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BRIEF REPORT

Comparison of a novel *Aspergillus* lateral-flow device and the Platelia® galactomannan assay for the diagnosis of invasive aspergillosis following haematopoietic stem cell transplantation

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Abstract
Purpose The detection of galactomannan in serum is a cornerstone for the diagnosis of invasive fungal disease (IFD). Because a delay in treatment initiation is associated with a poor outcome, the results have to be available promptly. However, due to methodological and economic reasons, the test frequencies of the commonly used galactomannan assays vary between daily to weekly, meaning that results may be available too late to be clinically useful. The novel *Aspergillus* lateral-flow device (*Aspergillus*-LFD) is a rapid test that may overcome these limitations.
Methods We compared the diagnostic performance of the *Aspergillus*-LFD and the Platelia® *Aspergillus* EIA (GM-EIA) in serum from 101 patients during and after allogeneic haematopoietic stem cell transplantation (HSCT). Clinical data and sera were collected prospectively and patients classified according to the European Organisation for Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) 2008 guidelines.
Results By the end of hospitalisation, one proven, nine probable and 20 possible cases of IFD were identified. Depending on the number of positive serum samples required for test positivity, the sensitivities, specificities and diagnostic odds ratios in patients with proven and probable IFD were as follows. One positive serum required: *Aspergillus*-LFD 40.0 %, 86.8 % and 3.03; GM-EIA 40.0 %, 89.0 % and 3.64. Two positive sera required: *Aspergillus*-LFD 20.0 %, 97.8 % and 11.13; GM-EIA 30.0 %, 98.9 % and 38.57. Although the GM-EIA was positive in a higher percentage of samples, this did not result in an earlier diagnosis.
Conclusions If used as a screening test (one positive serum required for test positivity) or to rule out IFD, the *Aspergillus*-LFD has shown a comparable diagnostic performance to the GM-EIA. However, if the results have to be confirmed by a second positive serum, the GM-EIA exhibited superior sensitivity. In terms of practicability, the *Aspergillus*-LFD has demonstrated to be a quick (15 min) and easy-to-use test for single-patient detection of *Aspergillus* antigens.

Keywords Aspergillosis · Haematopoietic stem cell transplantation · Galactomannan · Biomarker · Lateral-flow device · Invasive fungal diseases

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Introduction

Invasive fungal disease (IFD) is associated with significant morbidity and mortality [1]. Because a delay in treatment initiation is associated with a poor outcome, accurate and

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quick diagnostic tools are needed [2]. One cornerstone for the diagnosis of IFD is the detection of fungal antigens in serum. A widely used test for this purpose is the Platelia® *Aspergillus* EIA (GM-EIA), an enzyme immunoassay for detecting galactomannan, a component of the *Aspergillus* cell wall. The GM-EIA has been well studied and its performance for the diagnosis of invasive aspergillosis (IA) from serum samples was analysed in two meta-analyses [3, 4]. Using an optical density index (ODI) of 0.5 as the cut-off value, the investigators reported overall sensitivities of 79 and 78 % and specificities of 86 and 81 %, respectively. While the GM-EIA has an acceptable diagnostic performance, it exhibits some methodological drawbacks. Features like the assay turn-around time of ~3 h, the need for appropriately equipped facilities and last, but not least, economic reasons are responsible for test frequencies that are varying between clinical centres from daily to weekly, meaning that the results may be available too late to be clinically useful.

A diagnostic test that may overcome these limitations is the novel *Aspergillus* lateral-flow device (*Aspergillus*-LFD), developed at the University of Exeter, UK. The *Aspergillus*-LFD is an immuno-chromatographic assay that detects a glycoprotein antigen in serum or bronchoalveolar lavage (BAL) fluids secreted during the growth of *Aspergillus*. It is designed as a fast (15 min) point-of-care test that can be performed on a single-patient basis without complicated laboratory equipment [5]. Recent studies in animal models of infection and with human BAL fluids in haematological malignancy and solid organ transplant patients have shown the utility of the *Aspergillus*-LFD in detecting IA [6–8]. However, studies with relevant numbers of human sera have not been undertaken, notably in patients following allogeneic haematopoietic stem cell transplantation (alloHSCT). Our objective here was to compare the diagnostic performance of the *Aspergillus*-LFD and the GM-EIA in this patient population.

