# Utility and Drawbacks of Biomarkers in Diagnosis of Invasive Aspergillosis in Critically Ill Patients.

**Shaila Akhtar[1], *Shaheda Anwar[2], Taskin Jahan[3], Raisa Enayet Badhan[4], Ahmed Abu Saleh[2].**

**[1]Lecturer, Department of Microbiology, Green Life Medical College, Dhaka, Bangladesh.**

**[2]Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh.**

**[3]Lecturer (Department of Microbiology), Chattagram International Medical College and Hospital, Chattagram, Bangladesh**

**[4]Assistant Surgeon (Microbiology), Sheikh Hasina National Institute of Burn and Plastic Surgery, Dhaka, Bangladesh.**

***Corresponding Author**

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## ABSTRACT

**Background:** Hematopoietic stem cell transplant recipients, patients with hematological malignancies, and individuals experiencing neutropenia after anti-cancer medication are at high risk of developing invasive aspergillosis (IA). Furthermore, IA is being seen more frequently in different groups of people, including those receiving solid organ transplants, individuals with solid tumors or autoimmune disorders, and patients in intensive care units. High death rates are linked to diagnostic delays. The purpose of this study is to give a summary of the available data regarding the effectiveness of various tests of biomarkers and to talk about their benefits, limitations, and interpretation in a range of patient categories.

**Methods:** We searched the published English language literature using MEDLINE and EMBASE databases from January 2010 to October 2023. All studies about biomarker testing for the diagnosis of IA were included in the review.

**Results:** As valuable supplementary instruments for the early identification of IA, biomarker detection tests may play a part in the diagnosis of IA and tracking treatment response. Nevertheless, there are differences between patient populations and these biomarkers' sensitivity and specificity are not at their best. So, biomarker testing should be targeted at the right population and the right situation to offer the optional benefit in the approach of IA diagnosis and management.

**Conclusion:** Within the context of various clinical samples (serum, bronchoalveolar lavage fluid, cerebrospinal fluid), as well as distinct patient groups (once-hematological patients, recipients of solid organ transplants, and other patients at risk of IA), this review examines the importance, clinical utility and drawbacks of biomarkers testing for the diagnosis of IA in the critically ill patients.

**Keywords:** *Aspergillus fumigatus*; invasive aspergillosis; fungal biomarkers; galactomannan; 1,3-β-D-glucan

## INTRODUCTION

In patients with weakened immune systems, invasive aspergillosis (IA) poses a serious risk. The highest risk

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group includes those undergoing hematopoietic stem cell transplantation (HSCT), chemotherapy-induced neutropenia, and hematologic malignancies [1]. However, IA is increasingly seen in patient populations with different underlying disease types and immunosuppressive levels, including those undergoing solid organ transplants, solid tumor patients, auto-immune illnesses, congenital immunodeficiency, and chronic lung diseases [2,3].

With varying degrees of invasiveness, the pathophysiology and clinical presentation of IA may differ dramatically in these contexts. Conventionally, culturing and microscopy are used to diagnose infection-associated pneumonia (IA). These methods have drawbacks in terms of sensitivity, time to positivity, and difficulty in obtaining invasive specimens. For Aspergillus species, the sensitivity of culture is only 2–8%. It may now be possible to diagnose and treat IA more quickly thanks to the development of novel diagnostic techniques based on non-culture-based methodologies. As a result, non-culture-based techniques like PCR DNA identification or fungal biomarker measures in respiratory or blood samples are crucial supplementary tools. Table 1 provides an overview of the most popular fungal indicators for the diagnosis of invasive aspergillosis. The European Organization for Research and Treatment of Cancer (EORTC) and the Mycoses Study Group (MSG) have incorporated as microbiological criteria, among the biomarkers, galactomannan (GM) and 1,3-β-D-glucan (BDG) assays in their definitions of invasive fungal infections [4].

Due to their low sensitivity and specificity, there is ongoing discussion on the use and interpretation of these tests for the diagnosis of IA in different clinical settings and groups. The purpose of this study is to give a summary of the available data regarding the effectiveness of various tests and to talk about their benefits, limitations, and interpretation in a range of patient categories.

