Combining standard clinical methods with PCR showed improved diagnosis of invasive pulmonary aspergillosis in patients with hematological malignancies and prolonged neutropenia

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Abstract
Background: We assessed the diagnostic value of standard clinical methods and combined biomarker testing (galactomannan assay and polymerase chain reaction screening) in a prospective case–control study to detect invasive pulmonary aspergillosis in patients with hematological malignancies and prolonged neutropenia.

Methods: In this observational study 162 biomarker analyses were performed on samples from 27 febrile neutropenic episodes. Sera were successively screened for galactomannan antigen and for Aspergillus fumigatus specific nucleic acid targets. Furthermore thoracic computed tomography scanning was performed along with bronchoscopy with lavage when clinically indicated. Patients were retrospectively stratified to define a case-group with “proven” or “probable” invasive pulmonary aspergillosis (25.93 %) and a control-group of patients with no evidence for of invasive pulmonary aspergillosis (74.07 %). In 44.44 % of episodes fever ceased in response to antibiotic treatment (group II). Empirical antifungal therapy was administered for episodes with persistent or relapsing fever (group I). 48.15 % of patients died during the study period. Postmortem histology was pursued in 53.85 % of fatalities.

Results: Concordant negative galactomannan and computed tomography supported by a polymerase chain reaction assay were shown to have the highest discriminatory power to exclude invasive pulmonary aspergillosis. Bronchoalveolar lavage was performed in 6 cases of invasive pulmonary aspergillosis and in 15 controls. Although bronchoalveolar lavage proved negative in 93 % of controls it did not detect IPA in 86 % of the cases. Remarkably post mortem histology convincingly supported the presence of Aspergillus hyphae in lung tissue from a single case which had consecutive positive polymerase chain reaction assay results but was misdiagnosed by both computed tomography and consistently negative galactomannan assay results. For the galactomannan enzyme-immunoassay the diagnostic odds ratio was 15.33 and for the polymerase chain reaction assay it was 28.67. According to Cohen’s kappa our in-house polymerase chain reaction method showed a fair agreement with the galactomannan immunoassay. Combined analysis of the results from the Aspergillus galactomannan enzyme immunoassay together with those generated by our polymerase chain reaction assay led to no misdiagnoses in the control group.

Conclusion: The data from this pilot-study demonstrate that the consideration of standard clinical methods combined with biomarker testing improves the capacity to make early and more accurate diagnostic decisions.

Keywords: Invasive pulmonary aspergillosis, Biomarkers, Combination testing, Acute leukemia, Neutropenic fever

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Background
Patients suffering from hematological malignancies and prolonged neutropenia are significantly affected by life-threatening invasive fungal infections, particularly invasive pulmonary aspergillosis (IPA). Despite improvements in transplantation practices and antifungal therapy the treatment of patients with IPA is still challenging [1]. Diagnosis of IPA is difficult and delayed diagnosis contributes to the increasing severity of IPA associated high morbidity and mortality rates [2, 3]. Standard radiology as thoracic computed tomography (CT) scan and classical mycology of bronchoalveolar lavage (BAL) have low sensitivity and may be no more specific than fevers of unknown origin that are refractory to treatment with broad-spectrum antibiotics; especially in neutropenic patients [4, 5]. Despite many limitations empirical antifungal therapy is regarded as a standard treatment in these patients. The relatively low incidence of IPA is accompanied by the risk of over treating patients thus subsequently loading them with unnecessary drug toxicity [6]. On one hand these fever-driven approaches use a very limited diagnostic work-up in the need of avoiding deleterious delay in the initiation of therapy on the other hand they may miss IPA developing in the absence of fever [7]. An alternative management strategy could be preemptive antifungal therapy guided by IPA associated risk factors such as mycological tools, imaging techniques, detection of specific biomarkers by Aspergillus galactomannan enzyme immunoassay (GM-EIA) or sensitive polymerase chain reaction (PCR) [7, 8]. The routine use of non-culture based molecular assays might have the potential to fill the gap between over- and under treatment providing improved diagnosis compared to classical methods and single assay detection [9]. The primary aim of this non-randomized study was to assess the applicability of our enhanced patient surveillance strategy in routine clinical practice on our retrospectively stratified patient population. A further aim was to estimate the diagnostic utility of combined biomarker testing from the onset of febrile neutropenia by combining serum GM-EIA screening and facC-PCR detection. This could show how many patients would receive a misdiagnosis on the basis of a fever driven empirical approach without regarding results from sensitive and specific biomarker testing.

