) case was *Candida krusei*.

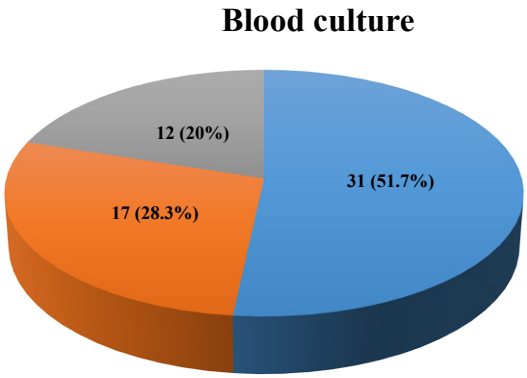

Figure 1: Results of automated blood culture among the study population (n=60)

Table 1: Laboratory test results of patients with clinical suspicion of invasive fungal infections (n=60)

	Patients of clinically suspected IFIs (n=60)	
Mycological findings	**No**	**%**
Blood Culture		
Positive	12	20.00%
Candida species	11	18.30%
Aspergillus species	1	1.67%
Negative	48	80.00%
Serum biomarkers		
Galactomannan	12	20.00%
1,3-β-D-Glucan	28	46.70%
Real-time PCR		
Positive	38	63.30%
Candida species	24	40.00%
Candida albicans	8	21.00%
Candida parapsilosis	9	23.30%
Candida glabrata	6	15.70%
Candida krusei	1	2.70%
Aspergillus species	14	23.30%
Aspergillus fumigatus	14	36.80%
Negative	22	36.60%

Table 2: Agreement between blood culture, serum biomarkers (1,3-β-D-Glucan and Galactomannan), and real-time PCR used for diagnosis of invasive fungal infections.

Test	Agreement %	κ* (Kappa)	*P* value
Blood culture vs real-time PCR	55.00%	0.211	**0.017**
Blood culture vs serum biomarkers	73.33%	0.444	**<0.001**
Real-time PCR vs serum biomarkers	81.67%	0.639	**<0.001**

*Value of κ
Strength of agreement <0.2: poor; 0.2-0.4: fair; 0.41-0.6: moderate; 0.61-0.8: good; 0.81-1.00: very good

Blood culture showed a 55.0% agreement with real-time PCR, and the kappa coefficient is 0.211, which was considered fair. On the other hand, blood culture exhibited a higher agreement of 73.33% with the serum biomarkers (BDG and GM) and, the kappa value is 0.444, which was statistically significant. Agreement between Real-time PCR and serum biomarkers (BDG and GM) was 81.67% and the kappa value was 0.639, which was considered good, described in Table 2. Table 3, shows clinically suspected IFI patients' classification according to EORTC/MSG criteria (Peter Donnelly *et al.*, 2020). According to this criteria, 11 (18.33%) cases were classified as proven cases, 12 (20.00%) cases were classified as probable cases, 16 (26.67%) cases were classified as possible cases and 21 (35.00%) cases were classified as no IFI cases. In Table 4, the sensitivity, specificity, PPV, and NPV are shown. Here updated EORTC/MSG criteria,2020 was used as the gold standard to calculate true positive and true negative cases. Proven and probable cases were considered true positive, and no IFIs cases were considered true negative. The sensitivity of GM, BDG, and real-time PCR were 67.65%, 92.00%, and 92.00% respectively. The specificity of GM, BDG, and real-time PCR were 100.00%, 80.77%, and 58.34% respectively. The PPV of GM, BDG, and real-time PCR were 100.00%, 82.14%, and 91.31% respectively. The NPV of GM, BDG, and real-time PCR were 65.63%, 91.31%, and 91.31% respectively. Table 5, describes the time taken by each method to diagnose invasive fungal infections. The automated blood culture method took the most time while PCR serum biomarkers took the least time.

