Review Article

Diagnosis of invasive pulmonary aspergillosis in the intensive care unit: what we should concern and how to do better

Hui Shen[1], Zhongheng Zhang[2], Yuetian Yu[3]

[1]Department of Laboratory Medicine, Shanghai East Hospital, Tongji University School of Medicine, Shanghai 200123, China; [2]Department of Emergency Medicine, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou 310020, China; [3]Department of Critical Care Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200001, China

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Correspondence to: Yuetian Yu. Department of Critical Care Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, 145, Middle Shandong Road, Shanghai 200001, China. Email: fishyyt@sina.com; Zhongheng Zhang. Department of Emergency Medicine, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, 310020, China. Email: zh_zhang1984@zju.edu.cn.

Abstract: Invasive pulmonary aspergillosis (IPA) is no longer a rare disease in the intensive care unit (ICU) on account of the increased number of immunocompromised hosts admitted, the application of invasive treatment procedures and the widespread use of broad-spectrum antibiotics. Different from those with agranulocytosis, symptoms and signs of IPA in ICU patients are subtle or non-specific which are unable to be distinguished from those with bacterial pneumonia or even with noninfectious diseases. Thus, accurately diagnosing of IPA is quite challenging. In this review, we will navigate through the IPA diagnostic tests currently available and how they apply to ICU population. Special attention is paid to the non-culture- based tests as well as have a look into what the future holds.

Keywords: Invasive pulmonary aspergillosis (IPA); intensive care unit (ICU); diagnosis; detecting techniques; biomarkers

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Background

Aspergillus is a large sort of saprophytic fungi which are present almost everywhere in the environment and more azole-resistant *Aspergillus fumigatus* have been detected in intensive care unit (ICU) patients these years (1-4). Invasive pulmonary aspergillosis (IPA) may lead to fatal illness if timely diagnosis and appropriate management is not done. What is more, it cannot be denied that IPA has been on the rise in the ICU during the last two decades due to the increased number of immunocompromised hosts admitted and the application of invasive treatment procedures (5,6). However, data regarding the incidence of IPA in the ICU is limited and IPA diagnosis accurately is a quite challenging job, because of the non-specific symptoms or signs, which may lead to the misdiagnosis of bacterial pneumonia or even non-infectious diseases (7-9).

For various reasons, the true incidence of IPA among critical ill patients is variable (10). First, biopsy is hard to perform in ICU due to the potential adverse effects like bleeding or pneumothorax (11,12). Second, postmortem examinations are not routinely performed in most of medical institutions (13,14). Third, it remains challenging to discriminate between infection and colonization with positive *Aspergillus* species culture results. Forth, the sensitivity and specificity of biomarkers for diagnosing IPA varies a lot, especially in the nonneutropenic ICU patients.

The current recommended criteria by European Society for Clinical Microbiology and Infectious Diseases (ESCMID) and the Infectious Diseases Society of America

(IDSA) still suggest collecting risk factors, chest imaging and biological data to support the diagnosis of IPA with a strong presumption (15,16). Recently, with the development of detecting technologies, non-culture-based methodologies are widely used in diagnosing IPA (17). Thus, we present the various arguments for the diagnosis of IPA in ICU patients, with a particular emphasis on the novel biomarkers and exploration techniques.

Epidemiology and risk factors of IPA in ICU

Previously, IPA was mostly observed in immunocompromised host in the department of hematology or oncology. It was once reported that the incidence of IPA could be as high as 59.2% in patients with hematopoietic stem-cell transplantation recipients (HSCT) whereas the incidence was relatively lower (1–6%) in those with solid organ transplantation (SOT) (18,19), even if the lung transplantation recipients are more at risk due to the early exposure of the spores in the contaminating environmental.

In the department of critical care setting, *Aspergillus* can harbour in the water and ventilation systems, as well as in various types of equipment. However, it is difficult to discriminate between true infection and colonization when *Aspergillus* is isolated from the lower respiratory tract specimens. The incidence of IPA in ICU ranges from 0.3% to 5.8% (20,21), and the overall mortality rate is over 80% (22,23).

The "classis" risk factor known as neutropenia was only observed in 10%-15% ICU patients while other factors were recognized in nonneutropenic ICU patients these years (24). Critical ill patients in ICU are subjected to multiple therapies to treat the diseases or even to maintain life (25,26). The therapeutic schedule may include insertion of central venous catheters (CVC), invasive mechanical ventilation (IMV), continuous renal replacement therapy (CRRT), parenteral nutrition (PN) or broad-spectrum antibiotics application that may affect the immunological defence system (27,28).

