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Validity of Combined Serodiagnostic Assays; Galactomannan, Mannan Antigens and Panfungal PCR in Diagnosis of Invasive Fungal Infection

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Authors' contributions

This work was carried out in collaboration between all authors. Author AZL designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AMAA managed the analyses of the study. Author AMZ managed the clinical part. All authors read and approved the final manuscript.

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ABSTRACT

Background: The development of reliable and rapid techniques for accurate diagnosis of invasive fungal infection (IFI) is an important goal that could help in effective treatment. Among the new promising methods used in diagnosis of IFI, pan-fungal PCR for detection of fungal DNA and measurement of serum Mannan and Galactomannan antigens.
Objectives: Our aim was to assess the performance of combined seroassays in diagnosis of IFI; Mannan plus Galactomannan antigens assays and the pan-fungal PCR with Galactomannan antigen tests compared to the culture based method.
Methodology: This study included 76 patients with suspected IFIs, (50 liver transplant recipients

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and 26 haematologic malignancy patients). Selected cases were subjected to complete diagnostic work-up (complete history, clinical, laboratory and radiological assessment). Blood samples were collected from all 76 cases, were divided into 2 parts; first part was inoculated into blood culture bottle for fungal isolation, serum was separated from the second part. Serum Mannan and Galactomannan antigens were measured by enzyme immunoassay (EIAs). Pan- fungal PCR technique was performed for molecular diagnosis.

Results: Among 76 of IFI patients, pure fungal growth was encountered in 16 cases (21.1%), which were 7 *Aspergillus* spp. and 9 *Candida* spp. Pan fungal PCR could diagnose 27/76 (35.5%) IFI cases; whereas, 29/76 (38.2%) cases were positive by combined GM (13.2%) plus MN (25%) EIAs. Sensitivity of combined antigen tests (100%) was higher than Pan- PCR (87.5%) assay alone and was the same as combined GM and PCR, while specificity was the same (78.3%). Blood culture method was considered as the gold standard. Sixteen (21%) patients were confirmed as proven IFI, ten (13.2%) were probable, 19 (25%) were possible and 31 (40.8%) were non-IFI. Unfavorable outcome was encountered in 9/76 (11.8%) patients (5 proven, 2 probable and 2 no-IFI), all of them were PCR positive.

Conclusion: Combinations of GM plus MN or PCR plus GM assays had high diagnostic performance in IFI patients. Both provided 100% sensitivity and NPV and 78.3% specificity. Consequently, these combinations will limit the time for deciding effective treatment strategies or empirical antifungal therapy.

Keywords: Fungal infection; Pan-fungal PCR; Galactomannan; Mannan.

1. INTRODUCTION

The incidence of invasive fungal infection (IFI) has flared up recently constituting a major threat especially in patients subjected to immunosuppressive conditions as in ; transplant recipients and patients with haematologic malignancies. *Candida* and *Aspergillus* spp. are the most commonly isolated fungi from patients with IFIs [1].

Conventional diagnostic methods of IFI usually rely on direct microscopy, culture and histopathology which require expert microbiologist. Moreover, these approaches are time consuming and may delay the initiation of therapy, or increase the consumption of empirical antifungal therapy before making a definitive diagnosis [2].

Recently, new techniques have been described which didn’t rely on cultures for diagnosis of IFI [3]. Among promising methods that aid in rapid and early diagnosis of IFI are, detection of fungal cell wall antigens Galactomannan (GM) and Mannan (MN) and molecular diagnosis with pan-fungal PCR [4].

Galactomannan antigen is a component of *Aspergillus* spp. cell wall which can be detected in patients’ blood as it is released during fungal growth in tissue. Mannan antigen is another cell wall component of *Candida* spp. that circulates in blood during infection. The detection of GM and MN antigens in patients serum can replace conventional methods. Both GM and MN antigens detection tests had a good sensitivity in diagnosis of IFI [4].

Pan-fungal PCR, primers were directed to conserved gene sequences located in ribosomal RNA genes. Pan-fungal PCR can be used to identify more than 90% of fungal pathogens involved in IFIs .Moreover, PCR offers the potentiality of being more sensitive than current culture-based method and can be applied to a variety of specimen types [5].

