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RESEARCH ARTICLE

Assessment of Galactomannan for the Diagnosis of Invasive Aspergillosis in Pediatric Aplastic Anemia Patients in Uttar Pradesh, India

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ABSTRACT

Invasive aspergillosis (IA) is a dangerous form of fungal infection, especially for those who already have weakened immune systems. Particularly pediatric patients with aplastic anemia (AA). This is very important for getting an early and correct diagnosis of IA so that treatment can begin quickly and patients have better results. The purpose of this research was to evaluate galactomannan's (GM) diagnostic value in detecting IA in children with AA in Uttar Pradesh (UP), India. A study involving children diagnosed with AA diagnoses and clinical characteristics that suggested they might have IA was done prospectively through observation. Serum samples were collected from each participant and analyzed using the Galactomannan assay. The galactomannan index (GMI) cut-off value of ≥0.5 was considered positive for IA. Additional diagnostic tests, including clinical evaluation, radiological findings, and microbiological investigations, were performed to determine the presence of IA and validate the GM results. Using extensive diagnostic criteria, 25 out of the 100 pediatric AA patients who participated in the research were diagnosed with IA. The GM assay's sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were all calculated, along with its diagnostic accuracy. The results demonstrated a diagnostic accuracy of 90%, the sensitivity was 80%, the specificity was 92%, the positive predictive value was 76%, and the negative predictive value was 94%. Furthermore, the study evaluated the association between GM levels and disease severity in IA cases. When comparing patients with localized infections to those with more severe types of IA, those with disseminated and invasive infections had considerably higher GM levels. This finding indicates that GM levels may serve as a potential prognostic marker for disease severity in pediatric AA patients with IA. In conclusion, highlights the potential of galactomannan as a valuable diagnostic tool for the detection of IA in pediatric aplastic anemia patients in Uttar Pradesh, India. The high sensitivity, specificity, and diagnostic accuracy observed that the GM assay can aid in the early identification of IA, enabling timely treatment initiation and potentially improving patient outcomes. In order to determine the best method for diagnosing IA in this particular patient population and scenario, more research and validation studies are required.

Keywords: Aplastic Anemia, ELISA, Galactomannan, Invasive Aspergillosis, Neutropenia

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INTRODUCTION

People with hematological cancers and people who are getting hematopoietic stem cell transplants often get invasive fungus infections (IFIs), which can kill or make them very sick. These infections can happen to both adults and children.[1] These factors which include extensive utilization of antibiotics with a wide range of activity. and dose-intensive regimens that harm mucosal barriers and cause profound anemia, are crucial in explaining the rise in frequency. Haematological cancer patients are highly to get IA, Criteria for treatment include chemotherapy, febrile neutropenia (< 500 neutrophils/ mm^3), non-responsiveness to broad-spectrum antibiotics for 5 days, and prolonged corticosteroid use. One significant factor contributing to the occurrence of sickness and mortality

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among individuals with compromised immune systems is a primary cause.[2-4] Although drugs, blood transfusions, or stem cell transfers can help treat AA, it can get worse over time. Every year, about 3.8 out of every million people in India are diagnosed with aplastic anemia.[4] and about 60% of them die from it.[4,5] In two large investigations, the most prevalent cause of IA was cancer (64 and 33%). This study focused on patients diagnosed with proven or likely invasive fungal disease (IFD). Also, hematopoietic stem cell transplantation (HSCT) and the use of indwelling central venous catheters make these patients probable to get fungal infections. In addition, more susceptible to developing fungal infections due to HSCT and the use of indwelling central venous catheters. For aplastic anemia to be diagnosed, it's required that a minimum of two of the following be true: (i) hemoglobin < 10 g/dl; (ii) platelet count < 50,000/μL; (iii) neutrophil count 1,500/μL, with a bone marrow (BM) that is hypocellular with no infiltration or fibrosis.[6-8]

AA patients are more likely to suffer from chronic infections. In spite of the fact that gram-positive (most often gram-positive *cocci*) and gram-negative [especially multi-drug resistance (MDR) negative bacilli] organisms account for the vast majority of infections, IFIs are still the greatest cause of death and are causing a rise in patient mortality in their respective fields.[4, 9-10] Patients with aplastic anemia frequently develop fungal infections, particularly aspergillosis and mucormycosis.[3, 10] Since the 1990s, IA has surpassed candidiasis as the leading cause of death from a fungal infection.[11] It is well known that some fungi are infectious in nature, like the yeasts *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, which are the most common causative agents of common infections.[12-14] According to recent studies.[15-16] *Candida* spp. is now the fourth most common causative microorganism in nosocomial sepsis. There have been cases in the past where individuals contracted serious infections from *Candida* and other yeast genera.[13, 17] Since May 2003, a double sandwich enzyme immunoassay (an extended version) has been used to find GM in serum samples to diagnose IA in the United States. In Europe, this test has been used for almost a decade. However, the double galactomannan enzyme immunoassay is not widely used in the United States, and despite its well-documented analytical performance in diagnosing IA in adult patients, India and the rest of the world don't have much information about IA in children with cancer. Few studies have looked at how the GM EIA can be used with children. Previous studies.[4,18,19] found that the false-positive results for GM EIA were up to 54% more common in children than in adults.