Methods
Study population
Between June 06, 2012 and May 08, 2013, 27 febrile neutropenia episodes (FNEs); 16 males with the median age of 42.5 (range 14—75) and 11 females with the median age of 45.5 (range; 19—66) (Table 1) were included at the onset of febrile neutropenia. The patients had different hematological malignancies (mainly acute leukemia) receiving stem cell transplantation and intensive chemotherapy (neutrophil count < 0.5 × 10⁹ cells/L; temperature > 38 °C of febrile neutropenia recorded twice or > 38.5 °C recorded once) in our local prospective case–control study. The study protocol was approved by the ethics committee of the University Hospitals of Debrecen, Hungary. Written consent was obtained from patients and the caretakers of the children in all of the cases. PCR testing was performed parallel with GM-EIA and needed no ethical approval. PCR results were sent back to clinicians however were not taken into clinical decision making.

Search strategy
For FNE, combined biomarker testing (CBT) was commenced. Serum samples were prospectively screened for the presence of genus and species specific biomarkers galactomannan (GM) and facC genes. The results were anonymously evaluated [10]. Screening for GM-antigenemia Platelia Aspergillus (GM-EIA) was used on the basis of the OD₄₅₀/₆₂₀ ≥ 0.5 cutoff value.

The real time PCR detection of A. fumigatus specific DNA was based on the Streptomyces factor C orthologous gene (facC-PCR): this was most likely horizontally transferred to a few filamentous fungi including A. fumigatus as previously described [11]. Since the facC gene that was acquired by horizontal gene transfer [12] is only present in a few fungal species [13] and they diverged millions of years ago, we were able to design species-specific assays that do not cross-react even with closely related species that carry this gene therefore the analytical specificity is increased as compared to other assays. At the onset of fever broad-spectrum antibiotic treatment was administered according to the published Infectious Diseases Society of America guidelines [14]. Furthermore standard chest CT and BAL analysis were performed as clinically indicated.

Antifungal therapy
Persistent fever, positive GM assay along with either positive CT and/or positive BAL was considered sufficient evidence to initiate broad spectrum antifungal treatment. In the case of fever that ceased after 48 h of antibiotic treatment, with negative CT and/or BAL results (when performed), we suggested extending the surveillance period. These cases were monitored for the presence of Aspergillus specific biomarkers (GM and the facC-orthologous gene) in parallel with thorough clinical examinations on a weekly basis.

Stratification of febrile neutropenia episodes
Cases were retrospectively stratified according to revised European Organization for the Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria in order to define a case-group of “proven” or “probable” IPA (7 patients) and a control-group possible IPA.
(20 patients with no EORTC/MSG evidence of invasive aspergillosis) [10, 15].

Ascertainment bias
Ascertainment bias can potentially overestimate the diagnostic odds ratio (DOR) for the benefit of the gold-standard GM-EIA method. By introducing false-positives it can erode the discriminatory power of the other diagnostic methods (e.g. facC-PCR).

