Diagnosis of Invasive Fungal Infections in Children

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Invasive fungal infections are important causes of morbidity and mortality particularly in high-risk patients. Recognizing such infections is often difficult because of non-specific symptoms and clinical signs. Timely diagnosis is also a challenge due to difficulty in obtaining adequate volume of samples, need for anaesthesia to perform certain diagnostic procedures, and insufficient data and experience related to fungal biomarkers and molecular detection tests. This results in widespread use of empiric broad spectrum antifungal agents with the consequent emergence of drug-resistant strains. This review focusses on the definition, clinical and microbial profile and diagnostic modalities for invasive fungal infections.

Keywords: *Fungal sepis, Invasive candidiasis, Opportunistic infections.*

Invasive fungal infections (IFI) are devastating opportunistic infections that cause significant morbidity and mortality, classically in children with a hematological malignancy like leukemia, or those who have undergone a hematopoietic stem cell or solid organ transplant. The incidence of IFI in these groups is between 5% and 25% [1]. Premature neonates or those with congenital or acquired immunodeficiency constitute a minor group of children who develop IFI [2,3]. Other patient groups at risk for IFI, including those with genetic predisposition [4], are mentioned in ***Box* I**.

Recognizing IFI is often difficult because of non-specific signs and symptoms where fever may be the only symptom. Therefore, there should be a high index of suspicion in patients at increased risk of IFI. Mortality is high in those who develop IFI and is influenced by the type of fungus, site of disease and presence of ongoing immunosuppression. Timely diagnosis of IFI is challenging due to difficulty in obtaining clinical sample, need for anaesthesia to perform certain diagnostic procedures, and insufficient robust data on usefulness of fungal biomarkers and molecular detection tests. Definitive diagnosis by tissue microscopy and culture is difficult in the early stages of infection. Using host factor and microbiological and clinical criteria, current definitions exist for proven, probable and possible invasive fungal infection [5]. In practice, IFI is often suspected in those with persistent fever and neutropenia who fail to respond to broad spectrum antibacterial agents within three to five days, and in whom a reasonable attempt has been made to exclude bacterial and viral infections. As mortality is high, early and aggressive treatment of suspected infections with empiric antifungal therapy is common. As many patients with persistent fever and neutropenia on empirical antifungal therapy do not have a fungal infection, there is increasing interest in biological markers and radiological imaging to identify those at highest risk of IFI [6]. There are currently insufficient data to support this approach in children. Conventional amphotericin B deoxycholate is a broad spectrum antifungal agent that has previously been the treatment of choice for most IFI. Its use is complicated by nephrotoxicity and infusion-related reactions. More recently, less toxic lipid formulations of amphotericin B have become available. Triazole derivatives (fluconazole, itraconazole, voriconazole, posaconazole and ravuconazole) and echinocandins (caspofungin, micafungin and anidulafungin) are the other available agents. The role of biological agents *e.g.* antiHSP90 monoclonal antibodies, Mycograb is being explored. The increasing use of combination antifungal therapy adds further complexity to treatment decisions [7]. Knowledge of predisposing condition, likely organisms, risk factors, relevant clinical signs and symptoms of IFI is important for enabling early diagnosis of IFI and improving disease outcomes [8] (***Web Table* I**).

DEFINITION

Invasive fungal infections cooperative group (IFICG) of European Organization for Research and Treatment for Cancer (EORTC) and Mycology Study Group (MSG) of National Institute of Allergy and Infectious Diseases (NIAID) have published standard definitions of IFI [5].

BOX I RISK FACTORS FOR INVASIVE FUNGAL INFECTION IN CHILDREN

Infections
- Cytomegalovirus (CMV)
- Inadequate treatment of superficial fungal infections
- Human immunodeficiency virus (HIV)
- Mycobacterium tuberculosis

Malignancy
- Hematological malignancy (predominantly acute myeloid leukemia)
- Solid tumors

Debilitated patients
- Critically ill patients on prolonged mechanical ventilation
- Neonatal and pediatric intensive care unit patients

Transplant patients
- Hematopoietic stem cell transplant (HSCT) / solid organ transplant

Non-Infectious causes
- Severe trauma
- Major surgical procedures
- Indwelling prosthetic devices
- Long-term use of antibiotics
- Prolonged hospitalization
- Extremes of age (preterm neonates)
- Immunosuppressive therapy

Immunological deficiency
- Neutropenia

Autoimmune diseases
- *Genetic predisposition*
- Impaired NADPH oxidase activity
- Abnormal synthesis of tumor necrosis factor α (TNF α), Interleukin 10 and other cytokines

Proven IFI: The presence of fungal elements either as mould/yeast in deep tissues of biopsy/needle aspirates that is confirmed on culture and histopathological examination.

