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# Serum galactomannan assay for the diagnosis of invasive aspergillosis in children with haematological malignancies

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## Summary

Diagnostic efficacy of Galactomannan (GM) assay for invasive aspergillosis (IA) is variably reported. Data from developing countries are scant. Children with haematological malignancies and fever were enrolled prospectively. Blood sample for GM was drawn on the day of admission; levels were measured with *Platellia Aspergillus* enzyme immunoassay. Diagnostic criteria were adapted from EORTC-MSG-2002. Proven, probable and possible episodes were considered as the disease group. One hundred febrile episodes in 78 patients were evaluated. The mean age was 6.1 years. Majority (75%) episodes were in patients with acute lymphoblastic leukaemia. One episode each was diagnosed with proven and probable IA, while 23 were diagnosed with possible IA. Best results were obtained with a cut-off value of 1.0, with sensitivity, specificity, positive and negative predictive value of 60%, 93%, 75 and 87 respectively. The sensitivity dropped to 40%, at cut-off value of 1.5 and specificity was 38%, at a cut-off of 0.5. A higher value of GM correlated with pulmonary nodules ($P = 0.037$) and mortality ($P = 0.001$). GM assay is adjunctive to clinical/radiological evidence. A negative GM assay may not reassure the physician against the use of amphotericin in patients with febrile neutropenia, as it does not exclude the diagnosis of clinically relevant other fungal infections, particular mucormycosis.

**Key words:** Acute lymphoblastic leukaemia, acute myeloid leukaemia, CT scan, febrile neutropenia, functional endoscopic sinus surgery, immunocompromised.

## Introduction

Invasive aspergillosis (IA) is the most common filamentous fungal infection in immunocompromised patients.[1] Diagnosis of IA is challenging, and often a frustrating experience for the treating physician. Conventional diagnosis is dependent on culture and histopathologic examination. Microscopy and culture of sputum and bronchoalveolar lavage samples are insufficiently sensitive.[2] A biopsy is often not feasible in patients with comorbidities. Radiology can provide diagnostic clues, however, lacks specificity. Blood, cerebrospinal fluid and bone marrow specimen rarely yield Aspergillus species.[3] Thus, in majority, diagnosis depends on a combination of clinical signs, radiological abnormalities and clinical experience.[4]

Galactomannan (GM) antigen detection test was introduced in the year 1995 and was approved by the FDA in 2003. GM is a heteropolysaccharide released from the cell wall of aspergillus.[5] The role of GM assay in diagnosis of IA is evolving. There are several studies that have evaluated the assay in adult population; however, studies in children, particularly from developing countries are limited. The degree of antigenemia that indicates IA is a subject of debate. Typical cut-off values of optical density index (ODI) of GM range from 0.5 to 1.5.[6,7] A cut-off of 0.5 is currently approved by FDA.[2] In

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a meta-analyses involving immunocompromised patients, the overall sensitivity of GM assay at an ODI of 1.0 was 75% (range: 59–86%) and the mean specificity was 91% (range: 84–95%).[7] This study was done to evaluate the role of GM assay in the diagnosis of IA in children on treatment for haematological malignancies and to identify the best cut-off value of the assay in our patient population.

## Subjects and methods

The study was conducted prospectively from July 2010 to December 2011 in a Pediatric oncology unit. Children, ≤14 years, on treatment for haematological malignancies and admitted with fever were enrolled. Patients who received piperacillin-tazobactam and/or amoxicillin-clavulanic acid were excluded as their administration has been associated with a false-positive GM assay.[8,9] Stem cell transplant (SCT) recipients were excluded as well. Management of the febrile episode was as per units policy and the discretion of the treating physician. The treating team was free to take decision for antibiotics/antifungals. Blood for GM assay was drawn on the day of admission along with the sample for blood counts and bacterial culture. Serial estimation of GM was performed once a week, till discharge or death in limited patients. The GM assay obtained at admission was considered for analysis. Investigations that aided in the diagnosis of IA, included chest x-ray, computed tomography (CT) scan lungs/sinuses, cultures of appropriate body fluids, bronchoalveolar lavage (BAL) and biopsy (ante- or postmortem) of suspected infected tissue were performed as clinically indicated. CT scan of lungs was typically requested in patients with (i) High unrelenting fever, despite antibiotics and no apparent focus of infection, (ii) Respiratory signs/symptoms in patients expected to have prolonged severe neutropenia. The decision was influenced by the haemodynamic and respiratory status of the patient, as the parameters affected the decision to transport the patient to the CT room. Decision for administration of amphotericin was typically based on persistent fever after 5 days of antibiotics or clinical/radiological suspicion of fungal Infection. Amphotericin B deoxycholate was used due to favourable cost profile.