Postmortem histology
The specificity of *Aspergillus* infection morphology was critically addressed in the histological samples obtained following autopsy. Samples were taken from the major organs according to a standard protocol, but no macroscopic signs of organ involvement were seen other than in the lungs. Lung sampling was performed from three independent parts of the potentially infiltrated lung parenchyma. Histological evaluation clearly proved the existence of invasive fungal infection. The detailed feature of the PAS and/or Hematoxylin and Eosin (H&E) stained hyphae supported the *Aspergillus* specific PCR assay based assumption of invasive aspergillosis in the lung although other filamentous fungi may resemble *Aspergillus* hyphae therefore definite identification is not possible on a morphological basis.

| Patient ID | Underlying condition | Sex, age (yr) | EORTC/MSG classification | No. of specimens per episode | No. of GM specimens | No. of PCR specimens | No. of PCR runs |
|------------|----------------------|---------------|---------------------------|-----------------------------|---------------------|----------------------|----------------|
| 1          | PTCL M, 18           | possible      | 8                         | 0 4 1 3                   | 3 9                |
| 2          | NHL M, 15            | possible      | 14                        | 0 7 2 5                   | 2 19               |
| 3          | OM M, 16             | possible      | 16                        | 0 8 1 7                   | 3 21               |
| 4          | AML M, 26            | proven        | 18                        | 0 7 10 1                  | 18 15              |
| 5          | OM F, 53             | possible      | 6                         | 1 2 0 3                   | 0 9                |
| 6          | AML M, 55            | possible      | 4                         | 0 2 1 1                   | 1 5                |
| 7          | AML M, 52            | proven        | 4                         | 2 0 2 0                   | 3 3                |
| 8          | AML F, 28            | proven        | 6                         | 1 2 3 0                   | 3 4                |
| 9          | MM M, 70             | possible      | 2                         | 0 1 0 1                   | 0 3                |
| 10         | ALL F, 27            | probable      | 8                         | 2 0 6 0                   | 13 1               |
| 11         | OM M, 57             | possible      | 2                         | 0 1 0 1                   | 0 3                |
| 12         | OM F, 66             | possible      | 6                         | 0 3 0 3                   | 0 9                |
| 13         | AML F, 55            | probable      | 8                         | 2 2 4 0                   | 8 4                |
| 14         | AML F, 46            | possible      | 2                         | 0 1 0 1                   | 0 3                |
| 15         | MM F, 44             | possible      | 2                         | 0 1 0 1                   | 0 3                |
| 16         | OM M, 56             | possible      | 6                         | 0 3 1 2                   | 1 8                |
| 17         | HL F, 26             | proven        | 4                         | 0 2 0 2                   | 0 6                |
| 18         | AML F, 50            | possible      | 2                         | 0 1 1 0                   | 3 0                |
| 19         | ALL M, 14            | possible      | 4                         | 0 2 0 2                   | 0 6                |
| 20         | AML F, 45            | possible      | 6                         | 0 3 0 3                   | 0 9                |
| 21         | OM M, 17             | possible      | 6                         | 0 3 0 3                   | 0 9                |
| 22         | OM F, 19             | possible      | 8                         | 0 4 3 1                   | 3 9                |
| 23         | NHL M, 57            | possible      | 2                         | 0 1 0 1                   | 0 3                |
| 24         | AML M, 36            | possible      | 6                         | 1 2 0 3                   | 0 9                |
| 25         | NHL M, 75            | proven        | 6                         | 2 1 1 2                   | 1 8                |
| 26         | AML M, 49            | possible      | 4                         | 0 2 0 2                   | 0 6                |
| 27         | OM M, 16             | possible      | 2                         | 0 1 0 1                   | 0 3                |
| **Total**  | 162                  | 77            | 85                        | 249                       |

*Neutrophil count < 0.5 × 10⁹ cells/L; University of Debrecen, Institute of Internal Medicine; AML Acute Myeloid Leukemia, ALL Acute Lymphoblastic Leukemia, MM Myeloma Multiplex, NHL Non-Hodgkin Lymphoma, PTCI Peripheral T-cell Lymphoma, OMs other malignancies, P present, A absent, NP not performed, CT thoracic computed tomography, BAL bronchoscopy with lavage*
Serum specimens
As part of our combined biomarker testing [9] consecutive serum samples (3 × 3 ml) were routinely drawn from patients at the onset of febrile neutropenia and sent to the Department of Medical Microbiology, Debrecen, Hungary for GM antigen monitoring. The GM assays required 300 µl of serum samples and the rest was stored at −20 °C. The two other intact serum tubes were forwarded to the Department of Human Genetics, Debrecen, Hungary for facC-PCR and opened only in our laboratory.