Methods

All adult patients that underwent alloHSCT at the University Medical Centre Freiburg, Germany in 2010 were enrolled. During that year, patient and clinical data were collected prospectively and sera were examined weekly for galactomannan and, in the most part, for (1 → 3)-β-D-Glucan. In the present study, we used these existing data to classify the patients according to the European Organisation for Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) 2008 guidelines for IFD [9]. To prevent incorporation bias, galactomannan was not used as mycological criterion for classification. Inexplicit radiological results were reassessed and missing (1 → 3)-β-D-Glucan data were completed as far as it was necessary for categorisation. (1 → 3)-β-D-Glucan (Fungitell® Assay, Associates of Cape Cod, Inc., USA) and galactomannan (Platelia® *Aspergillus* EIA, Bio-Rad Laboratories, USA) measurement was performed according to the manufacturer's instructions. The cut-offs used were 80 pg/mL for (1 → 3)-β-D-Glucan and an ODI of 0.5 for galactomannan. The persons who carried out the tests were blinded to the results of the EORTC/MSG classification.

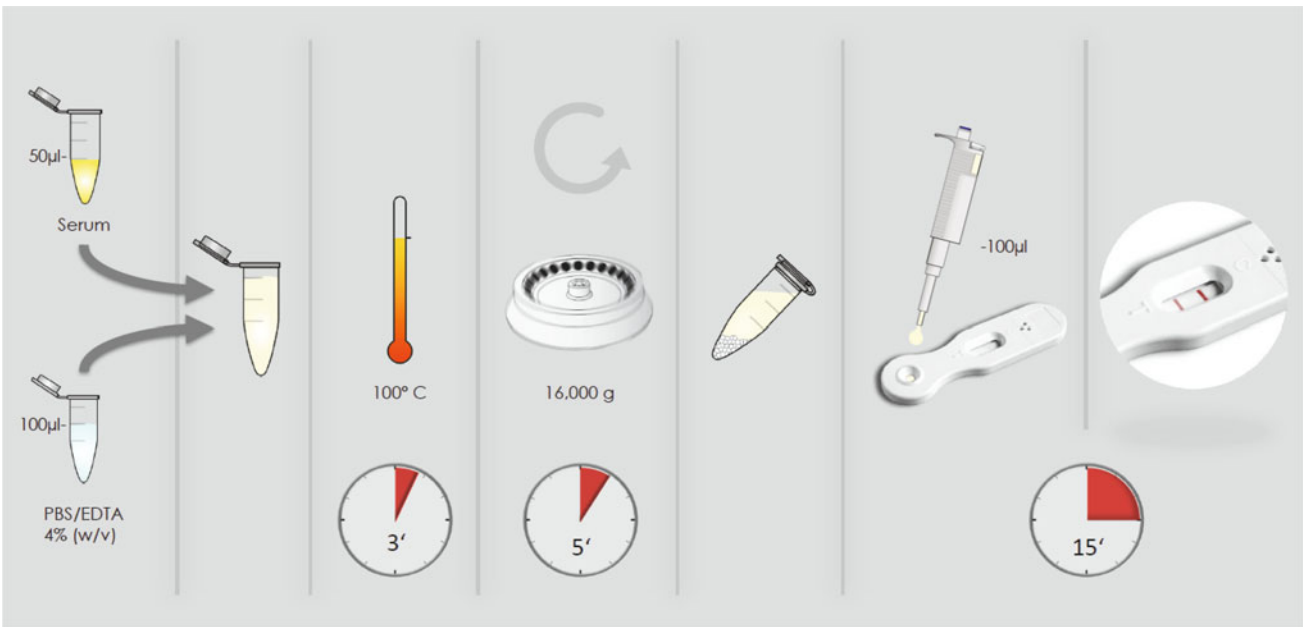

Fig. 1 *Aspergillus* lateral-flow device (*Aspergillus*-LFD) procedure

Fig. 2 Example of a weak positive *Aspergillus*-LFD test. The *upper band* (*C*) is the control band, indicating a valid test, while the *lower band* (*T*) is the sample band, indicating test positivity