Diagnosis of fungal infections was classified as proven, probable, possible or 'no' aspergillosis, based on criteria adapted from the 2002 European organization for research and treatment of cancer/mycology study group (EORTC/MSG) definitions.[10] For analysis, episodes with a proven, probable or possible disease were considered to have IA unless otherwise stated. The EORTC/MSG definitions permit the GM assay results to be used to meet microbiological criteria for IA. However, the GM values were not included in the criteria for classification of diagnosis of IA, as the assay was itself being validated.

Serum GM levels were measured using the Platelia Aspergillus enzyme immunoassay test (Bio-Rad, Hercules, CA, USA) as per the manufacturer's instructions. Results were recorded as the ratio of optical density of the sample to that of threshold control samples. A febrile episode was considered as an independent episode in a patient when it was >4 weeks apart from the previous one, with the patient being clinically well in between. The study protocol was approved by the institutional ethics committee and informed consent was obtained from parents/guardians.

### Statistical methods

Comparison of normally distributed quantitative variables, including age, absolute neutrophil count (ANC) and GM index between 2 or >2 groups was performed by independent *t*-test and one-way ANOVA respectively. For skewed data, quantitative variables were compared between 2 or >2 groups by Mann–Whitney *U*-test and Kruskal–Wallis test respectively. Categorical variables between groups were compared by Chi-squared test. All tests were two-tailed and *P*-value <0.05 was taken as significant. Analysis was performed on IBM SPSS Statistics v18 (SPSS Inc., Chicago, IL, USA).

## Results

One hundred and twenty-one febrile episodes in 91 patients were investigated. Twenty-one episodes were excluded due to incomplete information, administration of Piperacillin-Tazobactam or technical problems in the GM assay. One hundred episodes in 78 patients were available for analysis and further discussion will be restricted to them (Table 1). Of the 75 episodes in patients with acute lymphoblastic leukaemia (ALL), majority (33%) were during delayed intensification, followed by maintenance (23%). Sixteen (21%) episodes were during induction, followed by consolidation (12%) and interim maintenance (11%). Patients with acute myeloid leukaemia (AML) received oral itraconazole prophylaxis, while antifungal prophylaxis was not administered to other patients.

Invasive aspergillosis was proven in a single (1%) episode. This episode was in a patient with ALL in the first week of induction. The patient died of pneumonia; an autopsy confirmed pulmonary IA. A probable

**Table 1** Demographics of the study cohort ($n$ = 100 febrile episodes).

| | |
|---|---|
| Age | Mean: 6.1 years (range 1.5–13) |
| Male:Female ratio | 3.5:1 |
| Diagnosis (No. of episodes) | |
| Acute lymphoblastic leukaemia | 75 |
| Acute myeloid leukaemia | 17 |
| Biphenotypic leukaemia | 3 |
| Lymphoblastic lymphoma | 3 |
| Burkitt's lymphoma | 2 |
| Absolute neutrophil count ($\times 10^9$ per l) | |
| <0.2 | 11 |
| 0.2–0.5 | 60 |
| 0.5–0.75 | 29 |
| Duration of symptoms at admission | Mean: 2.5 days (range 1–30) |
| Duration of hospital stay | Mean: 14.1 days (range 4–60) |

diagnosis of IA was made in 1 (1%) patient with AML who developed pneumonia following induction chemotherapy; *Aspergillus* hyphae were demonstrated in the sputum. A possible diagnosis was made in 23 (23%) episodes on the basis of host-related and clinical criteria in the absence of microbiological evidence. Of the 23 possible episodes, clinical and/or radiological evidence of lower respiratory tract infection was observed in 21 (91%). In the remaining 2 (9%) episodes, infection was localised to the paranasal sinuses. Seventy-five episodes did not fulfil the criteria for diagnosis of IA. Additional fungal infections were proven in five patients with ALL: two with sinonasal mucormycosis and one each with renal mucormycosis, dermal fusariosis and widespread dermal *Candida tropicalis*.