METHODS& MATERIALS

Sample Collection

The study involved children with aplastic anemia between the ages of 1 and 17. The entire group of patients were taken to the pediatric hematology and oncology unit at King George's Medical University in Lucknow, India, from period of Feb 2016 to Dec 2016. The blood samples were obtained in accordance with recommended laboratory practices using either EDTA tubes or biphasic fungal blood bottles. According to Dinand *et al*.[20] and Ahmad *et al*.[4] there was no fungal spore contamination in the blood samples. Prior to testing, unopened samples were Stored within the temperature range of 2 to 8°C for a maximum duration of 5 days. When the sample is first opened, it can be kept at -2 to -8°C for 48 hours before testing. For further preservation, keep the serum at -20 to -70°C.

Data Analysis

A full record of each case was made, including details about the risk factors, the patient's health at the time of admission, and when the sample was taken. All of the patients were looked at to see if they met the new 2020 EORTC/MSGERC definitions of invasive fungal infection. In the context of invasive fungal infections, risk factors include a variety of conditions or situations that make a patient more susceptible to fungal infections. These included immunocompromised states (such as HIV/AIDS, chemotherapy, or organ transplantation), prolonged antibiotic use, neutropenia (low white blood cell count), and others. Categorized according to host factor criteria, clinical criteria, and mycological criteria, they were classified as either proven, probable, or possible IFIs.

AA patients' demographics, including age, gender, AA subtype, and treatment, were recorded. Data pertaining to infectious cases (IEs) were gathered up to the day of bone marrow transplantation (BMT) for individuals with AA undergoing BMT. The severity of AA was defined as significant if at least two of the following criteria were met: a neutrophil count below 500/mm³, a platelet counts less than 18,000/mm³, and anemia with a corrected reticulocyte count below 1% in the context of hypocellular bone marrow (less than 20% cellularity or 25 to 50% cellularity with less than 30% residual hematopoietic cells) and absence of fibrosis.[21] Additionally, the administration of immunosuppressive medications or immune modulators such as granulocyte-colony stimulating factor [G-CSF] and granulocyte-macrophage-colony stimulating factor [GM-CSF]) and the presence of coexisting conditions potentially linked to infection were documented.

GM Detection

The amounts of serum GM were measured by employing the Platelia aspergillus enzyme immunoassay test, manufactured by Bio-Rad and located in Marnes, La Coquette, France. The recorded results were expressed as indices in relation to the average optical density (OD) of the threshold controls. Samples with a GM index value of less than 0.5 were seen as positive. If a patient's second sample was collected more than a month after their first sample and the patient had improved clinically in that time, we counted it as a separate episode. At 0.7, 1.0, and 1.5 index values, all of these samples were also tested. After 4 days of treatment with broad-spectrum antibiotics and no improvement in fever, or based on clinical or radiographic suspicion of fungal infection, the decision to begin antifungal prophylaxis such as amphotericin B (AMB), fluconazole (FLU), or caspofungin (CAS) was made. HRCT

chest was done on people who had signs of a chest infection or who had a fever that didn't go away after taking antibiotics for 4 to 6 days. In some patients, HRCT of the paranasal sinus was also done.

The patients' guardians provided written consent after obtaining ethical approval from the institutional ethical committee.

Statistical Analysis

To assess the diagnostic efficacy of GM tests, we examined their sensitivity, specificity, PPV, NPV, positive likelihood ratio (PLR), and neutrophil-to-lymphocyte ratio (NLR) We employed the criteria set forth by the Mycoses Study Group of the European Organization for Research and Treatment of Cancer.[22] following the updated 2020 EORTC/MSGERC guideline as the benchmark to ascertain the count of true positive, true negative, false positive, and false negative cases. Instances of IA deemed likely or possibly were classified as true positives, while the absence of IA cases constituted true negatives. The determination of probable, possible, and no IFIs cases did not rely on GM results. The analysis was conducted using IBM SPSS Statistics v18 (SPSS Inc., Chicago, IL, USA).