The *Aspergillus*-LFD was performed from archived sera as follows (see also Fig. 1): 50 µL of serum were mixed with 100 µL of PBS/EDTA (4 % w/v). The solution was incubated for 3 min at 100 °C and then centrifuged for 5 min at 16,000*g*. 100 µL of the clear supernatant was applied to the LFD and the results were read after 15 min by three laboratory assistants in a blinded fashion. Serum from a healthy individual served as a control, against which test line results were interpreted as negative, weak positive, moderate positive or strong positive (Fig. 2). The devices used were from the same batch of prototypes, prior to the development of a commercially available test format.

For calculation of the diagnostic performance, MedCalc v12 was used. Comparison of positivity rates was done by the Fisher's exact test. The study was approved by the local ethics committee, application number 327/12.

Results

A total of 596 sera from 101 patients after alloHSCT were tested. By the end of hospitalisation, one proven, nine probable and 20 possible cases of IFD were identified. For the differentiation of patients with proven + probable IFD from patients with possible + no IFD, the sensitivities, specificities, positive predictive values, negative predictive values and diagnostic odds ratios were as follows: *Aspergillus*-LFD: 40.0 % [95 % confidence interval (CI): 13.7–72.6], 86.8 % (95 % CI: 77.7–92.7), 25.0 % (95 % CI: 8.3–52.6), 92.9 % (95 % CI: 84.7–97.1) and 4.39 (95 % CI: 1.08–17.86); GM-EIA: 40.0 % (95 % CI: 13.7–72.6), 89.0 % (95 % CI: 80.3–94.3), 28.6 % (95 % CI: 9.9–60.0), 93.1 % (95 % CI: 85.0–97.2) and 5.40 (95 % CI: 1.30–22.47). For the calculation of the predictive values, the prevalence of proven + probable IFD cases in our study population was used (9.9 %). All the diagnostic values are shown in Table 1.

Table 1 Diagnostic performance of the index tests for a diagnosis of proven + probable IFD

Criteria for positivity	Index test	TP	FP	FN	TN	Sensitivity	Specificity	PPV	NPV	LR+	LR−	DOR
One positive serum required	*Aspergillus*-LFD	4	12	6	79	40.0 % (13.7–72.6)	86.8 % (77.7–92.7)	25.0 % (8.3–52.6)	92.9 % (84.7–97.1)	3.03 (1.20–7.64)	0.69 (0.42–1.15)	4.39 (1.08–17.86)
	GM-EIA	4	10	6	81	40.0 % (13.7–72.6)	89.0 % (80.3–94.3)	28.6 % (9.9–60.0)	93.1 % (85.0–97.2)	3.64 (1.40–9.49)	0.67 (0.41–1.12).	5.40 (1.30–22.47)
Two consecutive positive sera required	*Aspergillus*-LFD	2	2	8	89	20.0 % (3.5–55.8)	97.8 % (91.5–99.6)	50.0 % (9.2–90.8)	91.8 % (83.9–96.1)	9.10 (1.43–57.76)	0.82 (0.60–1.12)	11.13 (1.38–89.88)
	GM-EIA	3	1	7	90	30.0 % (8.1–64.6)	98.9 % (93.2–99.9)	75.0 % (21.9–98.7)	92.8 % (85.2–96.8)	27.30 (3.13–238.38)	0.71 (0.47–1.06)	38.57 (3.53–421.08)

IFD invasive fungal disease, *TP* true-positives, *FP* false-positives, *FN* false-negatives, *TN* true-negatives, *PPV* positive predictive value, *NPV* negative predictive value, *LR+* likelihood ratio positive, *LR−* likelihood ratio negative, *DOR* diagnostic odds ratio, *LFD* lateral-flow device, *GM-EIA* Platelia® *Aspergillus* EIA