Chronic obstructive pulmonary disease (COPD) may lead to hypercapnia and sometimes IMV is need. Furthermore, COPD has become a considerable risk factor for IPA in ICU patients (29,30). It seems that *Aspergillus* colonization can easily be detected in the lower tract of airway which may lead to IPA due to lung structure alterations, reduced mucociliary clearance, impaired immunological response and mucosal lesions (31). Both compensated and decompensated cirrhosis have been described as risk factors for IA, and impaired phagocytosis has been proposed as a possible explanation for heightened risk in these groups (32). Diabetes has been observed as another risk factor due to the impaired innate and acquired immunity caused by hyperglycemia (33). Other risk factors like influenza and extracorporeal membrane oxygenation (ECMO) were also noted in the lecture (34-37).

IPA definition in ICU

Improve diagnostic accuracy of IPA in ICU is quite challenging. The criteria of IPA include proven, probable and possible diagnosis (38) which was only validated in immunocompromised host like HSCT or SOT.

Positive *Aspergillus* culture in respiratory tract samples is not uncommon in ICU and the upward tendency has been observed these years (39). However, to discriminate colonization and real infection remains a vexed question. To overcome this obstacle, the AspICU clinical algorithm has been proposed to discriminate IPA from *Aspergillus* colonization in ICU patients with higher diagnostic utility than existing tests (40) (*Table 1*). In Blot's multicenter observational study (40), this algorithm revealed 92% sensitivity and 61% specificity among 115 IPA proven patients. Although it seems to be an effective method to discriminate disease from colonization in critical ill patients, there still a number of shortages. This clinical algorithm includes at least one positive Aspergillus culture in a respiratory tract specimen as an essential inclusion criterion, while positive results are obtained for only approximately 50% of patients with IPA (41) that leads to misdiagnosis or missed diagnosis of IPA patients with negative culture results. Another flaw is that the algorithm does not include any antigen or DNA testing. Schroeder M tried to modify the algorithm by adding BALF GM value (42), and the study revealed that it could increase the diagnostic sensitivity for IPA in ICU patients. Anyhow, items of the algorithm really need to be modified since the development of new testing methods, and it is still a long way to go (43).

Diagnostic methodologies of IPA

Histological detection

Direct histopathological identification in lung tissue biopsies remains the gold standard for diagnosing IPA which is performed by CT-guided transthoracic biopsies, convex endobronchial ultrasound transbronchial needle

Table 1 AspICU criteria

Putative invasive pulmonary aspergillosis (all four criteria must be met)
(I) *Aspergillus* positive lower respiratory tract specimen culture.
(II) Compatible signs and symptoms (one of the following)
• Fever refractory to at least 3 d of appropriate antibiotic therapy
• Recrudescent fever after a period of defervescence of at least 48h while still on antibiotics and without other apparent cause
• Pleuritic chest pain
• Pleuritic rub
• Dyspnea
• Hemoptysis
• Worsening respiratory insufficiency in spite of appropriate antibiotic therapy and ventilatory support
(III) Abnormal medical imaging by portable chest X-ray or CT scan of the lungs.
(IV) Either IVa or IVb
(IVa) Host risk factors (one of the following conditions)
• Neutropenia (absolute neutrophil count 500/mm^3) preceding or at the time of ICU admission
• Underlying hematological or oncological malignancy treated with cytotoxic agents
• Glucocorticoid treatment (prednisone equivalent 20 mg/d)
• Congenital or acquired immunodeficiency
(IVb) Semiquantitative Aspergillus positive culture of BAL fluid without bacterial growth together with a positive cytological smear showing branching hyphae

aspiration or transbronchial biopsies in most cases (44-46) (*Figure 1*). According to Hoffer's retrospective study (47), 28 percutaneous biopsies revealed that IPA was diagnosed with 100% (18/18) specificity and 100% (10/10) sensitivity in the cohort of immunosuppressed children.

As is well-known, lung biopsy is not without risk in critical ill patients who are suspected IPA, especially in those with respiratory failure or even need invasive mechanical ventilation. According to Libby's meta analysis, the surgical complication rate of lung biopsy was about 22% which might lead to an overall 44% mortality rate (48). In patients with invasive mechanical ventilation, the incidence of complication could be as high as approximate to 60% (49).