RESULTS

The assessment of the test's sensitivity involved determining the ratio of patients exhibiting true positive results to those confirmed or likely to have aspergillosis. Specificity, on the other hand, was calculated by establishing the ratio of negative outcomes to the combined total of negative and false positive results. Among the 100 instances of aplastic anemia scrutinized, 54% were identified as female, while 46% were characterized as male. The study's median age stood at 12 years, encompassing an age range from 1 to 17 years.

The primary ailment manifested as acute myelogenous leukemia (AML) in 19 (19%) individuals, acute lymphoblastic leukemia (ALL) in 42 (42%), non-Hodgkin's lymphoma (NHL) in 5 (5%), Huntington disease in 9 (9%), neuroblastoma in 3 (3%), Rhobdosarcoma in 7 (7%), and diverse other conditions in the remaining cases (Table 1). Allogeneic HSCT was not administered to any of the patients. The unadjusted mortality rate during the hospital stay amounted to 18%, attributed to either the underlying malignancy or a combination of multiple infections/sepsis.

All the patients were categorized into proven 4 (4%), probable 32 (32%), and no IFIs 64 (64%) as per the new 2020 EORTC/MSGERC.[23] shown in Table 2. We collected blood samples from 100 aplastic anemia patients, and the following observations were made: Fungal infections are a significant contributor to both morbidity and death in juvenile patients diagnosed with aplastic anemia. In the present study, out of the total 100 cases, 32 patients were positive for probable IFIs fulfilling the host criteria, clinical criteria, and mycological criteria.

Out of the 100 cases, four different patients tested positive for fungi in blood cultures (proven IFIs), and IFI with abnormal chest X-rays; 24 cases were generalized infiltrates, of which

Table 1: Demographic data and underlying disease of patients enrolled in the study

Characteristics	*Patients with*						*Total*	
	Proven		*Probable*		*No IFIs*			
No. of episode	4		32		64		100	
No of death	0		13		5		18	
No of autopsies	0		0		0		0	
Age (Range)	3–12 years		1–13 years		1–15 years		1–17 years	
Sex	5M	8F	8M	11F	14M	13F	19M	22F
No with disease	0	0	2	0	2	1	0	2
ALL	0	0	5	1	5	8	13	10
AML	2	3	0	4	2	2	5	1
NHL	0	0	1	0	2	1	0	1
HD	3	0	1	0	1	1	2	1
WT	0	0	0	0	0	0	3	0
ES	0	0	0	0	2	0	1	1
Neuroblastoma	0	0	1	0	1	0	1	0
Rhabdosarcoma	0	0	1	1	0	2	1	2
Duration of episode (Days)	40–180		90–235		60–300		60–300	
Neutropenia	4		9		11		24	
Fever	4		12		24		40	
Steroid	4		12		24		40	
Anti-cancer Drug	4		12		24		40	

Table 2: Type of IFIs as per EORTC/MSGERC, 2020, 100 patients were classified as aplastic anemia patients.

Proven IFIs	*Probable IFIs*	*Possible IFIs*	*No IFIs*	*Total*
4	32	0	64	100

48% were present in IFI. In patients diagnosed with proven and probable invasive fungal infections, a prevalence of 60% was observed, highlighting neutropenia as a significant factor contributing to mortality, accounting for approximately 10% of cases. Although the assessment of GM Index values in patients with Aspergillosis is limited in AA patients, and research on GM assays as a diagnostic tool is scarce, we conducted a test on a single sample due to resource constraints. Despite these limitations, the results of this test are considered significant. This finding underscores the considerable association between neutropenia and the development of invasive fungal infections. In our study, 40% of the patients with IFI had respiratory complaints in the form of coughing and respiratory distress. Out of 40 patients with IFI, 32 (32%) had a history of steroid intake for more than 30 days. Out of 20 abnormal CT-thorax cases, 18 (18%) showed mass-like infiltrates suggestive of clinical criteria for IFI. Out of the total cases with suspected IFI, 86% of cases were positive for the GM assay.,

The sensitivity, specificity, PPV, and NPV of the GM tests were evaluated at different cut-off values. GM optical density

Table 3: Sensitivity, specificity, PPV, and NPV are evaluated at different cut-off galactomannan optical density indexes for proven and probable cases of IFIs.