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Table 3: Classification of cases according to EORTC/MSG criteria

	Clinically suspected invasive fungal infection cases (n=60)	
Class	**No**	**%**
Proven	11	18.33
Probable	12	20
Possible	16	26.67
No IFI	21	35

Table 4: Sensitivity, specificity, PPV, and NPV of galactomannan, 1,3-β-D-Glucan assay, and real-time PCR in detection of invasive fungal infections (n=60)*

Test	Sensitivity	Specificity	PPV	NPV
Galactomannan	67.65%	100.00%	100.00%	65.63%
1,3-β-D-glucan assay	92.00%	80.77%	82.14%	91.31%
real-time PCR	92.00%	58.34%	60.53%	91.31%

*Considering Updated EORTC/MSG criteria, 2020 as the gold standard

Table 5: Time taken by each method to diagnose invasive fungal infections

Method	Time
Automated blood culture	Up to 1 week
Serum biomarkers assay	3-4 hours
Real-time PCR	3-4 hours

Discussion

Despite new antifungal drugs, mortality and life-threatening complications of invasive fungal infections are still frequently reported in critically ill patients. The diagnosis of IFI is negatively affected by suboptimal culture results. Therefore, new tools are required to rapidly diagnose IFIs in patients in the ICU. In the present study, fungi were isolated in 12 (20%) automated blood cultures. Except for 1, all were *Candida* species. The single one was the *Aspergillus* species. This is consistent with Montagna *et al*. in Italy in 2013 who found 87.6% of yeast and 12.4% of mold in all infections [14]. The study was conducted on 5561 patients over 18 months. The large study population and different types of samples (e.g., blood, peritoneal and cerebrospinal fluid, bronchial aspirate, sputum) may be the reason for 12.4% of mold infections in their study. Apart from this, most *Candida* infections are endogenous while *Aspergillus* infection is mainly exogenous, this could be another reason for higher *Candida* species infection.

Candida was isolated in 18.33% of the current study population. A similar rate of 13.5% was reported by Yang, Cheng and Lo, in 2006 in Taiwan [15]. As only one case of *Aspergillus* species (1.67%) was identified it is difficult to compare with other studies, where also isolation rate of mold was relatively low. The single case of *Aspergillus* in our study was *Aspergillus fumigatus*. Binder and Lass-Flörl, in 2011 reported *Aspergillus fumigatus* as the most common species [16]. *Aspergillus fumigatus* is the most common mold in the environment, this could be the reason of most common species identified in blood culture. Even *Aspergillus fumigatus* is the most common contaminant in culture. In our case apart from culture, the patients were also positive for biomarkers (BDG and GM) and PCR from serum, which indicates case of invasive infection not contamination. BDG detection test, 46.7% of patients were positive in the study population. BDG detection test was positive in all the blood culture-positive cases and in 26.67% blood culture-negative cases. In the case of GM, 12 (20%) enrolled patients were positive including the single *Aspergillus* positive automated blood culture case in our study population. These are in agreement with Azab *et al.* [17]. Azab *et al.* in 2015 reported using BDG detection test 37.9% patients positive in their study [17]. Biomarkers detection test was positive in all the blood culture positive cases and 25% of the blood culture negative cases.

In the present study thirty-eight (63.33%) cases were positive by real-time PCR assay. Among them, 24 (40.00%) were *Candida* species and 14 (23.22%) were *Aspergillus* species. All the *Aspergillus* were *Aspergillus fumigatus*. Among the identified *Candida* species 8 (21.60%) were *Candida albicans*, 9 (23.30%) were *Candida parapsilosis*, 6 (15.78%) were *Candida glabrata* and 1 (2.70%) case was *Candida krusei*. Majority of the *Candida* infections 65.2% were caused by non-albicans *Candida* species, whereas 34.8% were caused by *Candida albicans*. Similar results were reported by Montagna *et al.* in 2013 in Italy [18]. They reported that 59.8% of IFI were caused by non-*Candida albicans* species, and *Candida albicans*, accounted for 40.2% of yeast infections [18]. About 38 (63.33%) of enrolled patients were positive by real-time PCR assay. However, 20.0% were positive by automated blood culture. This number increases to 45% by serum biomarkers detection assay and further increases to 63.33% cases out of the 60 study populations, with a statistically significant difference. These results are in accordance with the work of El-Sayed in 2012, who detected 57.7% cases positive by PCR [22]. 50% of PCR positive cases were missed by blood culture. Another study stated that 64.5% were positive by both culture and PCR, of these samples 19.4% showed no growth of fungi but were positive by PCR [23]. The diagnosis of patients with IFIs in the present study revealed a good agreement between serum biomarkers assay of fungal antigen in serum and real-time PCR. However, there is moderate agreement between the results of blood culture and serum biomarkers assay. Additionally, there is a fair agreement between the results of blood culture and real-time PCR. By the results, Azab *et al.* also detected a good agreement (94.4%) between the two non-culture-based methods [17].