DNA extraction
All DNA extraction steps were performed in a class II laminar-flow cabinet to avoid environmental contamination. Serum samples obtained from healthy volunteers were pooled and screened for Aspergillus fumigatus nucleic acid contamination prior to use as extraction negative controls (2.5 ml). In order to control the efficiency of the fungal nucleic acid extraction and potential PCR inhibition the QuantiFast Pathogen PCR+IC Kit (Qiagen, Hilden, Germany) was used to prepare extraction positive control samples (2.5 ml) containing 10 µl of internal control DNA. 2.5 ml of serum samples obtained from episodes were extracted along with the extraction positive and negative controls using the High Pure Viral Nucleic Acid Large Volume Kit (Roche Applied Science) according to manufacturer’s instructions. The elution volume was adjusted to 45 µl. 12 µl of eluate was used in each PCR reactions.

FacC-PCR based real-time detection
Species specific hydrolysis assays were used that targeted the facC orthologous genes present in A. fumigatus [11]. A. fumigatus specific duplex TaqMan-LNA hydrolysis assay was used targeting facC orthologous genes (AFUA_3G14910, and AFUA_5G00540) present in two copies (on Chr#3 and on Chr#5 respectively) in A. fumigatus genome. The duplex assay contained AFUA_3G14910, and AFUA_5G00540 specific primers (0.8 µM for each) and probes (0.5 µM for each). The primers and probe sequences were Afu3_Fw (5′-CTT TGGCATCGGACAGT-3′), Afu3_Rev (5′-GAGGGG GCAGAGAGATC-3′), Afu3_probe (5′-FAM-CAGCAT CC-DQD-3′), where DQD is dark quencher dye, Afu5_Fw (5′-GCTCCAACAGTCATTACCTT-3′), Afu5_Rev (5′-ATCGCGACGGTCTACATG-3′), Afu5_probe (5′-FAM- CGGCCGCC-DQD-3′). Amplification was carried out on LightCycler® 2.0 Carousel-Based platform (Roche Applied Science). 5× LightCycler TaqMan Master (Roche Applied Science) was used according to manufacturer’s instructions containing Uracil-DNA Glycosylase for carryover prevention during PCR and the reaction volumes were settled to 20 µl in glass capillaries where 12 µl of eluates were monitored for the presence of A. fumigatus genomic DNA. Each real-time run contained template-positive (PCR-IC spiked with 100–1000 GE Aspergillus fumigatus gDNA) and template-negative (PCR-NTC containing PCR grade nuclease free water) control samples.

Data interpretation
Discriminatory power of standard methods
The discriminatory power of the CT scans combined with either GM-EIA or with BAL were analyzed by comparison with patient status (cases and controls), which allowed the negative predictive values (PV−) to be calculated.

(i) Real time FacC assays. In this study triplicate facC-PCR reactions were performed. Single positive assay result from the triplicate reactions was used to define a sample as PCR positive [16–18].

(ii) Galactomannan ELISA. The Platelia Aspergillus GM-EIA (Bio-Rad Laboratories, Hungary) was used for galactomannan screening. GM-EIA cutoff values were determined using the OD450/620 value of sample/OD450/620 value of control. While evaluating single specimens any value above the OD450/620 ≥ 0.5 cutoff value was considered positive as requested for in vitro testing. While evaluating episode GM-EIA statuses the ratio of positive versus negative results were analyzed. In the case of equality, an episode was considered positive.

Statistical analysis of CBT
Along with reporting of descriptive statistics for categorical data; median, mean, standard deviation (±SD), diagnostic performance parameters (by doing the 2×2 contingency tables of cases and controls) with discriminatory properties of post-test probabilities (likelihood ratios) and accuracy indices (DORs) were calculated with 95 % confidence intervals (95 % CI) for GM screening (GM-EIA) and for the nucleic acid based diagnostic method (facC-PCR) respectively.