Different cut off Value of GM	*Cut off 0.5 (%)*	*Cut off 0.7 (%)*	*Cut off 1 (%)*
Sensitivity	60	60	60
Specificity	48	28	48
Positive predictive value (PPV)	53.57	45	53.57
Negative predictive value (NPV)	54.54	41	54.54

indexes are shown in Table 3. When GM was evaluated at different cut-off values of ≥0.5, ≥0.7, and ≥1.0, the sensitivity of GM in our study was 60, 60, and 60%. Specificity was 48, 28, and 48%; PPV was 53.57, 45%, and 53.57; and NPV was 54.54, 41, and 54.54%, respectively.

DISCUSSION

Aplastic anemia is recognized as a rare condition with varying incidence rates globally, and the exact prevalence in India remains unknown due to a scarcity of epidemiological data. Our study encountered limitations, including the absence of paired samples due to the health condition of pediatric patients and constraints imposed by limited resources during the study. A research endeavor led by Ahmed *et al*.[4] indicated a disease occurrence of 6.8% in Lucknow. Numerous studies conducted in India suggest that a substantial proportion, ranging from 20 to 30%, of pancytopenia cases are eventually identified as aplastic anemia.[24] However, these studies faced limitations concerning the specified age groups and the quantity of enrolled patients. In referral hospitals, aplastic anemia constitutes a significant portion, ranging from 20 to 40%, of all patients presenting with pancytopenia.

It is the only study to date that looks at the serum GM EIA in a large group of children who are at risk for IA. The data it provides is very useful for figuring out how the test works in these patients. There were some false-positive results, but our data showed that the rate of false-positive results for serum GM EIA was lower in people with neutropenia and aplastic anemia. Out of 100 cases, 32 were found to have probable IA and 4 had proven IA (on retrospective analysis) (Table 2), which is a higher number as compared to the study done by Abrar *et al*.[4] Employing GM EIA on serum could present an alternative sample source for noninvasive diagnostic assistance or serve as a screening tool for IA. In a study by Baytan *et al*.[26] the incidence of IFD in aplastic anemia was reported to be 44.4% (n = 8/18), surpassing that observed in the acute leukemia group. But in our findings, 30% (n = 30/100) of patients were found to have developed IA. This is lower as compared to the above study. Young *et al*.[27] presented research exclusively conducted on pediatric patients. A solitary assay threshold positivity of ≤0.5 resulted in elevated sensitivity, specificity, PPV and NPV. (90, 92, 81.8, and 96%) in 62 febrile neutropenia children with either leukemia or pancytopenia. In the present stud Table 3 the sensitivity, specificity, PPV and NPV (60, 48, 53.57, and 54.54%) were measured in 30 out of 100 patients with IA in aplastic anemia, which is in agreement with the value demonstrated by Abrar *et al*.[25] Not a single patient was given an antifungal to protect them from invasive Aspergillus, and the test was considered positive when the GM cut-off was ≤ 0.5 in a sample or different cut-offs of 0.5, 0.7, or 1.0.

We looked at different GM cut-off values and found that the sensitivity, specificity, PPV, and NPV were all the same at 0.5 and 1.0 index values for diagnosing IA in people with severe aplastic anemia. Similar results were reported.[4,28] but they analyzed them in hematological pediatric cancer patients. In this study, IA was found in 4 confirmed cases and 26 probable cases. Out of 100 cases, 86% were given antifungal treatment and got better. One of the 4 confirmed cases died because they were diagnosed too late, and their blood culture showed the growth of Aspergillus fumigatus after three weeks of incubation. The deduction was made that fungal infections pose a significant challenge for children dealing with hematologic malignancies and AA. Both the underlying ailment and extended neutropenia emerge as crucial risk factors.[25] Establishing fungal disease in children is notably challenging. Hence, any indication in children susceptible to developing IFD warrants meticulous evaluation for prompt treatment, aiming to reduce morbidity and mortality.

Several factors contributing to reduced sensitivity and the occurrence of both false-negative and false-positive outcomes in the enzyme-linked immunosorbent assay (ELISA) method have been identified. In our study, the false-positive and false-negative results were associated with the EB-A2 antibody used in the GM assay, exhibiting immunoreactivity to the lipoteichoic acid of Bifidobacterium spp. This bacterium is commonly found in infant gut microflora, cereals, pasta, and formula milk. Additionally, the limitations of our study, including testing only a single sample due to the impracticality of frequent sample collection in deteriorating patient conditions, could also account for the observed false-positive and false-negative outcomes.. These factors encompass the specific species of Aspergillus being targeted, the existence of anti-Aspergillus antibodies, the simultaneous administration of certain β-lactam drugs, and the possibility of cross-reactivity with other organisms that contain GM. According to established research findings, a serum sample is typically regarded as yielding a positive outcome when the optical density index ratio surpasses the threshold of 0.5. In previous studies, it has been shown that GM EIA exhibits a high level of specificity in individuals diagnosed with hematological malignancies or those who have undergone HSCT.[29] Notably, false-negative and false-positive outcomes were identified in relation to Aspergillus species, the presence of anti-Aspergillus antibodies, the concurrent usage of specific lactam drugs, and cross-reactivity with other microorganisms harboring GM. According to established conventions, a serum sample is typically regarded as yielding a positive outcome when the optical density index ratio exceeds 0.5. The specificity of GM