Probable and Possible IFI: Based on host factor and microbiological and clinical criteria and indirect tests [9].

Fungi having potential to cause IFI include Yeasts (*Candida spp, Cryptococcus spp*), moulds (*Aspergillus spp, Fusarium spp, Scedosporium prolificans, Mucor, Rhizopus,* and *Rhizomucor Absidia*), and dimorphic fungi (*Histoplasma capsulatum, Coccidioide simmitis, Blastomyces dermatitidis, Paracoccidioides spp, Sporothrix spp* and *Penicillium marneffii*).

DIAGNOSIS

High index of suspicion, early recognition and prompt antifungal treatment are the key factors to prevent invasive fungal diseases. Diagnosis of IFIs in pediatric population is challenging as clinical and radiological findings are often nonspecific, the volume of sample required to meet good sensitivity is lacking, culture-based methods have low yield, and histopathological examination can be hazardous. Recently introduced serological assays like galactomannan (GMN) and 1,3-β-D-glucan (BDG) antigens and molecular techniques have proved to be useful in improving the diagnosis of IFI.

Conventional Methods (*Direct Microscopy and Culture*)

Microscopy (direct and histopathology) and culture are the cornerstones for the diagnosis of IFI. Direct examination implies the use of 10% KOH–calcofluor mount. Microscopic examination may be less sensitive than culture and hence, a negative result does not rule out fungal infection. Positive direct microscopy of yeast or hyphae from a sterile site must be considered significant, even if the laboratory is unable to culture the fungus. Demonstration of septate hyphae from respiratory secretion in immunocompromised patient is suggestive of aspergillus, fusarium and scedosporlum, and broad aseptate hyphae for invasive mucormycosis. India ink staining of cerebrospinal fluid for diagnosing cryptococcal meningitis has a sensitivity of 60%. *Pneumocystis jirovecii* is detected by microscopy on Giemsa staining, which demonstrates the nuclei of trophozoites and intracystic stages, and silver stains, which display the cyst walls. The finding of 2- to 4 μm, oval, narrow-based budding yeasts allows a tentative diagnosis of histoplasmosis. Other organisms which can mimic the appearance of *H. capsulatum* in tissues includes *Candida glabrata*, *Penicillium marneffi*, *Leshmania donovani* bodies. Culture represents the gold standard in diagnosing fungal diseases.

Invasive candidiasis (IC) (both albicans and non-albican) is a frequent infection in hospital settings. Blood culture is considered as gold standard for diagnosis of candidemia [10,11] with sensitivity between 21% and 71%; lower in neonates and infants [12]. The European Society of Clinical Microbiology and Infectious Diseases / Fungal Infection Study Group ESCMID/EFISG

guideline recommends collection of three blood samples with a minimum volume of 2 mL each in children with a bodyweight <2 kg and 6 mL each between 2 and 12 kg to ensure a 50-75% sensitivity to detect *Candida* [13]. Blood cultures are further limited by slow turn-around time and are negative in deep seated *Candida* infections including hepatosplenic candidiasis. Aspergillus species are rarely cultured from blood samples, except *Apergillus fumigatus* and *Aspergillus terreus* [14]. The sensitivity of blood culture for invasive Aspergillosis is ~10% and ~40% in disseminated fusariosis. The culture positivity of broncho-alveolar lavage (BAL) is 34% - 50% [15,16]. The tissue culture positivity in mucormycosis is ~50% [17]. Histopathological examination helps in differentiating different classes of moulds with an accuracy of less than 80% and need of culture for confirmation. The antifungal susceptibility pattern of the isolates guide the therapy.