The European *Aspergillus* PCR Initiative (EAPCRI) optimized protocols for PCR routine clinical use for laboratory diagnosis of aspergillosis [5]. Recently, the PCR-based assays combined with fungal antigen detection tests for the diagnosis of IFI were recommended [1].

Thus, our study was designed to assess the performance of combined seroassays; Mannan plus Galactomannan and pan-fungal PCR plus Galactomannan compared to culture based method in diagnosis of IFI.

2. PATIENTS AND METHODS

This study was conducted at Menoufia university hospitals, Egypt. An informed consent was obtained from patients before enrollment. This study was conformed to the ethical guidelines of

the 1975 Declaration of Helsinki and approved by the local ethics committee of Menoufia University (2216/5/1/2016).

2.1 Inclusion Criteria

Patients who were at high risk of IFIs were prospectively studied during two and half years period and were included in the study. Patients essentially had fever refractory to antibiotics for > 96 hours, plus one or more of the following host factors; neutropenia (neutrophils counts<500/µl for >10 days), transplant recipient, patients on corticosteroid or immunosuppressant for more than 3 weeks.

2.2 Exclusion Criteria

Patients had proven bacterial infection, received immunoglobulins or plasma, were on antifungal therapy or less than 18years old.

2.3 Patients

This study included 76 patients (37 males and 39 females). They were admitted to ICU units as they had fulfilled the inclusion criteria as being at high risk to develop IFIs .The main body of the studied cases (50) were admitted to ICU after receiving liver transplantation and a further 26 patients were suffering from hematological malignancies (11 cases were acute lymphoblastic leukaemia, 9 cases were acute myeloid leukaemia and 6 cases were non-Hodgkin's lymphoma). Selected cases were subjected to complete history taking and diagnostic work-up including complete clinical, laboratory and radiological assessment.

2.4 Patients' Groups Classification

Using European Organization for Research and Therapy of Cancer and Mycoses Study Group (EORTC/ MSG) revised classification criteria, patients with IFIs were categorized as follow [4,6]:

Proven cases: were defined by positive culture that yielded fungus growth from sterile body fluid (blood).

Probable cases: were classified by presence of at least one host factor (neutropenia, fever, immunosuppressive therapy...), mycological evidence and one major, i.e., radiological evidence (or 2 minor) clinical criteria from site consistent with infection.

Possible cases: were defined by presence of appropriate host factors and with sufficient clinical evidence consistent with IFD one major, i.e., radiological support (or 2 minor) clinical criteria but for which there was no mycological evidenced.

No invasive fungus infection: referred to cases that had only host factors but neither microbiological nor clinical evidence of IFI were detected [1].

2.5 Samples Collection and Analysis

Blood (twenty milliliters) were collected from each patient within the first day of inclusion in the study and divided into 2 parts; first part (16 ml) was inoculated into blood culture bottles for fungal isolation (Bactec Plus Aerobic/F 25 Ml culture vials), second part transferred to sterile tube and left to clot then serum was separated, divided into aliquots and stored at -20°C until the time of Mannan, Galactomannan assay and pan-fungal PCR from serum samples.

1-Blood culture and fungal identification: Blood culture bottles were incubated aerobically at 37°C for 7 days, checked every day then checked twice a week for up to 4 weeks.Subculture was done on two Sabouraud's dextrose agar plates then incubated at 37°C for detection of Candida and at 25°C (room temperature) for up to two weeks for detection of filamentous fungi. *Candida* pp. was identified by culture characteristics and by using Candida chrome agar. *Aspergillus* spp. was identified according to standard microbiological methods [7].

2-Serum Galactomannan antigen assay: Serum GM levels were measured via Platelia Aspergillus kit EIA (Bio Rad, France).Test was performed according to manufacturer instructions. Samples that had GM index values ≥ 0.5 were recorded positive for IFI.

3-Serum Mannan antigen assay: MN was evaluated by Platelia Candida Ag plus kit (EIA; Bio Rad, France) . Samples with values of 125≥ pg/mL were considered positive for IFI.