Table 1: Biomarkers of Invasive Aspergillosis

| Biomarkers of Invasive Aspergillosis of Common Use |
|---|
| Galactomannan [Blood, BAL] |
| Aspergillus Lateral Flow Assay |
| 1,3-β-D-glucan |
| Nucleic acids |

# METHODOLOGY

Two investigators searched the published English language literature using the MEDLINE and EMBASE databases from January 2010 to October 2023. Search terms included “invasive aspergillosis,” “IA”, “(1-3)-β-D-Glucan,” “β-D-Glucan,” “β-Glucan,” “D-Glucan,” “BG,” “BDG”, “Biomarkers”, “GM” and “Galactomannan”. We screened the reference lists of included studies and related publications. Conference abstracts and letters were excluded because of the limited data. Results were arbitrated by a second investigator. Disagreements were resolved by a third author. The following information was obtained: population, study, and assay characteristics; reference standard; methodological quality; threshold for a positive result; and data for variability of results. When the same population was analyzed in several publications, the study’s results were accounted for only once.

# RESULT

### Galactomannan assay

The assay for galactomannan (GM) is an advantageous diagnostic means that tests the components of *Aspergillus* species’ cell walls, including galactomannan. The primary antigen found in IA patients is GM,

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which is easily found in serum, cerebrospinal fluid (CSF), bronchioalveolar lavage (BAL), and other fluids [5]. The overall ranges for this assay's sensitivity and specificity were 67 to 100% and 86 to 100%, respectively [6,7]. Unlike the pan-fungal BDG marker, GM is specific to *Aspergillus*, and most medical facilities utilize it for regular diagnosis and surveillance of patients at risk for IA. As of right now, patients' serum and bronchoalveolar lavage (BAL) specimens can only be used to identify Aspergillus GM using the single FDA-approved method (Platelia Aspergillus enzyme immune assay (EIA); Bio-Rad, Marnes-la-Coquette, France). Numerous attempts have been made since then to enhance or create brand-new serology-based diagnostics for the identification of IA. Torton *et. al*. [8] created the mouse monoclonal antibody JF5, which binds to a protein epitope on an extracellular glycoprotein antigen that *Aspergillus* releases during active growth. This antibody is used in the experiment.

Dichtl *et al.* developed a new JF5-based technique for IA detection called the galacto-mannoprotein (GP) ELISA. They then evaluated the assay's effectiveness against the standard Platelia Aspergillus antigen ELISA [9]. 267 samples from 49 potential (n = 4) or proven (n = 45) IA cases were used in the study. According to Pearson's correlation, the team saw a strong correlation, or R=0.82, between the measurement findings of the two tests. Furthermore, 156 sera samples (control group lacking Aspergillus) were utilized to ascertain the specificities of both GM and GP. The GM (cutoff 0.3) and GP ELISA (cutoff 0.2) had specificities of 96% and 76%, respectively. The specificity of the GM test was 99% with one false positive case when utilizing the artificial cut-off value of 0.5; the GP test, with a cutoff value of 0.4, had a specificity of 97% with five false positive cases. Thus, the sensitivity of the GM and GP tests was 40% based on the suggested and improved cutoffs (0.5 for GM and 0.4 for GP analysis). In summary, it was discovered that the sensitivity and specificity of the new GP ELISA were comparable to those of the Platelia GM ELISA. However, patients who are at risk of developing IA might need to undergo serial testing due to the limited sensitivity of the two tests. The novel GP ELISA can be used to diagnose and monitor IA in high-risk individuals, as it performs equally well as the GM ELISA. It is also precise and trustworthy.

One of the problems with Platelia GM-EIA is its low reproducibility and requirement for repetition. To provide a quick, user-friendly, reliable assay for diagnosing IA, Gallet and colleagues [10] assessed the performance of a novel single-sample fluorescent-based EIA assay for determining Aspergillus GM levels in a patient's serum. The group created a brand-new single sample test called VIDAS® GM-EIA (Biomerieux), which comes in a ready-to-use dispensable strip. They used 126 sera (44 fresh and 82 frozen) to evaluate the test's performance against Platelia GM-EIA. The VIDAS®GM and Platelia assays showed comparable diagnostic performance, as evidenced by the area under the curve (AUC) under the ROC curves, which were 0.892% and 0.894%, respectively. A VIDAS®GM cut-off of 0.36, which translates to a sensitivity of 95.7% and a specificity of 85.7%, was found using the ROC curves and the optimal Youden index, which measures the ideal balance between sensitivity and specificity. The main advantage of VIDAS®GM is that it's an easy-to-use tool with quick findings (70 min). This means that high-risk populations can be routinely screened or diagnosed quickly, allowing for the earliest possible start of intervention therapy.