Fungal Biomarkers

There are various nonculture methods available for diagnosis of IFI: Galactomannan (GM) for invasive aspergillosis; IA,1,3-β-D-glucan (BDG), a pan fungal marker for aspergillosis, candidemia and *Pneumocystis jiroveci* pneumonia; Mannan (Mn) antigen and anti-mannan antibody (a-Mn) assay for candidiasis; Latex agglutination and Enzyme immune assay (EIA) for Cryptococcus; Point-of-care test Lateral flow assay (LFA) for *Aspergillus* and *Cryptococcus*; and novel methods like T2 candida and molecular assay. In majority of studies, serum galactomannan assay and molecular methods have been evaluated in children.

Galactomannan: GM is a polysaccharide cell wall constituent of *Aspergillus* spp which is released during hyphal growth. It can be detected in serum, BAL and in CSF with immunoenzymatic microplate sandwich assay [18,19]. It serves as a useful tool in diagnosis of IA in adjunct to radiological findings and direct microscopy. Serum GM and high resolution CT chest imaging have been included in EORTC and MSG definition in pediatric patients [5,20]. The sample should be collected before starting the antifungal treatment in screw cap vial and stored at -20ºC if not processed on same day. The test should be performed inside the bio-safety cabinet to prevent any contamination. The results have to be interpreted cautiously as this antigen cross reacts with some of the endemic fungi, *Histoplasma capsulatum*, *Penicillium* sp and other fungi *Fusarium* spp, *Trichosporon* spp, patients on beta lactam/beat-lactamase inhibitor combi-nations like piperacillin-tazobactum and amoxicillin-clavulanate, patients receiving enteral nutrition, and in BAL-fluid specimens containing Plasma-Lyte with gluconate [21]. Currently available generic piperacillin- tazobactum preparations do not cross react. Non angio-invasive aspergillosis as seen in non-neutropenic immune compromised patients and systemic mould-active antifungal prophylaxis, are associated with false negative tests. The sensitivity varies from 78-90% and specificity 80-90% in adults with lower specificity in pediatric cases because of colonization with *Bifidobacterium* and soya food [22]. Screening twice weekly in children at high risk for IFI should be considered for early diagnosis. Although optimal cut-off value of GM in children is not well defined, there is an agreement on threshold of OD 0.5 in two consecutive samples or 0.7 in single sample (serum, CSF, BAL) [23]. The utility of CSF GM as a diagnostic tool for aspergillosis in CNS has not been supported by robust literature. The prognostic value of the test in predicting mortality has not been evaluated in pediatric populations. Presently, GM serves for prompt diagnosis of aspergillosis IA and can be used to monitor the effectiveness of antifungal therapy.

Lateral flow assay: A point of care assay has been recently FDA approved for diagnosis of invasive aspergillosis. The antigen is *Aspergillus* specific manoprotein that is detected by monoclonal antibody. It has a rapid turnaround time and does not cross react with other moulds [24]. According to a recent meta-analysis, the overall sensitivity and specificity of 68% and 87% in serum and of 86% and 93% in BAL is observed when compared with GM [25]. The test has not yet been evaluated in pediatric populations.

Beta D glucan (BDG): β D glucan is a panfungal marker (unlike the GM test which detects only IA) present in all fungi except *Mucormycetes* and *Cryptococcus.* There are four commercial kits available. The only FDA approved kit is Fungitell. The other three kits (Fungitec-G, Wako, Maruha*)* are mostly used in Japan. A cut-off value of <60 pg/mL is considered as negative, >80 pg/mL as positive and 60-80 pg/mL as borderline in adults with Fungitell. False positive may be seen with bacteremia with gram-positive or negative bacteria, mucositis and mucosal colonization with *Candida* species, administration of albumin and immunoglobulins, thrombocyte infusion with leukocyte removing filters, hemodialysis with cellulose membranes, use of blood products filtered through cellulose filters, *P. jirovecii* infection, administration of meropenem, cefepime, piperacillin tazobactam, or amoxicillin-clavulanate [26-29]. It has been observed that BDG levels are higher in healthy children as compared to healthy adults [30]. At present, the diagnostic utility of BDG in children is limited with poorly established cut-offs. It might help either as an adjuvant

with other diagnostic methods or as an indirect parameter to monitor IFIs.

Mannan antigen (Mn) and anti-Mannan antibody (a-Mn): Detection of mannan antigen may be helpful in serum of hepatosplenic candidiasis and Candida meningitis where blood culture is rarely positive [31]. The sensitivity and specificity of Mn/a-Mn antibodies combination is 83% and 86%, respectively. The sensitivity depends on type of *Candida* spp (species specific) *viz* 100% in *Candida albicans*, 50% in *Candida krusei* and *Candida kefyr* and 40% in *Candida parapsilosis* [32]. Presently, utility of this assay is limited because of several disadvantages like rapid clearance of the *Candida* Mn from serum, and cross-reactions due to colonization with *Candida* spp.