Air leak and pneumothorax are the most common lung biopsy complication while procedure related bleeding or hypoxia can also be observed in some patients (50-53). The question as to whether to perform lung biopsy in patients suspected IPA does not have a clear answer till now (54,55). The benefit of this invasive procedure must be weighed carefully against both the inherent risk and the risk of a result which might influence IPA therapy.

Direct microscopic examination and culture

The biological sampling is targeted to the anatomic site of *Aspergillar* disease development. The deeper and closer to the suspicious lesion initially observed on imaging sampling is made, the more it is pertinent (56). Direct microscopic examination is always performed on a fresh sample like bronchoalveolar lavage fluid (BALF), in a wet medium between a glass slide and cover slip (57-59). The morphological characteristics of *Aspergillus spp*. are the presence of hyaline and septate hyphae with dichotomous branches at angles of 45° and with uniform width (3–6 μm) (60,61). The direct examination is sometimes performed after fluorescent marking, which can increase the sensitivity of hyphae detection. Sometimes *Aspergillus* crown could be observed in KOH direct compression (*Figure 2*). However, it is really a hard work or impossible to distinguish the species of *Aspergillus* because of the difficulty in distinguishing the morphology of the different fungi species (62,63). What is more, *Pseudallescheria boydii* and *Fusarium* were reported to have the same morphological

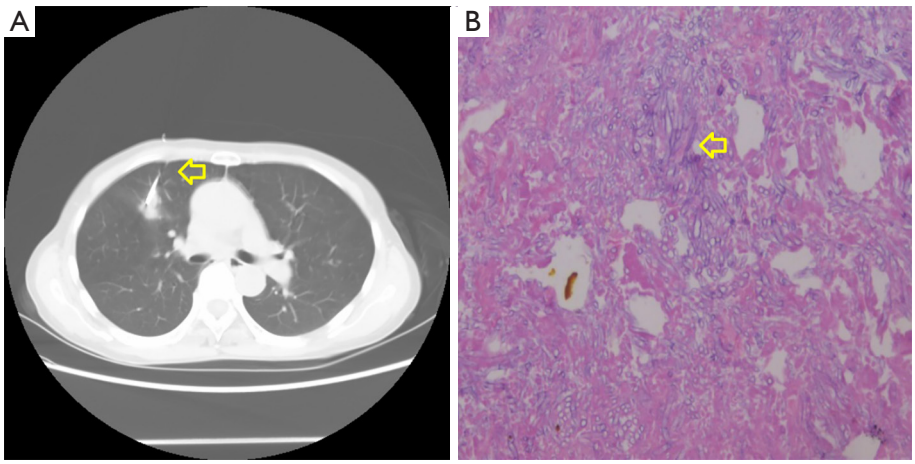

Figure 1 Lung biopsy. (A) Fine-needle aspiration was done in right middle lobe guided by CT findings to make a definitive diagnosis of IPA (yellow arrow); (B) typical *Aspergillosis* hyphae can be observed in lung tissues (HE, 200×) (yellow arrow).

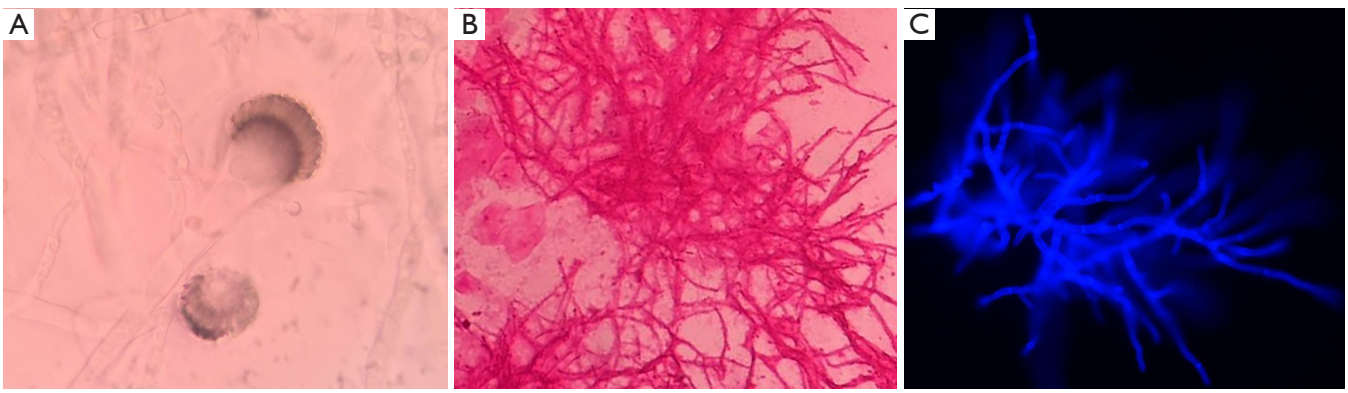

Figure 2 Direct microscopic examination of *Aspergillus*. (A) *Aspergillus* crown could be observed in KOH direct compression (400×); (B) *Aspergillus* hyphae in sputum smear (Gram stain, 100×); (C) *Aspergillus* hyphae in bronchoalveolar lavage fluid sample (Fluorescence staining, 400×).

characteristics (64).