### Blood culture

A blood culture was obtained prior to administration of antimicrobials; bacterial organisms were isolated in 18 (18%) episodes. The most common organism cultured was *E. coli*: 7 (39%), followed by *Pseudomonas* and coagulase-negative *Staphylococcus*: 3 (17%) episodes each. Two (11%) episodes, each had *Staphylococcus aureus* and *Acinetobacter*. Alpha-haemolytic *streptococcus*, *Klebsiella*, *Enterococcus* and methicillin-resistant *Staphylococcus aureus* were isolated in one (5.5%) episode, each.

### Radiological investigations

A chest x-ray was obtained in 96 episodes. Abnormal findings, suggestive of a pulmonary infection were observed in 39 (41%) episodes. A CT chest was requested by the treating physician in 23 episodes. The findings included consolidation (52%), ground glass opacity (35%), nodules (35%) and effusion (18%). CT of paranasal sinus with orbital cuts was suggestive of fungal infection in two patients. A functional endoscopic sinus surgery was performed in these two patients; culture of the secretions collected confirmed the diagnosis of sinonasal mucormycosis.

### Bronchoalveolar lavage/transbronchial lung biopsy

A BAL was performed in three cases to ascertain the aetiology of pneumonia. Microbiological examination of the BAL fluid was non-contributory in all the three. A transbronchial lung biopsy was performed in two patients. There was evidence of granulomatous inflammation in one. Special stains performed failed to narrow down the aetiology to any organism. The biopsy in second patient was similarly inconclusive.

### Postmortem investigations

A medical autopsy was performed in one patient and a diagnosis of pulmonary IA was confirmed. Postmortem biopsy of lung and liver were obtained in five patients; no specific infective aetiology could be established in any. Postmortem biopsy of kidney confirmed the diagnosis of mucormycosis in a patient; he had a hypoechoic lesion in the kidney detected on ultrasonography during life.

### Antimicrobials

Cefoperazone-sulbactam and amikacin was administered as the first line therapy in 89% of episodes. An inadequate response after 48–72 h was an indication to change to second line antimicrobials, vancomycin/meropenem, in 72 episodes. In 11 episodes, vancomycin/meropenem was administered upfront due to adverse patient profile. The mean duration of administration of intravenous antimicrobials was 13.3 days (range 3–30).

### Amphotericin

Amphotericin was administered in 72 episodes. It was administered in 20 (87%) and 45 (64%) episodes in the 'possible' and 'No IA' groups respectively. Both the patients with proven or probable IA received it as well.

### GM assay

The patients, with 'proven' and 'probable' diagnosis of IA had a GM index of 3.2 and 0.9 respectively. In the 23 episodes with 'possible IA', the index was >1 in 14

**Table 2** Galactomannan index in different patient groups.

| | Diagnosis of invasive aspergillosis | | | | |
|---|---|---|---|---|---|
| Galactomannan level | Proven *n* = 1 (%) | Probable *n* = 1 (%) | Possible *n* = 23 (%) | No IA *n* = 70 (%) | Other fungal infections *n* = 5 (%) |
| ≤0.5 | – | – | 5 (22) | 28 (40) | 1 (20) |
| 0.6–1 .0 | – | 1 (100) | 4 (17) | 37 (53) | 3 (60) |
| 1.1–1.5 | – | – | 4 (17) | 5 (7) | – |
| 1.6–2.0 | – | – | 4 (17) | – | – |
| >2.0 | 1 (100) | – | 6 (26) | – | 1 (20) |

**Table 3** Performance of galactomannan assay at varied cut-off values[1].

| | Cut-off values of galactomannan assay | | | |
|---|---|---|---|---|
| | 0.5 | 0.7 | 1.0 | 1.5 |
| Sensitivity (%) | 84 | 76 | 60 | 40 |
| Specificity (%) | 38 | 58 | 93 | 100 |
| Positive predictive value | 33 | 39 | 75 | 100 |
| Negative predictive value | 87 | 87 | 87 | 82 |

[1]The proven, probable and possible episodes are considered as the disease group.