Table 2 Characteristics and test results of patients with proven and probable IFD

No.	EORTC classification	Age/sex	Underlying disease	Site of IA	HRCT results	Microbiological criteria		Number of positive sera/total number		Highest result of test		First positive test (time lag in days)
						Culture	BDG_{MAX} (pg/mL)	LFD	GM	LFD (band intensity)	GM-ODI	
1	Proven	59/M	MM	Lung	Air crescent sign	*A. fumigatus* from pleural fluid	113	0/8	3/8	−	0.75	GM only
2	Probable	49/M	SAA	Lung	Nodular lesion + halo sign	Negative	>500	5/8	7/7	++	6.63	LFD and GM (0)
3	Probable	70/M	AML	Lung	Nodular lesion + halo sign	Negative	470	2/7	7/7	+	1.85	LFD and GM (0)
4	Probable	47/F	AML	Lung	Nodular lesion	Negative	>500	0/4	0/4	−	–	–
5	Probable	69/F	AML	Lung	Nodular lesion	Negative	>500	1/3	0/3	+	–	LFD only
6	Probable	63/F	AML	Lung	Cavity	Negative	>500	0/7	0/7	−	–	–
7	Probable	21/M	ALL	Lung	Nodular lesion	Negative	145	0/6	0/6	−	–	–
8	Probable	62/M	CLL	Lung	Nodular lesion	*A. fumigatus* from respiratory sample	113	0/11	0/11	−	–	–
9	Probable	21/M	AML	Lung	Nodular lesion and cavities	Negative	84	1/7	1/7	+	0.64	LFD and GM (0)
10	Probable	46/F	AML	Lung	Nodular lesion	Negative	326	0/12	0/12	−	–	–

LFD test interpretation: negative (−), weakly positive (+), moderately positive (++), strongly positive (+++)

IFD invasive fungal disease, *EORTC* European Organisation for Research and Treatment of Cancer, *HRCT* high-resolution computed tomography, *BDG* (1 → 3)-β-D-Glucan, *LFD Aspergillus* lateral-flow device, *GM* galactomannan, *ODI* optical density index, *MM* multiple myeloma, *SAA* severe aplastic anaemia, *AML* acute myelogenous leukaemia, *ALL* acute lymphatic leukaemia, *CLL* chronic lymphatic leukaemia

The one proven case of IA was detected only by the GM-EIA with an ODI of 0.75. The corresponding patient with multiple myeloma suffered from pulmonary aspergillosis. Chest computed tomography (CT) scan showed an air crescent-sign and *Aspergillus fumigatus* was cultured from a pleural aspirate. The characteristics of all patients with proven + probable IFD together with the test results are shown in Table 2.

Of 86 sera from patients with proven + probable IFD, the *Aspergillus*-LFD was positive with 9 (10.5 %) and the GM-EIA with 18 (20.9 %) of the samples. However, this difference was not statistically significant ($p = 0.092$). A direct comparison of the antigen-positive samples showed that sera with a GM-EIA ODI below 1.0 were only rarely positive in the *Aspergillus*-LFD. In the three patients with positive GM-EIA and *Aspergillus*-LFD results, both tests became positive at the same time, meaning that neither of the tests would have led to an earlier diagnosis.

Both the *Aspergillus*-LFD and the GM-EIA failed to detect six patients with probable IFD, all of which received either antifungal prophylaxis or antifungal therapy.