Biomarker measurement comparison
In this study 162 biomarker analyses from 27 febrile neutropenic episodes with the mean number of 6 ± 4.18 specimen/episodes (range; 2–18) were performed. For assessing sample concordance between the established gold-standard GM-EIA screening and our in-house facC-PCR method inter annotator agreement was calculated using the Cohen’s kappa test where values greater than 0.8 represent an excellent agreement, values of 0.61–0.8 represent a substantial agreement, values of 0.41–0.6 a moderate agreement, values of 0.21–0.4 a fair agreement and below 0.20 a slight agreement between two classifiers.
ROC analysis
To measure the diagnostic accuracy of the results from GM-EIA and facC-PCR testing were converted to qualitative (positive, negative) indexes. In the case of facC-PCR every single run was evaluated independently. After calculating the series of sensitivity (Se) and specificity (Sp) reports at every single decision threshold the receiver operating characteristic (ROC) curve was edited by plotting the true positive values (sensitivity values on y-axis) versus the false positive rates (1-specificity values on x-axis). Area under the ROC curve (AUC) was estimated with 95 % confidence intervals (95 % CIs) and with standard errors (±SD) where values greater than 0.91 represent excellent discriminatory accuracy, values of 0.81 - 0.9 represent good accuracy, values of 0.61 - 0.7 poor accuracy and values of 0.5 - 0.6 represent a diagnostically not useful test. Fisher’s exact test was used to generate two-sided P values with a P value of < 0.05 being considered significant. Furthermore likelihood ratios (LR+, LR-) and predictive values (PV+, PV−) were estimated.

Results
Classification of febrile episodes
Patient demographics with regard to EORTC/MSG classification are shown in Table 1.

Discriminatory power achieved by combining CT with GM-EIA and BAL
Concordant negative GM-EIA together with thoracic CT scans has been shown to have the highest discriminatory power to exclude IPA as a diagnosis. The calculated PV− proved to be high; 0.94 (17 controls, 1 case) (Fig. 1a). When combining BAL with thoracic CT scans the calculated PV− proved to be; 0.92 (12 controls, 1 case). BAL was performed in 77 % of all episodes. (6 cases and 15 controls). In the control group BAL excluded IPA in 93 % of the episodes (14 of 15). BAL was able to diagnose IPA in one case only (patient ID 10). In this case CT failed to detect IPA (Fig. 1b).

Diagnostic performance of the biomarker assays
Performance parameters such as sensitivity (Se), specificity (Sp), likelihood ratios (LR+, LR−), predictive values (PV+, PV−) and diagnostic odds ratio (DOR) were estimated for GM-EIA serology and facC-PCR by using the current cutoff index for the gold-standard GM-EIA (OD450/620≥0.5) and by considering a single positive PCR run as significant for facC-PCR. Performance parameters with and without stratification correction are shown in Table 2.

Data on CBT
On completion of combined biomarker testing categorical data; median, mean, standard deviations (±SD) were calculated for GM screening (GM-EIA) and for the nucleic acid based diagnostic method (facC-PCR). Data are shown in Fig. 2.

Sample concordance
Sample concordance was assessed between the two classifiers; Platelia Aspergillus GM-EIA and facC-PCR showing a fair agreement generating an observed ratio of 70.13 % (agreement; 54 of 77) and a kappa statistic of 0.258 (95 %