Markers for Cryptoccocosis and Histoplasmosis: Diagnosis of Cryptococcal meningitis or disseminated cryptococcosis is possible by demonstration of encapsulated yeast cells on India ink, culture or detection of Cryptococcal antigen (CrAg). The detection of CrAg in serum or CSF can be done by latex agglutination test (LA) and enzyme immunoassay (EIA). The sensitivity and specificity of LA varies from 93%-100% and 93%- 98 %. False-positive findings have been reported in cases of *Trichosporon* spp., *Capnocytophaga* spp., or *Stomatococcus* spp. infections or detergents. Recently,a point of care assay, an immune chromatographic lateral flow assay (CrAg LFA; Immuno-Mycologics, Norman, OK, USA) has been designed which has a sensitivity and specificity of 98% [21]. It is cheaper, has shorter turnaround time (15 min) and can also detect *C. gattii*, an additional advantage in comparison to other CrAg tests available [33].

Histoplasma takes several weeks to grow on culture. Detection of Histoplasma antigen from serum or urine is non-invasive, and is a rapid test for diagnosis in disseminated histoplasmosis. The sensitivity is highest in disseminated histoplasmosis followed by acute and chronic pulmonary and least in subacute pulmonary histoplasmosis. Rising antigen levels can also be used as early predictors for clinical relapse or treatment failure. A limitation of antigen testing is the significant cross-reactivity of the assay in the presence of other fungal infections, including blastomycosis, paracoccidioidomycosis, penicilliosis, aspergillosis, and coccidioidomycosis [34].

Molecular Methods

Molecular methods are potential alternative options for early diagnosis of IFI, ideal being broad-range/pan-fungal PCR. European Fungal PCR Initiative group (FPCRI) have standardized and validated protocols for *Aspergillus* PCR which can be used as a screening tool because of its high negative predictive value. SeptiFast PCR, a commercial mutiplex realtime PCR (Roche diagnostics, Germany) available for 20 clinically relevant pathogens including six fungi *i.e*. five *Candida* spp and *A. fumigatus* proved to be helpful where culture was negative. Rate of positivity was 14.6% as compared to culture (10.3%) [35]. PCR positivity depends on site and amount of clinical specimen. The *Asper Genius* assay detects and differentiates wild type from pathogenic *Aspergillus fumigatus* with (four) azole resistance associated mutations in the *cyp51A* gene. FKS1-echinocandin resistance for *Candida* spp can also be done directly from blood [36].

Recently FDA approved T2 candida method which is a rapid test to detect five species of *Candida* (*C. albicans, C. tropicalis, C. parapsilosis, C. krusei and C. glabrata*) directly from blood sample. Principle of the assay is initial nucleic acid extraction amplification and hybridization of the product. The detection limit is 1 CFU/ml (compared 100 -1000 CFU/ml in NAATs). It is fully automated and results provided within 3-5 hours. However, this technique has not yet been evaluated in pediatric population [21].

Radiological Diagnosis

The role of imaging in invasive fungal infections is multi-dimensional. Imaging helps in identification of the focus of infection, establishing a possible diagnosis, and detection of serial changes. Plain radiographs often prove inconclusive. Other imaging modalities include computed tomography (CT), ultrasound and magnetic resonance imaging (MRI).

Contrast enhanced CT scan remains the most important imaging modality in the diagnosis of invasive fungal infections. Evaluation of the pulmonary pathology requires a reconstruction of images into routine lung window and high resolution lung window images. With the present day fast CT scanners and a volumetric acquisition of data, good quality image acquisition in a very short time frame is made possible. This is especially crucial in acutely ill febrile or dyspneic children. The appearance of new abnormalities on CT chest not responding to broad spectrum antibiotics should be considered as possible IFI in these children.

Detection of any abdominal focus of infection requires a meticulous ultrasound examination with additional high resolution ultrasonography, using high frequency linear transducer of 5-12 MHz, of the solid

organs such as liver, spleen and kidneys.Transcranial USG using a small footprint sector probe is useful in neonates and young infants.