The introduction of a unique GM-EIA assay created by IMMY is another advancement. A single rat monoclonal antibody known as EB-A2 has been utilized in the Bio-Rad GM EIA; it binds to the fungus galactomannan. comparable to the IMMY lateral flow kit, the new IMMY GM-EIA assay uses two monoclonal antibodies: one binds to a novel target, while the other attaches to a GM epitope that is comparable to that of EB-A2. White *et. al.* [11] conducted a retrospective case-control study to assess the recently introduced IMMYGM-EIA. The group found that, at a positive threshold of 0.5, the IMMY GM-EIA had a sensitivity of 71% and a specificity of 98%, respectively. However, the generated sensitivity and specificity were reached by reducing the threshold to 0.27, 90%, and 92%, respectively. The IMMY and BioRad GM-EIA demonstrated good agreement, with an observed sample agreement value of 94.7% and a kappa statistic of 0.820. It appears that the IMMY GM-EIA is a competitive substitute for blood sample

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analysis. Additionally, the assay's plate-based design facilitates large batch testing and offers the potential for automation to further minimize human error. To get more information on the IMMY-GM-EIA assay and its clinical performance, this suggests doing more multi-center evaluations and prospective cohort studies.

Without defining a threshold to determine a positive result, the EORTC-MSG panel included a positive GM value as a microbiological criterion of invasive fungal infection (IFI) [12]. A single value ≥0.7 or two consecutive values ≥0.5 in serum should be taken into consideration by the laboratory working group of the European Conference on Infections in Leukemia (ECIL) as the cut-off that should trigger additional diagnostic work-up (such as a CT scan) for presumed IFI [13]. For BAL and CSF samples, a higher cut-off (single value ≥0.8 or 1.0) ought to be taken into account.

**1,3-β-D-glucan assay**

The majority of fungal species have a major cell wall component called 1,3-β-D-glucan, which is detected by the various BDG tests. However, some fungal species, such as Malassezia spp. and those belonging to the Mucoromycotina subdivision, have less 1,3-β-D-glucan in their cell wall and are therefore typically not detected by these tests. As a result, the BDG assay is utilized as a pan-fungal marker and is not specific for the diagnosis of invasive aspergillosis.

The Fungitell Assay (Associates of Cape Cod, MA, USA) is the only BDG assay approved by the FDA. The BDG assay has been incorporated into the EORTC-MSG criteria for fungal infection and is performed in colorimetric or turbidimetric formats [14]. Other than Fungitell, there are currently several BDG assays available. These include Fungitec-G, Beta Glucan-BGStar, and Beta-Glucan test (Mauha, Japan). The fungal strain, patient demographic, and assay platform all affect the assay's cutoff values, sensitivity, and specificity.

The Fungitell assay yields results for IA with sensitivity and specificity values of 69.9–100% and 73–97.3%, respectively; for other assay results, the corresponding values are 81–93% and 77.2–99.5% [15,16]. For the past 20 years, the Fungitell assay has proven a useful supplementary test for the identification of IFIs [17]. The test is conducted using 21 sample batch tests in a single run using a fast microtiter plate technique. A low batch format is similarly necessary, even if this would be advantageous for use in major universities or reference labs that run high sample numbers every day [18]. Fungitell STATTM, a straightforward single-patient method for measuring serum BDG levels in an index value format, was developed with this objective in mind. It enables patients to be promptly classified as positive, negative, or inconclusive. Similar to the traditional Fungitell assay, the new format measures the rate at which para-nitroaniline (pNA) is released following hydrolysis by activated BDG-sensitive protease zymogens using reagents based on Limulus amebocyte lysate (LAL).