Sabouraud's dextrose-agar is the most used medium in culture method. The global yield of the positive culture results are rather moderate, including for IPA proven, at 50% (65). *Aspergillus spp*. usually grows in 2 to 5 days, or less for some species if the culture medium is kept at 37 ℃ (66). In addition, the experience and knowledge of the microbiologist is of great importance to identify the colonies correctly.

Bronchoscopy

The development of bronchoscopy and bronchoalveolar lavage (BAL) has led to an increased use in ICU, where their applications for differential diagnosis of pulmonary diseases make them indispensable instruments for intensivists (67-69). Invasive aspergillus tracheobronchitis (IATB) is no longer rare only in immunocompromised host or those with malignancy in ICU which may be an early period of IPA (70). It was reported that lung parenchyma was usually involved together with IATB which led to a poor prognosis and high mortality (71).

Intraluminal lesions could be divided into four types according to the relevant bronchoscopic features (*Figure 3*). (I) Full-layer involvement type: tracheobronchial lesions infiltrating through the matrix layer of bronchi with extensive pseudomembrane formation; (II) ulcerative type: superficial ulcer and inflammatory infiltration in

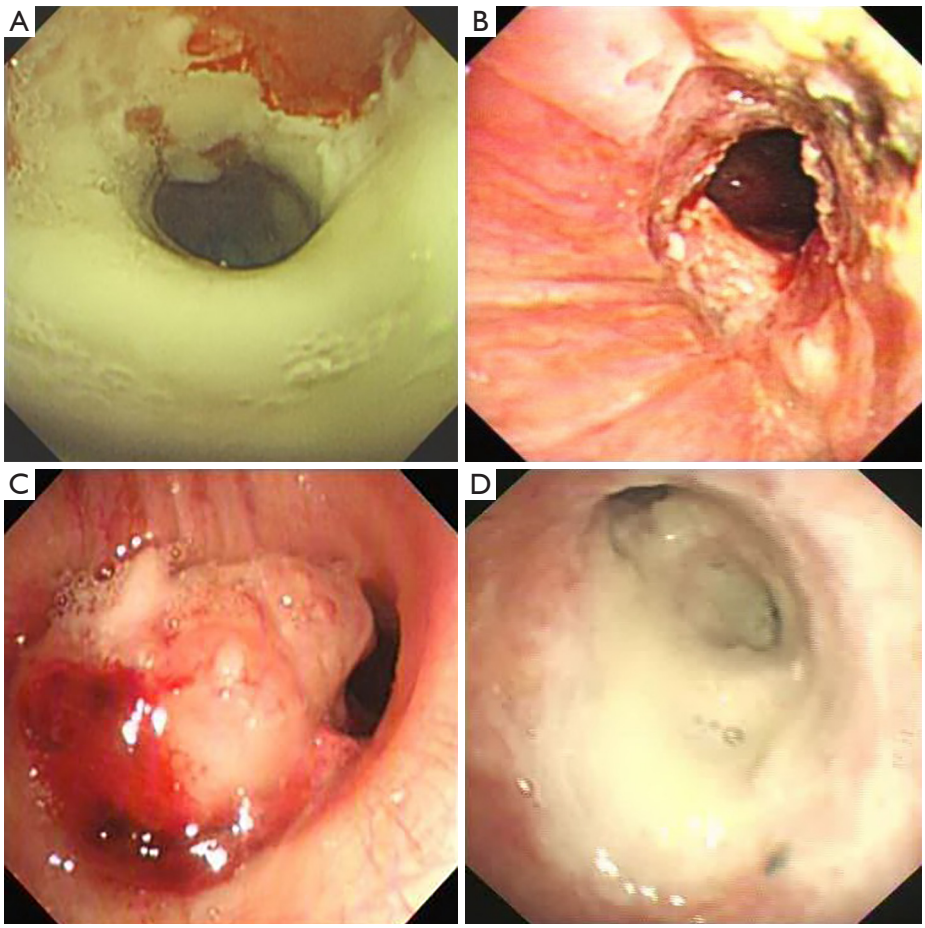

Figure 3 Morphological features of intraluminal lesions of IATB. (A) Full-layer involvement type; (B) ulcerative type; (C) occlusion type; (D) mixed type. IATB, invasive aspergillus tracheobronchitis.