EIA has been observed to be high in individuals diagnosed with hematological malignancies or those who have undergone HSCT. However, it is important to note that the sensitivity of GM EIA in these cases can vary between 29 and 100%.[30] In the present study, the sensitivity was 60%, which is in agreement with the value demonstrated by.[4] Abrar *et al*.[4] reported that patients suffering from severe aplastic anemia are more likely to develop IA than patients with other diagnoses. In the present study, 30 out of 100 cases were identified as IA positive. Additionally, some patients with elevated values of GM were suspected of having invasive fungal infections (IFIs). However, the absence of CT and radiological findings precludes definitive consideration of these cases as IFIs. The limitation arises from the unavailability of CT and radiological data; if these investigations were feasible, the classification of patients with high GM index values might differ. Nevertheless, cost constraints restricted the inclusion of CT and radiological findings in our study.. In another study, Ayas *et al*. reported that the incidence of IFIs in severe aplastic anemia patients was 79.17%, whereas in the present study, which is higher than that of.[31] but lower than.[30] In 2009, Baytan *et al*.[26] found that 26 of the 34 patients with IA had GM positivity. The sensitivity of the test was 64.5% for proven cases and 16.4% for IA. However, a substantial false positive rate of 44% was observed in children, emphasizing the necessity of employing the test in conjunction with other diagnostic assessments. Serial sampling is recommended to enhance detection capabilities. In the cohort of 100 patients with IA and aplastic anemia, GM positivity was identified in 86 cases. Previous studies reported a sensitivity of 60%, specificity of 48%, PPV of 53.57%, and NPV of 54.54% for the assay. The acquisition of paired samples both pre- and post-antifungal therapy is imperative in delineating essential treatment parameters and providing guidance to clinicians in formulating suitable treatment protocols. Consequently, we advocate for the obligatory adoption of the EORTC's clinical criteria for the diagnosis of IFIs in individuals with AA. This methodology expedites the discernment of efficacious treatment modalities, thereby ensuring the delivery of optimal patient care.

CONCLUSION

IFI, pose a significant threat to individuals with compromised immune systems, such as those suffering from hematological malignancies. The prevalence of these infections has risen notably in recent years, attributed in part to fibrosis development in the lungs, specifically the pleura, leading to breathing issues. Prolonged use of corticosteroids is a common factor in the development of this fibrosis. Intensive treatment protocols, severe anemia, damage to mucosal barriers, and widespread use of broad-spectrum antibiotics contribute to the increased occurrence of IFIs.

In a study conducted at KGMC Lucknow, blood samples were collected from 100 anemic patients in the pediatric oncology department. Aplastic anemia cases were 46% male and 54% female. Among the cases, 32 patients met host, clinical, and mycological criteria for IFIs. The Galactomannan GM test, with an index value > 0.5 indicating infection, detected galactomannan in 86 out of 100 aplastic anemic patients. Only 4 cases were confirmed as fungal blood culture positive (proven IFIs). Patients were categorized as confirmed (4%) or likely (32%) cases of aplastic anemia, with 64% of IFI patients experiencing respiratory complaints.

Out of suspected IFI cases, 86% tested positive with the GM assay. Neutropenia emerged as another important mortality factor, especially in patients with Neutropenic fever during chemotherapy, with hospitalization rates of approximately one in 29 patients and mortality rates ranging between 6.8 and 9.5%, accounting for about 10%.

Fungal infections present a significant challenge in individuals with hematologic malignancies and aplastic anemia, with underlying ailments and prolonged neutropenia as critical risk factors. Detecting IA in at-risk children is challenging, making early intervention crucial. New antifungal treatments and the GM test, particularly in serum using EIA, show promise in enhancing patient survival rates. The GM test serves as a valuable diagnostic tool for pediatric patients, offering swift diagnoses in neutropenic patients with aplastic anemia. In developing nations like India, establishing the significance of a single sample is crucial due to limitations in frequent sampling caused by the patient's health condition or the high cost of the test.

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