MRI is invaluable in the evaluation of infection of the central nervous system and musculoskeletal system. MRI is helpful in follow-up of pulmonary fungal infections as it is non-ionizing radiation, though its sensitivity in the detection of small pulmonary nodules in infection is less than CT.

CNS infections: CNS infections in IFI vary depending on the organism. CNS aspergillosis may be seen on imaging as (*i*) multiple areas of embolic infarcts secondary to meningitis and involvement of perforating vessels, (*ii*) multiple ring enhancing lesions or (*iii*) contiguous involvement of the dura associated with adjacent sinusitis/skull base osteomyelitis [37]. CNS mucormycosis is usually associated with sinusitis and bone erosion. Often there is associated cavernous sinus thrombosis and contiguous CNS spread of infection (***Web Fig.* 1**). Disseminated hematogenous candida infection may result in meningoencephalitis. The imaging findings include meningeal enhancement and multiple ring enhancing or nodular lesions in the brain parenchyma.

Screening patients with probable and proven invasive pulmonary aspergillosis by MRI of brain is recommended even in absence of neurologic signs/symptoms. Dissemination of pulmonary aspergillosis to CNS has been reported in 14% and clinical features of CNS infection occur late in the course of disease.

Sinonasal infection **(*Web Fig.* 2)**: Sinonasal invasive fungal infection on imaging appears as soft tissue opacification of the sinonasal cavity with mucoperiosteal thickening; with variable degree of bony erosion. Skull base osteomyelitis and contiguous CNS spread of infection can occur as a complication.

Pulmonary infection: Pulmonary candidiasis can present as a part of disseminated candidiasis. The imaging features can manifest as lobar consolidation and mimic bacterial pneumonia or may present as multiple nodules similar to aspergillosis. Invasive aspergillosis (***Web Fig.* 3**) usually shows multiple pulmonary nodules often with perinodular ground glass opacity 'halo sign'. The imaging signs of improvement include cavitation (crescent sign) within the nodule. Pulmonary mucormycosis can present as airspace consolidation or multiple nodules. The nodules may show halo sign or reverse halo sign. Reverse halo sign refers to an area of peripheral consolidation and a central ground glass opacity. Bird's nest sign is seen as central ground glass opacity with multiple intersecting or irregular lines [38]. Both the signs can be seen in angioinvasive aspergillosis, mucormycosis, cryptogenic organizing pneumonia, Wegener's granulomatosis and other causes.

Abdominal infection: Disseminated candidemia may show focal lesions in liver, spleen and other abdominal organs. The lesions appear hypoechoic, target lesion or as small abscesses on USG. CT has superior sensitivity than USG in detecting focal hepatosplenic lesions which appear hypodense (***Web Fig.* 4**).

Musculoskeletal: Musculoskeletal involvement in disseminated fungal infection may manifest as osteomyelitis and arthritis. Vertebral and costal fungal osteomyelitis can develop from contiguous pulmonary infection as in aspergillosis, or by hematogenous dissemination and traumatic inoculation. The imaging findings are similar to other forms of osteomyelitis and include osteopenia, erosions and periosteal reaction.