tracheobronchial mucosa; (III) occlusion type: bronchus obstruction >50% of the original caliber which caused by extensive pseudomembrane formation or necrotic tissues and (IV) mixed type: two or more different forms of typical bronchoscopic features coexisting in one patient which sometimes might lead to tracheoesophageal fistula (72-74). As the most direct and effective method in IATB diagnosis, bronchoscopy plays a crucial part in differential diagnosis of immunocompromised patients in ICU. Early detection of airway lesions might improve the prognosis of those with IATB (75).

Chest imaging

Over the past few decades, chest radiographs and thoracic computed tomography (CT) scan have become the most important diagnostic tools for lung diseases since they are non-invasive procedures (76). However, chest radiography is nonspecific in ICU patients with IPA, especially in nonneutropenic patients (*Figure 4*). The classic air crescent sign and halo sign are less often present and their sensitivity is relatively low (less than 25%) than in neutropenic patients in ICU (77). Moreover, even if the halo sign is seen in the chest CT, the specific of IPA is not satisfactory. For example, the halo sign may be observed in pneumonia due to highly virulent bacteria like *pseudomonas aeruginosa*, community-acquired methicillin-resistant *staphylococcus aureus* or *klebsiella pneumoniae*. Sometimes it can be seen in other pathological states due to neoplasm metastasis, Wegener's granulomatosis or dermatomyositis (78-80). What is more, dual infection is not uncommon in ICU patients which may complicate the clinical diagnosis of IPA based on chest CT alone (*Figure 5*).

Besides the classic halo sign and air crescent sign,

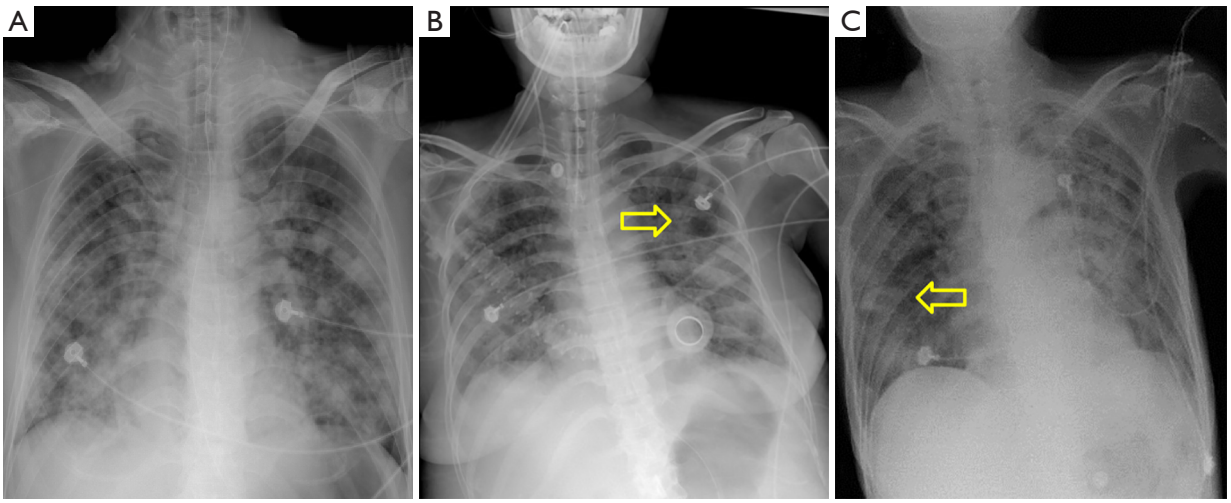

Figure 4 Chest radiographs for patients with IPA which were verified by histopathology. (A) 53-yo female with dermatomyositis and interstitial pneumonia who was receiving steroids. Bilateral lung diffuse lesions were seen with nodules and patchy shadows. (B) Chest radiograph for a 55-yo male with chronic obstructive pulmonary disease (COPD) who was receiving steroids. Bilateral pulmonary patchy clouding opacity with thin wall cavernous lesion (yellow arrow). (C) Bilateral pulmonary patchy clouding opacity with thick wall cavernous lesion (yellow arrow) could be seen in a 35-yo female after liver transplantation (6 months) who received steroids and immunosuppressive agents.

other findings from chest CT have been reported with various frequencies, especially in nonneutropenic patients in ICU: cavitary lesions, pleural effusion, pulmonary macro-nodules (>1 cm) and micro-nodules (<1 cm), areas of alveolar consolidation with or without infarction, etc. (81-83). According to Caillot *et al.*, the multiple lesions of lung seems to be one significant prognosis factor of 90-day mortality in patients with IPA (RR =4.9, P=0.001) (84).