4-Pan-fungal PCR assay: DNA extraction was done from serum using [QIAamp DNA Mini Kit (Qiagen, Germany)] [8]. The 18S rRNA gene of various fungal pathogens were amplified by PCR using universal primers, ('5-ATT GGA GGG CAAGTC TGG TG) and ('5-CCT ATC CCT AGT

CGG CAT AG) which hybridizes to conserved regions of fungal 18S rRNA gene leading to generation of a PCR product of 450 bp. Cycling conditions was adjusted as follow; 3 minutes of initial denaturing at 94°C, followed by 30 cycles of denaturing at 94°C, annealing at 58°C for 60 seconds and extension at 72°C for one minute, then final extension was performed at 72°C for 7 minutes[9].

2.6 Statistical Analysis

The data were analyzed using IBM SPSS advanced statistics. Diagnostic values of GM, MN and PCR assays were evaluated using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), blood culture method was considered as the gold standard. Kappa (κ)-test was done to measure the agreement between two tests (<0=poor, 0.21–0.40=fair, 0.41–0.60=moderate t, 0.61–0.80= good, and 0.81–1.00= perfect agreement).

3. RESULTS

In this study, 76 patients had fulfilled IFI inclusion criteria, of these, 50 cases were liver transplant recipients and 26 cases had hematological malignancies. They were 37 males and 39 females with ages ranged between 20 and 52 years. Fever not responding to antibiotics was the common sign in all cases while neutropenia was noticed in 53/76 of cases, groups classifications criteria were summarized in Table 1.

Distribution of blood culture, GM, MN and PCR tests results were shown in Table 2. Among 76 IFI patients, pure fungal growth was encountered in 16 cases (21.1%), which were 7(9.2%) *Aspergillus* spp. and 9(11.9%) *Candida* spp. Pan fungal PCR could diagnose 27/76 (35.5%) of IFI cases; whereas, 29/76 (38.2%) cases were positive by combined GM (13.2%) plus MN (25%) EIAs.

Notably , among positive PCR (27/76) cases, 14/16 cases (9 *Candida* spp. and 5 *Aspergillus* spp.) were also blood cultures positive, however PCR assay failed to detect two *Aspergillus* spp. in blood samples of proven IFI patients. Moderate agreement between PCR and blood culture techniques was detected, Table 3. All 16 positive blood culture IFI patients, were positive by GM plus MN EIAs, moderate agreement (κ =0.604) was seen between both techniques as shown in Table 4. Both combined GM plus MN antigens and pan-fungal PCR tests could diagnose 27/76 (35.5%) of IFI patients, additionally GM plus MN EIAs detected the two blood culture positive (PCR negative) cases, a perfect agreement was detected between combined GM plus MN antigen detection and pan-fungal PCR (κ =0.943), Table 5.

Table 6 summarized sensitivity, specificity, positive and negative predictive values of combined MN plus GM, pan-fungal PCR and combined GM plus PCR. Blood culture method was considered as the gold standard. Sensitivity of combined antigen tests (100%) was higher than pan fungal- PCR(87.5%) assay alone and was the same as combined GM and PCR, on the other hand, specificity was the same (78.3%) with nearly similar accuracy (>80%). This results highlighted the idea of our study as an enhanced sensitivity of both combined GM plus MN and GM plus PCR assays was seen.

Distribution of three assays results among different groups with respective to EORTC/MSG revised guidelines 2008 of IFI were summarized in Table 7. Sixteen (21%) patients were confirmed as proven IFI, all were positive by combined GM and MN assays but only 14 cases were PCR positive and two cases were PCR negative (false negative). Additionally, ten (13.2%) patients were probable IFI, 19 (25%) were possible and 31 (40.8%) were non-IFI.

Interestingly, 7 PCR positive cases were also positive by combined Galactomannan and Mannan assays in probable (9 cases), possible (2) and non-IFI (2) groups. Among 31 patients of non-IFI who didn’t develop clinical criteria, only 2 patients were found to be PCR and antigen detection tests positive, those two cases represented 2/27 (7.5%) of total PCR positive patients and 2/29 (6.9%) of cases detected by antigen detection tests (false positive). Unfavorable outcome was encountered in 9/76 (11.8%) patients, 5 proven, 2 probable and 2 patients classified as no-IFI, all of them were PCR positive.