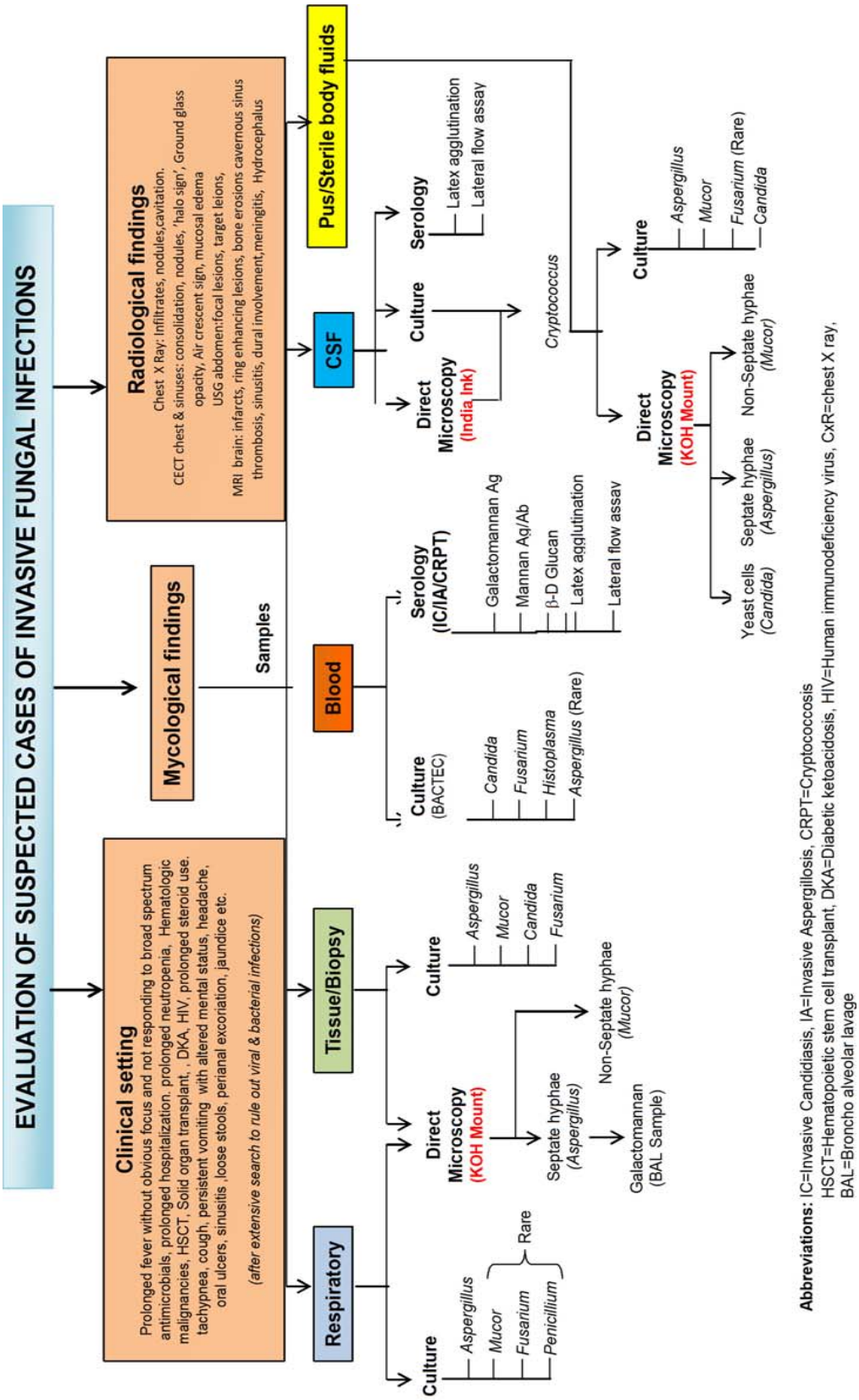
Approach to a Suspected Case of Fungal Infection

The risk factors for occurrence of fungal infections must be kept in mind while evaluating a patient of pyrexia of unknown origin or unusual signs/symptoms at usual/unusual sites. If signs /symptoms of localized fungal infection are present (oral ulcers/skin ulcers/pneumonia/sinusitis) sample must be sent as soon as possible for direct microscopy and culture for definitive diagnosis. The treating team must interact with the mycologist and radiologist when evaluating such a case. The investigations must be ordered judiciously and interpreted rationally as definite evidence is often lacking and antifungals have significant side effects and require prolonged administration. They should be chosen depending on the organism identified, host characteristics, site of infection and local epidemiological data of fungal infections including resistance.

A suggested algorithm for evaluation of a suspected case of fungal infection is depicted in ***Fig* 1**.

CONCLUSION

Diagnosis of IFIs in children remains challenging. The validation and utility of various currently available fungal diagnostic tools are lacking, particularly in the pediatric group. Although tissue diagnosis remains the gold standard, other tests like Galactomannan assay and PCR can be used as adjunct for diagnosis of IFI in children. Newer methods like T2 candida and lateral flow assay need validation in children with candidemia and invasive aspergillosis. Role of radiology in diagnosing IFI needs further exploration.



Abbreviations: IC=Invasive Candidiasis, IA=Invasive Aspergillosis, CRPT=Cryptococcosis HSCT=Hematopoietic stem cell transplant, DKA=Diabetic ketoacidosis, HIV=Human immunodeficiency virus, CxR=chest X ray, BAL=Broncho alveolar lavage

FIG. 1 *Evaluation of suspected invasive fungal infection.*

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