In a recent study, D'Ordine and colleagues [18] compared the linearity of response across the Fungitell range, the analytical reproducibility (inter and intra-lab variance), and the positive and negative percent agreements (NPA) calculated with and without the indeterminate zone, as well as the performance characteristics of Fungitell STAT$^{TM}$ and Fungitell on 488 patient samples. The number of positives and negatives that a test finds that agree with another test performed on the same samples is indicated by the PPA and NPA values. Using Fungitell STAT$^{TM}$, over 250 distinct patient samples and lab-spiked samples showed good linearity. In comparison, NPA was 91% with an indeterminate value and 98% without one. PPA's value was 74% with an inclusion of indeterminate and 99% without one. Because of this, Fungitell STAT$^{TM}$ can effectively discriminate between positive and negative samples whether or not Fungitell ambiguous samples are present. For low-batch routine testing, the Fungitell STAT$^{TM}$ assay is a viable choice because of its great performance, low false positive rates, and high reproducibility.

Being a non-specific pan-fungal biomarker with low sensitivity values and a high rate of false positive

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results due to cross-reactivity is another drawback of BDG. According to Racil *et. al.* [19], 75% of false positive results in patients were linked to concomitant bacteremia, hemodialysis, or immunoglobulin therapy. A second commercial assay for identifying BDG in plasma samples was released in 2018. To compete with Fungitell in the European market, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan, invented the Wako-glucan test (GT). De Carolis and colleagues' study [20] involving sera of patients with IA (n = 40), IC (n = 78), and PJP (n = 17) in comparison to sera of control patients (n = 187) revealed that reducing the cutoff value when utilizing the Wako test increased sensitivity while maintaining the same level of specificity (97.3%). For the GT, the sensitivity and specificity were 80.0% and 97.3% for the diagnosis of IA, respectively, when the cutoff was lowered to 7.0 pg/mL. Therefore, in a clinical situation, the Wako-glucan test performed almost as well as the Fungitell once the GT threshold value was optimized for positive. The study also noted that GT could run up to 16 samples in parallel in addition to being able to run a single patient option. It was also easier to perform and analyze than the Fungitell.

Additionally, colorimetry using Limulus reagent is employed along with the widely utilized Goldstream® Fungus (1–3)-β-d-Glucan Detection Kit (ERA Biology, Tianjin, China) in clinical applications for BDG detection. We examined the efficacy of Goldstream® and Wako for IFD diagnosis in cases of *Pneumospora* , *Aspergillus*, and *Candida* infections. Overall, Goldstream®'s IFD diagnostic sensitivity and specificity (39.6% vs. 43.8%, 83.5% vs. 94.9%) were lower than Wako's [21]. For labs with varying samples, Goldstream® matches the IGL-800/IGL-200 (Fully Automatic Kinetic Tube Reader, ERA Biology, Tianjin, China). The FungiXpert® Fungus (1–3)-β–d-Glucan Detection Kit (CLIA) (ERA Biology, Tianjin, China) was introduced in 2020. It uses chemiluminescence immunoassay technology, which reduces detection time to 50 minutes and has automatic detection. Despite a gradual introduction into clinical use, the FungiXpert® Fungus (1–3)-β-d-Glucan Detection Kit (CLIA) still requires additional evidence to validate its diagnostic performance for IFD.

According to the EORTC-MSG panel, BDG detection is appropriate for diagnosing likely IFD in the right clinical context. Patients with hematologic malignancies, both with and without neutropenia, neutropenia after hemodialysis-sparing chemotherapy, and some ICU patients who have gastrointestinal surgery associated with recurrent anastomotic leaks, upper gastrointestinal tract perforations, or necrotizing pancreatitis when there is a clinical suspicion of infection are among those at higher risk (>10%) for infection [22,23]. It is advised to use the Fungitell test (Associates of Cape Cod, Falmouth, MA) with a single threshold (>80 pg/mL) [24]. Although the test has been utilized for CSF samples with some success to support a diagnosis of central nervous system (CNS) IFD in some situations when other diagnostic tests are negative or unclear, it was decided that this test should only be performed on serum samples [25].