4. DISCUSSION

Conventional methods used in diagnosis of IFI, such as blood culture, had poor sensitivity and delay the detection of fungal etiology in IFI patients. Therefore, evaluation of non-culture-based methods for the detection of IFI is mandatory [10]. Serodiagnostic assays, including detection of fungal cell wall antigens and fungal

DNA are new techniques can aid in diagnosis of IFI [11].

In this study *Candida* spp. (9 isolates) was detected by blood culture method more than *Aspergillus* spp. (7 isolates), this correlates with the findings of Shi et al. (2015), as he reported that Candida spp. was the predominant fungal etiology in transplant recipient individuals who developed IFI [12]. Also, similar rates of IFI among immunecompromised patients or even higher were reported in Egypt [9,13].

Table 1. Demographic data and risk factors of patients enrolled in the study, classified according to EORTC/MSG criteria for invasive fungal infection

Patients characteristics and risk associated	Proven IFI	Probable IFI	Possible IFI	No IFI	Total IFI
Episode n.(%)	16(21 %)	10(13.2%)	19(25%)	31(40.8%)	76(100%)
Age, years (range)	20–43	38-46	30-52	20-47	20-60
Sex n.(%)					
Male	10	6	10	11	37
Female	6	4	9	20	39
Liver transplant recipient	12	8	14	26	50
Hematologic malignancy	6	7	2	11	26
Neutropenia	12	10	10	21	53
Fever	16	10	19	31	76
Immune-suppressive therapy	12	8	10	20	50
Steroid	12	8	10	20	50
Chemotherapy	6	7	2	11	26
Cannula site infection	5	2	2	1	10
Dead transplant cases	5	0	0	2	7
Dead malignancy cases	0	2	0	0	2

Table 2. Results of blood culture, Mannan, Galactomannan and pan-fungal PCR assays for 76 patients with suspected IFIs

Tests	n (%)
Blood culture(76)	
Positive	16(21.1 %)
Candida	9(11.9%)
Aspergillus	7(9.2%)
Negative	60(78.9%)
Antigen detection tests (76)	
Total positive assays	29(38.2%)
Galactomannan	10(13.2%)
Mannan	19(25%)
Negative assays	47(61.8%)
Pan fungal PCR(76)	
Positive	27 (35.5%)
Negative	49(64.5%)

Table 3. Agreement between blood culture and pan fungal PCR in patients with suspected IFIs

	Blood culture					
PCR	**Positive (16)**		**Negative (60)**		**Total (76)**	
	No	**%**	**No**	**%**	**No**	**%**
Positive	14	87.5	13	21.7	27	35.5
Negative	2	12.5	47	78.3	49	64.5
Total	16	100	60	100	76	100
κ-Test	**0.526 moderate agreement**					

Table 4. Agreement between blood culture and combined Mannan plus Galactomannan in patients with suspected IFIs

	Blood culture					
Ag detection test	**Positive(16)**		**Negative (60)**		**Total (76)**	
	No	**%**	**No**	**%**	**No**	**%**
Positive	16	100	13	21.7	29	38.2
Negative	0	0	47	78.3	47	61.8
Total	16	100	60	100	76	100
κ-Test	**0.604 moderate agreement**					

Table 5. Agreement between combined Mannan plus Galactomannan and pan fungal PCR in patients with suspected IFIs

Ag detection test	**PCR**					
	Positive (27)		**Negative (49)**		**Total (76)**	
	No	**%**	**No**	**%**	**No**	**%**
Positive	27	100	2	4	29	38.2
Negative	0	0	47	96	47	61.8
Total	27	100	49	100	76	100
κ-Test	**0.943 perfect agreement**					

Table 6. Sensitivity, specificity, accuracy, positive and negative predictive values of antigen detection assay and pan-fungal PCR in76 patients with suspected IFIs

	Combined MN plus GM	**Pan fungal PCR**	**Pan fungal PCR plus GM**
Sensitivity	100%	87.5%	100%
Specificity	78.3%	78.3%	78.3%
Positive predictive value	55.2%	51.8%	55.2%
Negative predictive value	100%	95.9%	100%
Accuracy	82.9%	80.2%	82.9%