Fig. 1 Representation of the discriminatory power of Aspergillus GM-EIA (a) and BAL (b) when combining these with chest CT. a CT−GM+: episodes proved to be positive when testing both by CT and Aspergillus GM-EIA, CT−GM+: episodes proved to be positive when scanning by CT and negative when testing by Aspergillus GM-EIA, CT−GM+: episodes proved to be negative when scanning by CT and positive when testing by Aspergillus GM-EIA, PCR−GM+: episodes proved to be negative when testing both by CT and Aspergillus GM-EIA. Black symbols represent controls while red symbols represent cases. Contoured symbols represent episodes with ceased fever, while shaded symbols represent episodes with present or recurrent fever refractory to broad spectrum antibiotic treatment. Small sized symbols represent children. Numbers denote patient ID numbers. b CT−BAL+: episodes proved to be positive when testing both by CT scanning and BAL, CT−BAL+: episodes proved to be positive when testing by thoracic CT and negative when testing by BAL, CT−BAL+: episodes proved to be negative when testing by thoracic CT and positive when testing by BAL, CT−BAL+: episodes proved to be negative when testing both by thoracic CT and BAL. Black colored symbols represent controls while red colored symbols represent cases. Contoured symbols represent episodes with ceased fever, while shaded symbols represent episodes with present or recurrent fever refractory to broad spectrum antibiotic treatment. Small sized symbols represent children. Numbers denote patient ID numbers.
Table 2  Diagnostic performance of GM-ELISA and facC-PCR when testing serum samples

| Parameter | Platelia Aspergillus-EIA | facC-PCR |
|-----------|--------------------------|----------|
| % Se (95 % CI) | 52.63 (28.90-75.51) | 69.23 (48.21-85.63) |
| % Sp (95 % CI) | 98.31 (90.88-99.72) | 68.85 (55.71-80.09) |
| LR+ | 31.05 | 2.22 |
| LR- | 0.48 | 0.45 |
| PV+ % | 90.91 | 48.65 |
| PV- % | 86.57 | 84.00 |
| DOR (95 % CI) | 64.44 (7.342-565.694) | 4.974 (1.81-13.434) |
| % Se (95 % CI) | 37.5 (17.97-57.48) | 84 (65.46-93.19) |
| % Sp (95 % CI) | 96 (87.02-99.54) | 83.02 (70.19-91.91) |
| LR+ | 9.5491 | 4.85 |
| LR- | 0.6651 | 0.21 |
| PV+ % | 89.4268 | 75.68 |
| PV- % | 62.9298 | 88.00 |
| DOR (95 % CI) | 15.33 (3.117-75.420) | 28.667 (9.048-90.823) |
| P value | P < 0.0001 | P < 0.0001 |

Calculated diagnostic performance for Platelia Aspergillus GM-EIA and facC-PCR at the standard cutoff OD450/620:0.5 for GM-EIA and by considering ≥1 positive Cq as significant/triplicate. Performance parameters calculated after stratification corrections based on autopsy data are highlighted with bold letters. Performance parameters calculated after stratification corrections based on autopsy data are highlighted with bold letters.

Diagnostic accuracy

Area under the ROC curve (AUC) was estimated for both classifiers; GM-EIA and facC-PCR. Values represent for GM-EIA a fair while for facC-PCR a good discriminatory property. The area under the ROC-curve (AUC) for GM-EIA was 0.7283 (95 % CI 0.6031-0.8535); P value of < 0.0012 ± 0.0639 while that for the facC-PCR was 0.8004 (95 % CI 0.6976-0.9031); P value of < 0.0001 ± 0.05243. ROC curves with the calculated AUCs are shown in (Fig. 3).

Combined biomarker assay performance by section analysis

Testing control serum specimens with GM-EIA and facC-PCR yielded a high specificity (96 %) (Fig. 4b). Only two control specimens from episodes with no EORTC/MSG evidence of IPA were false-positive when testing with GM-EIA. GM-EIA failed to detect the presence of Aspergillus GM antigen in 63 % of the cases and sensitivity proved to be only 38 % (Fig. 4a). In the case population facC-PCR was able to detect the presence of Aspergillus fumigatus specific nucleic acid target molecules in 84 % of cases by considering a single positive run from triplicate reactions as significant. Conversely facC-PCR failed to detect 16 % of the samples from cases (Fig. 4a). With regard to specificity, 83 % of control specimens were negative when tested with facC-PCR ensuring a relatively high false-positivity rate of 17 % for PCR (Fig. 4b). Comparison analysis was done by examining the different sections of Venn-diagrams shown in Fig. 4. Only paired results were considered and those that were superior in numbers (in the cases of patient ID 4 and ID 10 for the benefit of facC-PCR) were excluded from the section analysis. In doing so we can see that 50 % of specimens from cases were detected only by facC-PCR but not with GM-EIA thus 8 % of specimens from cases were missed with either of the two methods. It is noteworthy that in 83 % of all cases when testing control specimens (from episodes with no EORTC/MSG evidence of IPA) both assays remained consistently negative furthermore there were no false-positive cases when testing with both methods.