[18F]FDG-PET has been applied to improve the radiological detection of IPA. However, up taking of the tracer during IPA is indistinguishable from those seen during bacterial infections, inflammatory reactions, or even during cancer. The specificity of PET for diagnosing IPA has been dramatically improved these years through the application of *Aspergillus* siderophores, and monoclonal antibodies conjugated to radionuclides. What we should note is that all these studies to date have been conducted only in animal models of IPA (85).

Biomarkers detection

Pentraxin 3 (PTX3)

PTX3 is a soluble pattern recognition receptor (PRR) which is produced by nonimmune cells and phagocytes at the sites of injury or inflammation (86). Therefore, it has been recognized as a biomarker of sepsis in the last decade (87,88). Some recent studies showed that PTX3 had a non-redundant role in modulating various effect or pathways involved in immune resistance to *Aspergillus fumigatus*, including activating innate immune cells and driving protective adaptive immunity (89).

As Kabbani reported (90), the concentrations of PTX3 in BALF was higher in IPA patients compared with those in the state of *Aspergillus* colonization [439.20 (IQR, 168.18–778.90) *vs.* 68.93 (IQR 13.67–156.74) pg/mL, P<0.001]. What is more, it might help to identify false-positive galactomannan (GM) values in BALF samples. Another study by Li revealed that BALF PTX3 had a satisfactory diagnostic efficacy (91). When the cutoff value of BALF PTX3 was set at 1.9 ng/mL, the sensitivity was 86.3% while the specificity was 82.5%, respectively.

PTX3 plays an important role in antifungal immunity and it was once thought that genetic PTX3 deficiency might be a reliable method to predict the incidence of IPA. However, the real effect is still controversial. The genetic deficiency in homozygous haplotype (h2/h2) of PTX3 was first reported by Cristina Cunha in 2014 (92). In 268 patients with HSCT, it revealed that PTX3 gene polymorphisms associated with an increased risk of IPA in the discovery study (adjusted HR, 3.08; P=0.003) and