(61%). In the 70 episodes with 'No IA', the index was ≤1 in majority (93%) (Table 2). The patient with dermal fusariosis had GM index of 4.2, followed by 3.9 a week later. The index was <1 in the three patients with mucormycosis. The performance of GM assay was evaluated at varied cut-offs (Table 3). The highest specificity and positive predictive value was observed with a cut-off value of 1.5. The highest sensitivity and negative predictive value was observed with an index of 0.5. When GM assay >1.0 was considered as the cut-off, the probability of a positive test to be true positive was 0.71 (95% CI 0.48–0.88). The probability that it would be false positive was 0.28 (95% CI 0.12–0.52). For a negative test, the probability of true negative was 0.87 (95% CI 0.78–0.93) and false negative was 0.13 (95% CI 0.16–0.22). The conventional positive and positive (weighted for prevalence) likelihood ratios were 7.5 (95% CI 3.3–17.2) and 2.5 (95% CI 1.2–5.2) respectively. The conventional negative and negative (weighted for prevalence) likelihood ratios were 0.43 (95% CI 0.27–0.7) and 0.41 (95% CI 0.08–0.26) respectively. When the GM assay of >1.0 was considered as the cut-off, 5 (7%) episodes in the 'No IA' group were false positive. In retrospect, the clinical profile of these five patients was not suggestive of fungal infection. The patients were administered amphotericin for ≤5 days; all had recovered and were well in the follow-up period.

### Mortality data

Twenty (20%) episodes were fatal. These included 1 (100%) proven, 13 (57%) possible and 3 (4%) 'No IA' episodes. Three of the five patients with other fungal infections died.

### Correlation of GM values with various variables

There was no correlation of the GM with age ($P = 0.32$), type of leukaemia ($P = 0.71$), ANC ($P = 0.13$) or a positive blood culture ($P = 0.13$). The mean GM in febrile episodes with a finding of multiple pulmonary nodules on CT scan was 1.88 ± 1.58. This was greater than the value (0.93 ± 0.47) in episodes where CT scan lacked the finding of nodules ($P = 0.03$). Higher GM values were observed in febrile episodes that were fatal (2.03 ± 2.15), versus the ones which recovered (0.75 ± 0.59) ($P = 0.001$).

### Serial GM

A total of 136 samples in 100 febrile episodes were prospectively collected for GM assay. A sample was obtained in the second and third week in 30 and six episodes respectively. Persistent high levels (>1.0) were observed in five episodes with a possible diagnosis of IA; four of them died. Persistently, low levels (<1.0) were observed in 15 episodes; the majority (11) belonged to the 'No IA' or 'other fungal infection' group.

## Discussion

Several cases of IA remain undiagnosed until it is too late for clinical utility or diagnosed only at autopsy.[11] There is a pressing need for development of a rapid and accurate diagnostic test. In a review of autopsies from our Institute, a tertiary care center (~1500 beds) in north India, systemic fungal infections were detected in 2.4% of all autopsies performed (15 040 deaths autop-

sied over 26 years) and IA was detected in 49% of the fungal positive cases.[12]

In this study, of the 100 febrile episodes investigated, merely one case, each, was categorised as 'proven' and 'probable IA'. A large majority (75%) of episodes did not fulfil essential host/clinical criteria and microbiological evidence consistent with IA as per the EORTC/MSG, and were classified as 'No IA'. This reflects the difficulty in establishing a diagnosis of IA. It remains a dilemma whether the 23 episodes with a 'possible' diagnosis, truly had IA or not. Unfortunately, this is a major limitation of the EORTC/MSG criteria; a large number of patients get classified as 'possible' IA.[13] The gold standard investigation, that is culture or tissue diagnosis is often not performed for varied reasons or is non-contributory. It thus becomes a challenge to evaluate the performance of a new diagnostic tool. It is plausible that the number of patients with a 'proven' diagnosis could have increased with greater number of invasive investigations or autopsies.