Table 7. Distribution of GM, MN and PCR assays results alone and in combination with respective to EORTC/MSG classification of 76 IFI patients

Patient classification	**No. of patients positive for indicated test n**					
	Positive by GM	**Positive by MN**	**Positive by combined GM and MN**	**Positive by PCR**	**Positive by PCR and GM tests**	**Negative by both combinations**
Proven (16)	4	12	16	14	14	0
Probable (10)	3	6	9	9	9	1
Possible (19)	2	0	2	2	2	17
No IFI (31)	1	1	2	2	2	29
Total	10	19	29	27	27	47

In this study, 27/76 (35.5%) of IFI cases were diagnosed by panfungal PCR, however, PCR results were negative in two proven culture positive IFI (12.5%) cases (false negative), this finding is in accordance with Al-Ashry and Raggab (2017), they reported that PCR was negative in three blood culture positive cases [13]. In our study, a moderate agreement between blood culture and PCR results (kappa=0.526) was detected, this degree of agreement was previously reported by Azab et al. [4]. In this work, the sensitivity of pan-fungal PCR was found to be 87.5%, specificity was 78.3%, 51.8% PPV and 95.5% NPV, this result is nearer to Gupta et al. and El-Mahallawy et al. as they reported a limited sensitivity of pan-fungal PCR (75% and 82.7% respectively) in diagnosis of IFI [1,9].

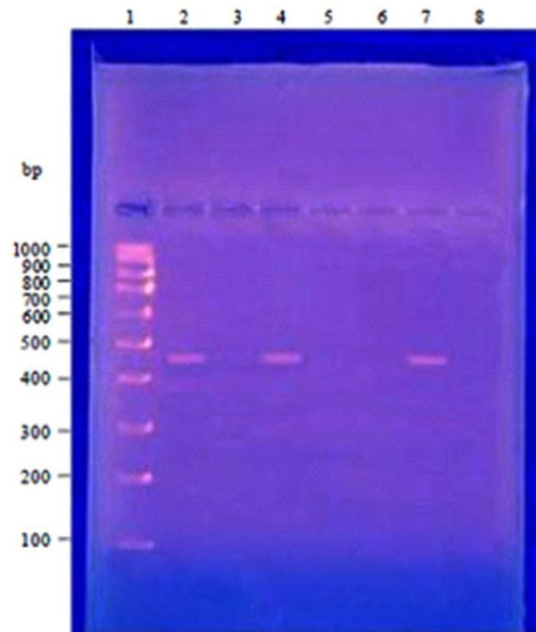

Fig. 1. Pan fungal PCR showing fungal DNA at 450 bp; lane (1) is for molecular ladder, lanes 2,4 and 7 are positive cases, while lanes 3, 5,6 and 8 are negative cases

PCR technique is very helpful in screening for fungal DNA in sterile fluid samples especially blood samples; it can detect dead or even a piece of fungi. However, false negative results and low sensitivity of PCR assay in this work may be attributed to the small number of fungal cells in serum samples compared to whole blood; the type of sample can affect PCR assay results [14]. Neutrophil in blood samples are considered to contain greater amounts of fungal DNA. Even though 100% sensitivity of PCR assay for IFI has been reported [15]

Combined biomarkers seroassays is recently used in IFI diagnosis. In this study two combinations have been evaluated, GM plus MN antigens EIAs and GM antigen plus PCR assays. A remarkable 100% sensitivity and NPV values were detected in GM plus PCR assays compared to PCR assay alone (87.5%, 95.9%), this correlates with the work of Loeffler et al., (2016).High negative predictive values of those combinations assays indicates its validity in ruling out the disease [16].

Also studies by El masry et al. 2005 and Azab et al. 2015, correlate with our finding as they highlighted the perfect diagnostic performance of combined two non-culture based methods; PCR plus ELISA and Galactomannan plus Mannan assays [17,4]. In Gupta et al. study, the best combination that aid in diagnosis of IFI was GM plus PCR assays with higher sensitivity compared to each test alone, however, he reported a lower specificity (64%) of such combination [1].