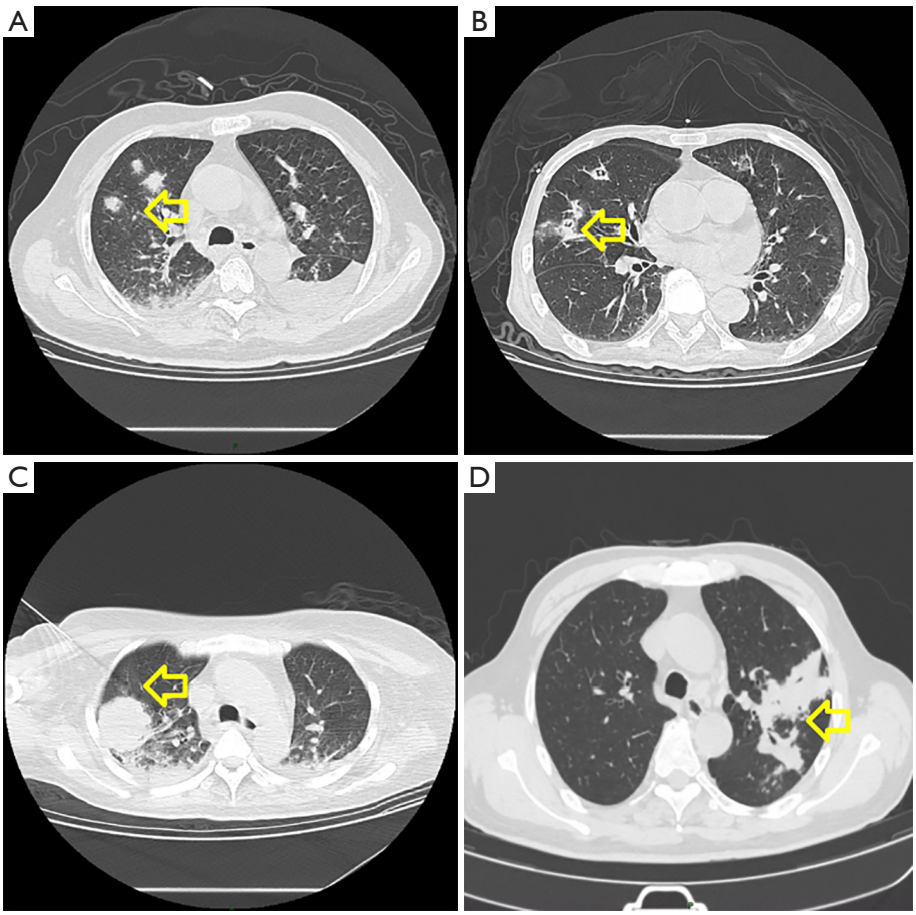

Figure 5 Thoracic computed tomography (CT) for patients with IPA which were verified by histopathology. (A) Halo sign which consists of an area of ground-glass opacity surrounding pulmonary lesions in a patient with systemic lupus erythematosus (yellow arrow); (B) air crescent sign is surrounding a small nodular opacity in a patient with liver transplantation; (C) pulmonary macronodular in the right upper lobe in a patient with dermatomyositis. Fine-needle aspiration was done to make a definitive diagnosis (yellow arrow); (D) tuberculosis and IPA co-infection in a patient with kidney transplantation. Pulmonary lesions with thin wall cavity are seen in the left upper lobe (yellow arrow).

the confirmation study (adjusted HR, 2.78; P=0.03). It was presumed that the instability of messenger RNA might lead to impaired phagocytosis and clearance of the aspergillus. However, the results could not be confirmed by de Boer (93). Forty-four patients with HSCT and 68 controls were enrolled in their case-control study. No association of donor PTX3 variants (+281A→G SNP, GG genotype; +734A→C SNP, AA genotype; Haplotype h2/h2) with the risk of IPA was found during their study. What is more, many other studies about different hosts have come to different conclusions these years (94-97) (*Table 2*). This creates challenges in generalizability. It is recognized that many single-nucleotide polymorphisms (SNPs) in innate immune genes have been linked to an increased risk of IPA, but the disease probably may not develop unless other immune deficiencies are present. Whether the genetic deficiency of PTX3 contributes to the incidence of IPA needs further study, and a systems-biology approach may help in understanding the clinical consequences of genetic polymorphisms affecting innate immunity.

Triacetylfusarinine C (TAFC)

In order to overcome iron restriction by the host and obtain iron for growth, *Aspergillus* species always secrete low-molecular mass iron chelators, which is called siderophores. The main category of siderophore of

Table 2 Pentraxin 3 gene polymorphisms and invasive pulmonary aspergillosis

Author	Host	Number of patients	Donor/patients PTX3 Variant	HR/OR (95% CI)	P value
2014, Cristina Cunha *et al.* (92)	HSCT	268	+281A/G SNP, GG genotype	2.92 (1.69–5.05)	<0.001
			+734A/C SNP, AA genotype	2.62 (1.52–4.54)	<0.001
			Haplotype h2/h2[b]	3.08 (1.47–6.44)	0.003
2014, Mark G.J. de Boer *et al.* (93)	HSCT	112	+281A/G SNP, GG genotype	0.61 (0.20–1.92)	0.4
			+734A/C SNP, AA genotype	0.70 (0.25–1.94)	0.49
			Haplotype h2/h2[b]	0.65 (0.21–2.06)	0.47
2015, Cristina Cunha *et al.* (94)	Lung transplant	102	Haplotype h2/h2[b]	6.69 (1.57–28.5)	0.01
2015, A. Wójtowicz1 *et al.* (95)	SOT	1,101[a]	+281A/G SNP, GG genotype	2.29 (1.04–5.03)	0.04
			+734A/C SNP, AA genotype	3.18 (1.45–6.98)	0.004
			Haplotype h2/h2[b]	2.43 (1.11–5.34)	0.03
2017, Cynthia E. Fisher *et al.* (96)	HSCT	2,565	+281A/G SNP, GG genotype	1.33 (1.09–1.64)	0.005
	HSCT	2,609	+281A/G SNP, GG genotype	1.54 (1.02–2.34)	0.039
			+734A/C SNP, AA genotype	1.82 (1.11–2.94)	0.017
2018, Qian He *et al.* (97)	COPD	162	+1449A/G, AA genotype	5.94 (2.31–15.31)	0.0004
			+281A/G SNP, GG genotype	1.44 (0.44–4.73)	0.52
			+734A/C SNP, AA genotype	1.86 (0.19–18.65)	0.49

[a], IMI: invasive mold infection,81% caused by Aspergillus species; [b], the Haplotype h2/h2 refers to the G-A/G-A homozygous haplotype of +281G (rs2305619) and +734A (rs3816527). COPD, chronic obstructive pulmonary disease; HSCT, hematopoietic stem cell transplantation; SOT, solid organ transplantation.