IFIs in this study were defined and classified using EORTC/MSG Consensus Group criteria [13], and according to these criteria 11 (18.33%) cases were classified as proven, 12 (20%) were probable, 16 (26.67%) were possible, and 21 (35.00%) cases were classified as no IFI cases. This is consistent with a study [24]. This study results of BDG are in agreement with the study conducted by Giacobbe *et al.* [25]. In which the sensitivity, specificity, PPV, and NPV of BDG were 92.00%, 81.00%, 79.00%, and 93.00%. A meta-analysis by Karageorgopoulos *et al*. showed an overall pooled sensitivity and specificity of serum BDG assay for diagnosis of invasive fungal infections 76.8% and 85.0% respectively [26]. However, marked statistical heterogeneity was noted. In this study, BDG assay gave false positive results in only 5 patients. False positive results in ICU patients may be due to many clinical variables and conditions such as surgical gauzes, renal replacement therapy, albumin transfusion, and broad-spectrum antibiotics [28,30]. Specificity and PPV can be increased by two consecutive BDG testing without significant impact on NPV [28]. The result of GM is consistent with many studies. A meta-analysis by Leeflang *et al.* showed the sensitivity and specificity of GM assay 49% to 77% and 89% to 97% respectively [29]. In our study, the GM assay gave no false positive result. GM showed false negativity in 11 proven cases but these cases were *Candida* blood culture positive, so GM obviously should be negative in these cases. Another cause of false negative results may be steroid intake [30].

In this study, the sensitivity, specificity, PPV, and NPV of real-time PCR were consistent with the study conducted by Gupta *et al.* [31] . This result is also consistent with the study conducted by Springer *et al*. where sensitivity was 85.10% and specificity was 64.50% [32]. Here, real-time PCR gave 15 false positive results. Compared to gold standard as observed by several other authors, these false positive results could be due to subclinical infection, fungal colonization, contamination by airborne fungal spores, fungal PCR product carryover, and cross-reactivity with nonfungal DNA. The risk of contamination is relatively low as the positive material (specimens and positive control) was stored separately from all other reagents. Workstations were wiped with 5% hypochlorite and 70% ethanol at every stage. The extraction of DNA, preparation of PCR products, mixing of PCR products, and amplification were carried out in biosafety hoods. All the reagents were mixed and dispensed in a pre-mix area. Jordanides *et al.* commented on the difficulty in determining an actual 'false-positive' result from an early 'true positive' result, reflecting the fact that PCR may be a more sensitive indicator of early invasive fungal infections [33]. In this study the specificity of real-time PCR is not good because of statistical analysis many of the PCR positive cases were considered as false positive. But we could not consider them as false positive because these patients were clinically suspected cases of invasive fungal infections. They had the signs and symptoms of invasive fungal infections and most of them had radiological and others laboratory evidences. Blood culture revealed no bacteremia also. So, we could not consider them as false positive cases.

In the study, false negative results were observed in 2 out of the 60 patients evaluated. The real-time PCR assay for *Candida* species detection used in this study can detect only four species of *Candida*. Several many species of *Candida* can cause invasive fungal infections in intensive care unit patients. In addition, in the study, samples for PCR were taken only once and no subsequent samples were taken. This might have led to the false negative reports in some of the cases as sequential positive PCR reports have been shown to increase the sensitivity of PCR in the studies by Landlinger *et al*. [34].

Conclusion

Real-time PCR and serum biomarkers detect more cases of IFIs than blood culture in blood from clinically suspected cases of IFIs in the ICU. BDG is positive in all the *Candida* and *Aspergillus* culture-positive cases and GM is positive in one *Aspergillus* culture positive case. Concerning the time consumed to diagnose IFIs, real-time PCR and serum biomarkers take the least time. The diagnosis of patients with IFIs reveals a good agreement between real-time PCR and serum biomarkers.

Conflict of Interest

The authors have none to declare.

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