Even though, Okuturlar et al. 2015 reported 100% sensitivity and NPV of GM assay alone, and reported lower GM test specificity (27.1%) [18]. In our study, combined GM plus MN specificity (78.3%) was higher than that reported concerning GM assay alone (27.1%) [18] or combined GM and PCR tests (64%) [1]. Specificity of the antigen detection assays may be raised by increasing diagnostic cut-off index values. Finally, no single screening test can be used alone, in IFI diagnosis, especially with one serum sample [1].

In this study, a perfect agreement was detected between the results of combined GM plus MN antigen and panfungal PCR assays (κ =0.943). On the other, hand, a moderate agreement was observed concerning the results of blood culture with either pan-fungal PCR or with GM plus MN antigens detection assays. Fortunately, this combination may decrease invasive techniques and also directing different strategies in IFI treatment plane and in empirical use of valuable antifungal.

Here, combined GM plus MN antigens detection assays have been evaluated, the same sensitivity and NPV (100%) was observed. Mannan antigen has been evaluated before alone or in combination with Mannan antibodies as a screening tool in invasive candidiasis. In similar work, by Mohamed et al. (2018), they reported that serum Mannan had 100% sensitivity in diagnosis of IFI among liver transplant patients compared to blood culture method (85.7%) [19].

Our findings point to the potential clinical utility of these combinations, so if single sample is available, it must be evaluated either by GM plus MN antigens or GM antigen plus PCR assays.

Standardized protocols of antigen detection assays allowed their inclusion in the diagnosis of IFIs by EORTC/ MSG in 2008, but PCR not yet included. So, we advised the use of GM plus MN antigens. Moreover, antigen detection tests are costless, simple, rapid and required less special experience compared to PCR assay.

All 76 patients enrolled in our study, were classified according to EORTC/MSG revised guidelines 2008 diagnosis criteria of IFIs into, sixteen (21%) proven IFI, ten (13.2%) probable, 19 (25%) possible and 31 (40.8%) were non-IFI. All 16 proven IFI were positive by combined GM plus MN assays.

However, only 14/16 (87.5%) of them were diagnosed by PCR, as PCR assay results were negative in two proven culture positive IFI (12.5%), (false negative). False negative results rarely encountered with combined GM plus MN assays. Even though, in Azab et al. 2015 and Gupta et al. 2016 studies all proven IFI cases were PCR and combined antigen test positive, moreover those assays were earliest diagnostic tool of IFI ; their results preceded blood culture results and in some cases preceded clinical findings and consequently can direct the use of empirical antifungal [4,1].

False positive results is one of the main obstacles that we met in this work and previously reported in similar studies both in PCR and antigen detection assays. In this study, only 2 patients of non-IFI who didn't develop clinical criteria were found to be PCR and antigen detection tests positive, those two cases represented 2/27 (7.5%) of total PCR false positive results and 2/29 (6.9%) of antigen detection tests false positive. However, these false positive results may reflect hidden aspergillosis, intestinal colonization or from macrophages releasing DNA measurable by PCR [9]. Additionally, high false positive results occur with GM assay may be due to cross reaction with antibiotics, particularly beta-lactams [20].

In our work, PCR and antigen detection tests fulfilled the need for rapid diagnosis which is mandatory in our patients, as they took less time (2 days) compared to culture method. Hence, their potential clinical utility for improved prognosis and survival.

Even though, species identification and the availability of susceptibility testing provided by conventional blood culture method is a valuable advantage in treatment strategies. The opportunity for fungus isolation is not always easy or available in each hospital as it requires expert microbiologist.

5. CONCLUSION

In this study, we advised GM plus MN or PCR plus GM assays in diagnosis of IFI. As these combined assays yielded higher diagnostic accuracy, 100% sensitivity and NPV and 78.3% specificity compared to PCR alone. The choice will be according to feasibility of equipment and specialized personnel. Moreover, these combinations will limit the time for deciding effective strategies whether in treatment plan or in the use of empirical antifungal therapy.

CONSENT AND ETHICAL APPROVAL

This study was conducted at Menoufia university hospitals, Egypt. An informed consent was obtained from patients before enrollment. This study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the local ethics committee of Menoufia University (2216/5/1/2016).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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