Survival and mortality data

The overall mortality rate was 48.14 % (Additional file 2: Figure S2). 13 of 27 patients died during the surveillance period; 4 of 13 were cases (30.77 %) and 9 were controls (69.23 %). Post mortem histology (PMH) was pursued in 53.85 % (7 of 13 patients) of fatalities. 10 of 23 people (43.47 %) with patient ID 4, 9, 11, 14, 15, 17, 18, 19, 22, 24 (Fig. 1a) from the GM-EIA negative group and 3 of 4 people (75 %) with patient ID 7, 10, 13 from the GM-EIA positive (Fig. 1a) group. In the case of one episode of the latter group PMH proved to be negative (Additional file 2: Figure S2; patient ID 10).

Stratification correction at autopsy

In two cases major discrepancies were observed between the clinical and postmortem findings.

(i) Autopsy proved hyphal tissue invasion in only one patient (Additional file 2: Figure S2; patient ID 4). Interestingly this patient’s fever ceased in response to antibiotic treatment and provided negative CT result along with 7 consecutive negative GM-EIA tests but generated positive PCR results in 10/11 runs (Fig. 4). This case was thus classified “possible” and remained undiagnosed. It was only at autopsy that this patient was confirmed to have had IPA. The presence of fungal hyphal invasion in the lung tissue was confirmed by PAS and H&E staining and the episode was subsequently classified “proven” (Additional file 3: Figure S3).

(ii) One episode (patient ID 24) was originally classified “probable” based on persistent fever refractory to broad spectrum antibiotic treatment, positive
thoracic CT, and one positive GM-EIA result out of three but definite negative PCRs (9 of 9 runs). As postmortem histology could not demonstrate fungal involvement, episode was reclassified possible (Additional file 4: Figure S4).

Discussion
The incidence of life threatening invasive fungal infections (IFI) caused by filamentous fungi is continuously increasing [19, 20]. Aspergillus species are among the most common human pathogens causing serious infections in severely immunocompromised patients and those suffering from prolonged neutropenia [21, 22]. Patients with acute myeloid leukemia (AML) are considered to be at the highest risk for developing neutropenia and consequent IFI [23]. Adequate antifungal therapy initiated early in the course of disease could mitigate devastating effects of IFI. Despite advances in the field of antifungal therapy and improvements in diagnosis through the use of rapid methods to screen for Aspergillus genus (GM by sandwich ELISA) or species specific (nucleic acid detection by PCR) biomarkers, estimation of the risk of infection has remained difficult thus clinicians rely on empirical treatment. The limitations of conventional diagnostic methods such as cultures and radiology

Fig. 2 Data on CBT in fevered neutropenia episodes at the University Hospitals of Debrecen. (*) Neutrophil count less than 500/mm³. (†) persistent fever for more than 96 h, refractory to antibiotic treatment, (group I patients); (‡) lack of persistent fever more than 96 h, (group II patients); (*) according to European Organization for Research and Treatment of Cancer criteria, (FNE); febrile neutropenia episode. There were in total 162 tests (mean; 2.93 ± 2.14/FNE) performed. 77 serum specimens (mean; 2.79 ± 1.89/FNE) were screened for the presence of GM antigen and 85 PCRs (mean; 3.07 ± 2.39/FNE) were performed supported by 249 runs (mean; 9 ± 7.02/FNE) and targeting special fungal nucleic acid marker genes (facC-PCR) of prokaryotic origin. Reporting of descriptive statistics with mean values and standard error (±SE) are shown.