A. fumigatus is triacetylfusarinine C (TAFC) which is produced only by actively growing cells and cannot be detected in conidia (98). It is reported that TAFC can be tested in serum and BALF samples in patients with IPA using ultra performance liquid chromatography tandem mass spectrometry (LC MS/MS) (99,100). Meanwhile, TAFC is also a small molecule (905,323 g/mol) with a relatively short half-life in blood which leads to a rapid clearance (99). Thus, it can be detected in kidneys, bladder as well as urine samples. It has been demonstrated in Martin' study that TAFC/creatinine index determination in urine had a high sensitivity (86%) and specificity (88%) in diagnosing IPA which may be a promising biomarker because of the non-invasive sampling (101).

Metabolites detection

A great large number of secondary metabolites can be produced by *Aspergillus fumigatus*, and almost all of them are volatile (102). Thus, they might be detected as biomarkers like volatile organic compounds (VOCs) in exhaled breath. It seems to be a promising diagnostic method due to the non-invasive techniques and the demand rates of BAL or lung biopsy could be decreased.

On the other hand, it has been demonstrated that a great variety of characteristic VOCs could be produce by *A. fumigatus in vitro*. The metabolites may vary a lot depending on the antifungal treatment or respiratory microbiota. Therefore, a set of metabolites rather than a very specific one is much preferred as the "breath-signature" of IPA. Four metabolites (trans-geranylacetone, α-trans-bergamotene, β-trans-bergamotene and β-vatirenene) have been proven to diagnose IPA with a higher sensitivity (94%) and specificity (93%) (103). Another study by Chambers revealed that 2-pentylfuran might be a relatively specific marker of IPA (104). Anyhow, further investigation needs to be carried out and the specific set of metabolites should be reintegration.

Table 3 Non-culture-based-tests of IPA diagnosis

Targets of detection	Methods
Antigen	
BDG	Chromatometry/turbidimetry
GM	EIA
Glycoprotein	LFD
Antibody	
Aspergillus IgM antibody	EIA
Aspergillus IgG antibody	EIA
DNA/RNA	Real-time PCR
	PCR-ESI-MS
	NGS
	PNA-FISH
Proteins	MOLDI-TOF MS
Metabolites	
VOCs	GC-MS
Biomarkers	
PTX3	EIA
TAFC	MS

BDG, β(1,3)-D-glucan; GM, galactomannan; VOCs, volatile organic compounds; PTX3, Pentraxin 3; TAFC, triacetylfusarinine; LFD, lateral flow devices; NGS, next-generation sequencing; EIA, enzyme immunoassay; PCR-ESI-MS, PCR-electrospray ionization-mass spectrometry; PNA-FISH, peptide nucleic acid-fluorescence in situ hybridization; MOLDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; GC-MS, gas chromatography-mass spectrometry; MS, mass spectrometry.

Proteins detection

Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a rapid and accurate technology for the identification of bacteria as well as yeast and mold species in recent years. Filamentous fungi can be identified using MALDI-TOF MS, although *Aspergillus* species demonstrate variable phenotypes as a result of which protein spectra may vary (105). However, MALDI-TOF MS application for the identification of *Aspergillus* species is limited till now due to the insufficient fungal databases and a good quality of mass spectra is hard to obtain. Fungal cell wall is thicker and more robust than that of bacteria which also makes it more difficult to use this technology (106,107). Anyhow, the novel method will be a great advance in reducing the time of IPA diagnosis in ICU.

What the future holds

In the last century, identification of *Aspergillus* species was routinely based on the morphological characteristic from direct microscopic examination. Although it requires expertise and with low sensitivity, it can detect the *Aspergillus* hyphae in less than 15 minutes. Thus, it should not be forgotten as a traditional technique with high feasibility.

A great number of novel non-culture-based-tests of IPA diagnosis have been well developed in the last few years (*Table 3*). Detection technology has entered the era of molecules and genes. However, there is no perfect diagnostic method for IPA till now and each technique has its own limitation which must consequently be confronted in the future. Combination of detection methods is recommended to elevate the sensitivity and specificity so as to improve the prognosis of IPA patients in ICU.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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