Both tests produced false-positive results in nine patients out of 71 without evidence of IFD. In three of these patients, both the *Aspergillus*-LFD and the GM-EIA were positive with the same serum sample, while another two of the patients tested positive with different serum samples. Three of these five patients were positive for all three fungal biomarkers [*Aspergillus*-LFD, GM-EIA, (1 → 3)-β-D-Glucan] with at least one of the serum samples. Of the remaining four patients, either the *Aspergillus*-LFD or the GM-EIA was positive. With all but one of the false-positive patients, no second serum sample was positive. If two consecutive positive sera per patient were used for the diagnosis of IFD, the *Aspergillus*-LFD detected two and the GM-EIA detected three patients. In this case, the sensitivities, specificities, positive predictive values, negative predictive values and diagnostic odds ratios were as follows: *Aspergillus*-LFD: 20.0 % (95 % CI: 3.5–55.8), 97.8 % (95 % CI: 91.5–99.6), 50.0 % (95 % CI: 9.2–90.8), 91.8 % (95 % CI: 83.9–96.1) and 11.13 (95 % CI: 1.38–89.88); GM-EIA: 30.0 % (95 % CI: 8.1–64.6), 98.9 % (95 % CI: 93.2–99.9), 75.0 % (95 % CI: 21.9–98.7), 92.8 % (95 % CI: 85.2–96.8) and 38.57 (95 % CI: 3.53–421.08).

Discussion

The *Aspergillus*-LFD is a novel rapid test for the detection of *Aspergillus* antigens in serum and BAL fluids. While there is a lack of information on the diagnostic performance in human serum samples [5], Hoenigl et al. [8] evaluated the *Aspergillus*-LFD using BAL fluids from 37 patients with haematological malignancies or after solid organ transplantation. They found an excellent sensitivity of 100 % and a specificity of 81 % for the diagnosis of probable IA.

Our study using serum samples from alloHSCT patients showed that the *Aspergillus*-LFD and the GM-EIA had comparable diagnostic performance on a per-patient basis if a single positive serum was defined as a positive test. However, the 40 % sensitivity of both assays was clearly below the average galactomannan sensitivity of 79 and 78 % as reported in the meta-analyses by Pfeiffer et al. [3] and Leeflang et al. [4]. One reason for this could be that we used (1 → 3)-β-D-Glucan as mycological criterion for EORTC/MSG classification. (1 → 3)-β-D-Glucan is part of the fungal cell wall of most medically relevant fungi except for the *Mucorales* and *Cryptococcus* species. It is, therefore, elevated in invasive infections like aspergillosis, candidosis, fusariosis, scedosporiosis or pneumocystosis. In contrast, the *Aspergillus*-LFD and the GM-EIA are essentially specific for *Aspergillus* species. The discrepancy in sensitivity might, therefore, be due to non-*Aspergillus* infections detected by (1 → 3)-β-D-Glucan but not galactomannan. In fact, only two of the ten patients with proven and probable IFD had *A. fumigatus* cultured from clinical samples. The remaining eight patients were classified as probable IFD on the basis of positive (1 → 3)-β-D-Glucan results (Table 2). If this hypothesis is correct, the sensitivity of the *Aspergillus*-LFD should increase by using the *Aspergillus*-specific GM-EIA instead of (1 → 3)-β-D-Glucan for EORTC/MSG classification. By doing so, one proven, five probable and 24 possible cases of IA were identified. The sensitivity and specificity of the *Aspergillus*-LFD increased to 50.0 % (95 % CI: 13.9–86.1) and 87.4 % (95 % CI: 78.6–93.0), respectively. Obviously, infections by non-*Aspergillus* species are one but not the only reason for the lower sensitivity in the present study, and the moderate increase in sensitivity reflects the low prevalence of non-*Aspergillus* infections in haematologic patients. Another explanation could be that all of our IFD patients received antifungal prophylaxis or therapy, and it is well known that galactomannan sensitivity in such patients is markedly reduced [10]. In a guinea pig model of IA, it has recently been shown that the administration of antifungals results in reduced sensitivity of the *Aspergillus*-LFD using serum samples but not BAL fluids, providing a possible explanation for the discrepancy between the Hoenigl BAL study and our data [11].