**Lateral flow device assay:**

In a different method, researchers have demonstrated that testing urine specimens can be sensitive and specific for the diagnosis of IA. They have done this by using a novel galactofuranose-specific anti-*Aspergillus fumigatus* monoclonal antibody in a lateral flow immunodiagnostic device in dipstick format. In histoplasmosis patients, they did see some cross-reactivity, comparable to previous immunoassays that identify the common β-galactofuranose epitopes. The whole patient cohort's specificity was 92% (95% CI, 74 to 99%). The assay's sensitivity varied depending on the patient population; for instance, in the cohort of patients with proven/probable IA as a whole, the sensitivity was 80% (95% CI, 61.4 to 92.3%); however, in patients with bone marrow transplants, hematological malignancies, or neutropenia (n=50), it was 89.5% (95% CI, 66.7 to 98.7%); in noncancer patients, it was 63.6% (95% CI, 30.8 to 89.1%) [26]. This is in line with the inconsistent results of the Platelia GM immunoassay, which in a Cochrane Library assessment of 54 papers published before 2014 [27] ranged from 61% to 82% sensitivity and 81% to 93% specificity. Importantly, this new assay is especially promising in the field of IA diagnoses because of the minimal sample preparation required, the ability to visually assess results within 30 minutes of testing [26], and the

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume X Issue XII December 2023

relative convenience of collecting urine specimens.

**Nucleic acid amplification (NAA):**

For the precise and prompt identification of fungus isolated from cultures as well as the direct detection of IA from clinical specimens, NAA is a promising method [28,29]. While species- or genus-specific PCR allows the detection of fungi at a particular phylogenetic/taxonomic level, consensus/broad range/pan fungal PCR allows the identification of all fungi as these target conserved areas in the rRNA gene cluster [30]. Multiple fungi can be detected at the same time thanks to multiplex tests. To measure the fungal load and detect amplified products in real-time, any of these methods can be modified [30]. PCR has been thoroughly studied in relation to Aspergillosis [30]. PCR has a sensitivity range of 70 to 80%, and PPV increases with testing two consecutive samples and is dependent on the infection prevalence. Commercial tests can concurrently detect antifungal resistance by identifying point mutations in azole-resistant *Aspergillus fumigatus,* and they also provide consistent methods and enhanced quality control [29].

## DISCUSSION

A combination of clinical and paraclinical factors are used to diagnose IA; however, the majority of these indicators are just suggestive and do not provide absolute evidence of infection. GM and BDG values should only be evaluated in conjunction with other clinical, radiological, and microbiological criteria of IA as established by the EORTC-MSG consensus [14] due to their poor sensitivity and specificity. One of the mycological parameters used by EORTC/MSGERC to categorize likely IFD cases is GM positive [14]. In high-risk asymptomatic patients with a high probability of IA (neutropenia, hematological malignancies, bone marrow/hematopoietic stem cell transplant (HSCT), lung/solid organ transplants) who are not receiving active antifungal prophylaxis, the serum GM test is advised as a screening test for early identification of IA [31]. Serum/BAL GM is advised only in symptomatic patients receiving antifungal prophylaxis to identify breakthrough infections [31]. Positive genetic testing results have low positive predictive values (PPV) to "rule in" the disease even in high-risk groups, but negative genetic testing results have high NPV to "rule out" the disease [32]. Weekly or twice weekly serial serum GM testing enables early identification of IA, the start of AFT, and the tracking of clinical results [33,34]. Children's test results and diagnostic thresholds for IA are comparable to those of adults [14]. Only in certain situations, such as hematologic cancer patients with or without neutropenia, neutropenia after allogeneic stem cell transplant, and ICU patients who are 10% more likely to develop IFI (complicated gastrointestinal surgery, recurrent perforation or hepatobiliary anastomotic leakage, necrotizing pancreatitis, or Candida score ≥3), is the initiation of antifungals guided by a positive BDG test [35]. Due to low PPV in these patients, a positive BDG test is not advised when initiating AFT in solid organ transplant patients, patients receiving long-term immunosuppressive medication, or patients experiencing short-term (10-day) neutropenia brought on by chemotherapy [35]. Diagnostic certainty is increased by repeating BDG tests or combining it with GM [34]. Withdrawing or holding off on antifungal medication is not possible in patients receiving solid organ transplants or immunosuppressive therapy unless the pre-test risk of infection is low to moderate. A negative BDG has a low net positive value. Its applicability for therapeutic monitoring is debatable, and rising titers don't always indicate that clinical symptoms are getting worse [35]. Since *Aspergillus* PCR is more sensitive than blood culture, it is advised as a screening test for the early identification of infection in high-risk individuals who are not receiving antifungal prophylaxis, as well as for the diagnosis of established infection when symptoms and signs are present [36]. Because NAA tests have a high NPV (98–100%), they can be used as a test to "rule out." The EORTC/MSG definitions of mycological evidenceof IA now include two or more consecutive positive *Aspergillus* PCR results in blood, plasma, serum, or BAL, or one positive PCR result in plasma, serum, or whole blood with one positive PCR result in BAL[14].