The original EORTC/MSG-2002 criteria for the diagnosis of IA have been utilised in this study. They have been extensively applied in trials that have evaluated the role of GM assay.[9,14] The criteria were revised in 2008, with the objective of minimising the number of cases previously classified as 'possible' invasive fungal disease.[15] The significant changes in 2008 included the elimination of the 2002 category of minor clinical criteria and restriction of the clinical criteria for lower respiratory tract infections to very specific findings in CT scan.[15,16] In the revised classification, fever no longer constitutes a host factor. In a retrospective audit of 589 high-risk patient episodes from UK, 125 of 155 'possible' (81%) and 12 of 16 'probable' (75%) cases of IA changed to 'non-classifiable' when the new criteria were applied.[16] We preferred the original criteria for our study, as otherwise the large majority of the possible episodes would have been rendered 'non-classifiable'. For a meaningful analysis, episodes with a diagnosis of proven, probable or possible disease were considered to have IA. It may be debated whether the episodes with a 'possible' diagnosis should be included in the positive group or not. When sensitivity and specificity were calculated with proven and probable episodes as the disease group, the results were vague and ambiguous as merely two episodes were included in the positive group (data not shown). Indeed, in the retrospective review of 589 episodes, by Tsitsikas *et al.* [16] the low levels (0.01%) of proven/probable cases, contrasted with 44% of patients who received antifungal treatment for suspected invasive fungal disease. Thus, there is a wide dichotomy between 'clinical decision making' and the guidelines, largely as establishing a definitive diagnosis of invasive fungal disease continues to be a major challenge.

Tanriover *et al.* [17]. from Turkey evaluated the applicability of GM in 58 episodes in adult patients with haematological malignancies. The episodes were classified as proven: 1 (1.7%), probable: 4 (7%), possible: 20 (34%) and as no IA: 33 (57%). Sulahian *et al.* [18]. from France evaluated the specificity and sensitivity of GM in 347 children from pediatric hematology service and 450 patients from SCT unit during a 4-year prospective study. A diagnosis of confirmed or probable IA was made in 44 (9.8%) and 9 (2.6%) patients from the SCT and the pediatric hematology units respectively. Tanriover *et al.* [17] reported low sensitivity (60%) and specificity (21%) for an ODI of 0.5, when proven and probable group were considered as diseased. When the possible group was included in the disease category, sensitivity (84%) and specificity (27%) improved. Suankratay *et al.* [19] from Thailand evaluated GM antigenemia in 50 episodes in 44 adult neutropenic patients with haematological disorders receiving chemotherapy or a SCT. The sensitivity and specificity were 94% and 79% respectively, at a cut-off value of 0.75. The study considered proven (10%) and probable (24%) groups as diseased. The authors did not include 'possible' cases due to the relatively less specificity of the 'possible' group. Herbrecht *et al.* [20] from France studied the GM assay in 3294 serum samples during 797 episodes in adult and paediatric oncohaematologic patients. Of the 153 episodes with IA, 67 were probable, while 31 and 55 were proven and possible respectively. Higher sensitivity was reported in the proven group (64.5%), as compared with probable (16.4%) and possible (25.5%) groups. Marr *et al.* [6] examined the performance of the GM in 67 recipients of SCT with a mean age of 40 years. They observed higher sensitivity (61.5%) in the proven as compared with probable group (45.5%).

We analysed the sensitivity and specificity at varying cut-off values. A cut-off value exceeding 1.0 was observed to be best suited in our cohort. The specificity (93%) at the cut-off value of 1.0 was appropriate. The sensitivity was, however modest at 60%. The sensitivity significantly dropped with a higher cut-off and the specificity was low at a lower value. In general, lower sensitivity rates have been obtained in paediatric patients.[21] Herbrecht *et al.* [20] reported a higher specificity in adults (98.2%) than in children (47.6%) ($P < .0001$). False-positive results were observed more frequently in children (44%) than in adults (0.9%)

($P < 0.0001$). Hayden *et al.* [22] studied the expression of GM antigen in 990 serum samples from 56 paediatric oncology patients; a GM index ≥0.5 was considered positive. Tanriover *et al.* [17] observed improvement in sensitivity and drop in specificity when cut-off value was reduced from 1.5 to 0.5. Sulahian *et al.* [18] concluded that two consecutive samples with GM values >1.5 were required for assessing positivity. Marr *et al.* [6] demonstrated that decreasing the cut-off to 0.5, increased sensitivity, with minimal loss of specificity in a cohort of SCT recipients.