As mentioned above, the diagnostic performance of the index tests was comparable if a single positive serum was sufficient for classification as probable IFD. However, this was not true if two consecutive positive serum samples were required for test positivity. In this case, the GM-EIA would detect three patients as compared to two patients

detected by the *Aspergillus*-LFD. Sensitivities (30 vs. 20 %), positive predictive values (75 vs. 50 %) and diagnostic odds ratios (38.57 vs. 11.13) would then suggest the GM-EIA to be substantially more sensitive than the *Aspergillus*-LFD. It is, however, important to note that the higher sensitivity of the GM-EIA did not result in an earlier diagnosis because the first positive serum sample of patients with IA was nearly always highly positive. While the assay sensitivities in our patient cohort were unsatisfactory, both tests showed good specificities, comparable to the results of former studies [3, 4]. This is also reflected by the negative predictive value of 93 % for both tests, highlighting the fact that, with a negative test result, the existence of IA is unlikely.

Another noteworthy point is that, in about 50 % of patients with false-positive serum samples, more than one fungal biomarker was elevated. This could be due to the administration of substances known to interfere with fungal antigen measurement (e.g. piperacillin/tazobactam) or to a laboratory contamination of the serum with *Aspergillus* spores. However, in the case of the LFD, cross-reactivity with piperacillin/tazobactam has not been reported and quiescent spores are not detected by it [5].

Tests for fungal antigens are an important element in the diagnosis of IA. The analytical principle behind most of these assays is an enzyme immunoassay. Characterised by a high sensitivity, a common drawback of these test formats is that they are labour-intensive, time-consuming and require laboratories equipped and accustomed to these tests. Because of the necessity to include a standard curve and a variable number of controls in every test run, the per-sample costs are reduced the greater the number of samples analysed simultaneously. As a consequence, test frequencies range from daily in large centres with many high-risk patients to weekly, or not at all, in smaller hospitals. However, IA is not only restricted to high-risk populations and may, for example, affect patients in intensive care units, after surgery, in infancy or in alcoholism [10]. Therefore, fungal antigen tests should be easily accessible. For this purpose, a simple, single-patient test would be ideal. The novel *Aspergillus*-LFD fulfills these requirements, being fast (15 min) and easy to perform, without the need for extensive laboratory facilities. Because of the necessity to heat and centrifuge the sera, the *Aspergillus*-LFD is not a bedside test but is well suited to rudimentary laboratories. Using sera from alloHSCT patients, we experienced a faint, unspecific band that appeared in a considerable number of sera, and required the use of serum from a healthy individual for the setting of the threshold intensity and establishment of test line positivity. However, it is important to note that the LFD that we used is a prototype test and the background interference is currently being eliminated during the development of a CE-marked commercial format.

Taken together, the advantages of the *Aspergillus*-LFD in terms of practicability and the characteristics of its diagnostic performance (high specificity, comparable sensitivity only if a single positive serum is required for test positivity), an appropriate way of utilisation would be as a screening test in resource-limited settings. Because of the high negative predictive value, a single negative test would make an IFD unlikely and the *Aspergillus*-LFD could be used exclusively without disadvantage compared to the GM-EIA. A positive test, however, should be confirmed by a second, different assay. This second assay may be the GM-EIA or, rather, an *Aspergillus*-specific polymerase chain reaction (PCR), because a recent study provides evidence that the combination of antigen assays with PCR may be superior to the combination of two antigen assays [12]. Diagnostic laboratories that do not perform *Aspergillus*-specific PCR or GM-EIA would then send the samples for confirmation to a laboratory accustomed to these tests. In the meantime, antifungal therapy could be started, depending on the radiology results and the clinical presentation of the patient.

In summary, the novel *Aspergillus*-LFD is a fast and user-friendly diagnostic device with a comparable performance to the GM-EIA if used as a screening test or to rule out IFD. It may be a valuable diagnostic alternative, especially in situations where the GM-EIA is not performed on a daily basis. However, the number of IA cases in our study is limited and further work with a larger number of patients is necessary in order to fully evaluate a refined, CE-marked *Aspergillus*-LFD and to corroborate our results in the setting of alloHSCT.

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Conflict of interest Christopher R. Thornton developed the *Aspergillus*-LFD and is involved in its commercialisation. All other authors declare no conflict of interest.

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