When screening asymptomatic individuals on antifungal prophylaxis, the GM test is not appropriate. When

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it comes to non-neutropenic people and illnesses confined to the respiratory system, serum GM ELISA is less sensitive [31]. False Positives (FP) happen when a patient is infected or colonized by other fungi or bacteria that have genetically modified organisms (GM) in their cell walls; when they are treated with beta-lactam antibiotics (amoxicillin-clavulanate); when they are treated with chemotherapy or graft-versus-host disease; when they are in the first 100 days after hemodialysis or transfusions of blood products collected in cellulose bags; etc. [31]. BDG's sensitivity of less than 40% makes it unsuitable for use as a screening test in patients at moderate risk [34,37]. Additionally, the test is non-specific (it cannot distinguish between different fungi) and is not advised to be used as mycological proof of likely IFI [14]. Due to its low specificity, it is not verified in BAL. Mean BDG levels are often greater in immunocompetent children than in adults, there is a lack of data about its usage in the pediatric population, and optimum cutoffs in neonates and children are uncertain [37]. Hemodialysis, immunoglobulin replacement therapy, surgical gauze packing of serosal surfaces, intravenous antibiotic therapy (amoxicillin-clavulanic acid) or blood products, specific bacteremia (*Pseudomonas aeruginosa*), and contact with laboratory reagents containing glucan are among the factors that might cause FPs [32]. The most frequent causes of false positives and false negatives results of BDG are summarized in Table 2. Because IA is uncommon in non-neutropenic individuals, *Aspergillus* PCR in blood is not advised for screening purposes or for use in conjunction with antifungal prophylaxis because of decreased sensitivity and decreased positive predictive value (PPV). Fungal PCR in-house is difficult to standardize because of contamination, primer/probe cross-reactivity that causes FP and subpar DNA extraction, analytical procedures, and the existence of PCR inhibitors that cause FNs.

Table 2. Some causes of false positive and false negative results in the BDG test

| False Positive | False Negative |
|---|---|
| Contamination of laboratory material with glucans. | Hyperpigmented serums (bilirubin, triglycerides). |
| Bacteremia due to Streptococcus species or some Gram-negative bacilli such as Pseudomonas species. | Antifungal treatment (prophylaxis, empirical). |
| Contact with surgical sponges and gauzes. | Azithromycin or pentamidine IV. |
| Hemodialysis patients with cellulose-containing filters. | |
| IV treatment with immunoglobulins, albumin, or coagulation factors. | |
| Antibacterial IV treatment with antibiotics such as amoxicillin-clavulanic or piperacillin-tazobactam. | |
| Antineoplastic treatments with Lentinane or Polysaccharide k. | |

## CONCLUSION

The presumptive diagnosis of IA is increasingly using fungal biomarkers due to the difficulty in obtaining microbiological proof of IA. Because they help determine when to start or stop antifungal medication, biomarkers are essential to antifungal stewardship. Their function in predicting, tracking, and directing the length of antifungal therapy is yet unclear, though. Antifungal prophylaxis, pretest illness probability, and patient features all have a significant impact on the accuracy of the diagnosis. Even in people who are at high risk, GM and PCR are far more appropriate for "rule-out" diseases than "rule-in" diseases. BDG is only helpful in particular clinical contexts. The sensitivity and specificity of illness detection are increased by running a test again and combining two or more biomarkers. The inability to distinguish between colonization and infection and the lack of isolates for susceptibility testing and species identification are the

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main drawbacks of biomarkers.

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