Five (5%) episodes in the study were confirmed to have other fungal infections, including three with mucormycosis. Cross-reactivity of *Fusarium* spp. with Aspergillus GM observed has been documented earlier and constitutes a drawback with respect to the specificity of the immunoassay.[23] Notably, a negative GM test may not reassure the treating physician against the use of amphotericin in patients with febrile neutropenia, as it does not exclude mucormycosis, which can have a similar presentation to IA.

The mortality was 20%. The high figure is, in part, biased, as patients with high-risk features were preferentially recruited. Ghosh *et al.* [24] from New Delhi, evaluated GM assay in 150 neutropenic episodes among inpatients aged ≥15 years, with a diagnosis of acute leukaemia and recipients of SCT. Fatal outcome was reported in 23 (15.3%) episodes.[24] Suankratay *et al.* [19] from Thailand reported a mortality of 16% while evaluating GM assay in 50 neutropenic episodes in adult patients.

There was a significant correlation of higher GM value with CT scan evidence of multiple pulmonary nodules ($P = 0.03$). The results indicate multiple pulmonary nodules to be suggestive of IA. Typical signs of pulmonary invasive fungal disease (halo sign, air crescent sign and cavities) were not observed in any patient. The presence of a nodule with a halo sign has been shown to be highly suggestive of IA in neutropenic patients.[25] Park *et al.* [26] evaluated CT findings and their prognostic value in 50 non-neutropenic transplant recipients and 60 neutropenic adult patients with pulmonary IA. Macronodules (≥1 cm in diameter), but not micronodules, were independently associated with 90-day mortality. It is increasingly being recognised that radiographic findings in immunocompromised children with proven pulmonary invasive fungal disease are often non-specific.[27]

Patients who died had a significantly higher GM level ($P = 0.001$), indicating that IA was more likely in patients with a fatal outcome. Suankratay *et al.* [19] similarly reported persistently high GM values in patients who died. Woods *et al.* [28] evaluated serial serum GM in 56 adult patients treated for haematological malignancies with chemotherapy or SCT. The survival of patients whose GM titres normalised was significantly better compared with those whose titres remained persistently positive ($P < 0.0001$). GM assay predicted a higher risk of death in the study by Ghosh *et al.* [24] as well. The analysis of serial GM values is restricted due to limited number of patients in whom serial evaluation was performed due to resource constraints. However, the numbers indicate that death was more common in those with persistently high level. It was not feasible to correlate the efficiency of antifungal treatment with serial GM level as there were merely two cases in the proven/probable categories.

Similar to the experience of Tanriover *et al.* [17] the limitations of our study include low number of probable and proven cases. This might have led to worse results than some other studies in the literature. However, it is a valuable experience to share, as it demonstrates the difficulty of establishing a confirmatory diagnosis of IA in clinical practice as well as the intricacy of implementing a GM- and CT-based diagnostic strategy in the milieu of a developing country. In recently published guidelines, prospective monitoring of serum GM, twice per week has been suggested in high-risk hospitalised children for early diagnosis of IA. The strength of recommendation was weak and the evidence was moderate quality.[27]

To conclude, the most reasonable cut-off value for GM assay for the diagnosis of IA in children with haematological malignancies in our study population was >1.0. The sensitivity, specificity, positive and negative predictive values of GM assay at the cut-off index of 1.0 were 60%, 93%, 75 and 87 respectively. The sensitivity and specificity dropped significantly at higher and lower cut-off values respectively. The role of GM assay is adjunctive to clinical and radiological investigations for the diagnosis of IA. A negative GM test may not reassure the treating physician against the use of amphotericin in patients with febrile neutropenia, as it does not exclude the diagnosis of clinically relevant other fungal infections, particularly mucormycosis.

## Conflict of interest

Authors have nothing to declare.

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