Fig. 3 Diagnostic performance (receiver operating characteristic curves) for Aspergillus GM-EIA and facC-PCR methods. ROC curve illustrating the diagnostic performance of GM-EIA and facC-PCR at different discriminatory thresholds by plotting true positives (sensitivity) against false positives (1-specificity)
contribute to delayed treatment of disease. IPA occurs mainly among people with prolonged neutropenia, and only few data are available on IPA in non-neutropenic patients [24]. Our prospective study introduces a combined biomarker monitoring strategy for the management of IPA in patient groups developing neutropenia by using a nucleic acid based real-time diagnostic adjunct to more established diagnostic techniques to enable early and more accurate diagnostic decisions.

An aspergillosis surveillance group was established at the University Hospitals of Debrecen comprising geneticists, molecular biologists, microbiologists and physicians, to assess the discriminatory power of combined biomarker testing by considering facC-PCR results together with those of the GM-EIA in our local patient population including patients with different hematological malignancies. The study was observational, thus no diagnostic or treatment decisions were based on the study’s results. According to our inclusion criteria patients with febrile neutropenia were considered from the appearance of fever. FNEs underwent combined biomarker testing by screening of sera for the presence of GM antigenemia (GM-EIA) and the facC-PCR with the mean duration of 3.11 ± 2.36 days.

During patient surveillance thoracic CT scans were performed on FNEs and IPA was confirmed in 57 % (4 of 7) of cases; 3 episodes with proven and one episode with probable IPA thus generating a low PV⁺ of 0.57 but a relatively high PV⁻ of 0.85. In one case of a patient (ID 16) with persistent fever, clinical diagnosis of invasive mold infection (IMI) was supported by CT even though BAL culture and GM-EIA (3/3) were consistently negative. Bronchoscopy with lavage was performed in 78 % of episodes (20 of 27 patients), which yielded an extremely low PV⁺ (0.16). False positivity was observed in only one case (patient ID 5) ensuring a high PV⁻ (0.93). Interestingly this patient provided misleading CT results and gave a positive (1/3) GM-EIA result.

It was noticeable that co-analyzing the GM-EIA results with that of CT scans (Fig. 1) provided sufficient strong discriminatory power to exclude IPA (PV⁻ was 0.94). There was only one case (patient ID 4) with a fever that ceased that proved to be false-negative with both of the primary diagnostic methods (GM-EIA and CT). Analysis of BAL was also negative in this case and the patient did not receive antifungal treatment. However during the extended follow-up period facC-PCR showed consecutive positive results and a high PV⁺ of 0.91. In this episode we extended the biomarker surveillance period to 17 days gathering 11 facC-PCR and 7 GM-EIA samples which gave contradictory data; 10 positive facC-PCRs compared to 7 negative GM-EIA.

PMH was pursued in 7 cases and 2 episodes were reclassified after autopsy (Table 2). In both cases facC-PCR convincingly supported the presence (patient ID 4) and the absence (patient ID 24) of Aspergillus hyphae. By comparing our in-house facC-PCR method with GM-

![Fig. 4 Venn-diagrams with section-analysis comparing the outcomes of Platelia Aspergillus GM-EIA and facC-PCR. TP true-positive, FN false-negative, TN true-negative, FP false-positive. Cases (proven and probable IA) with positive GM-EIA and PCR results were regarded as true positive (TP) while those with negative outcomes were considered to be false negative (FN). Controls (unclassified patients with no EORTC/MSG evidence of IA) with negative GM-EIA and PCR results were coded to true negative (TN) and those with positive outcomes false positive (FP). a 27 of 32 (84 %) and 9 of 24 (38 %) serum samples from cases (proven/probable) proved to be true positive (TP) when testing with facC-PCR and GM-EIA respectively. GM-EIA failed to detect 15 of 24 and facC-PCR 5 of 32 samples. Section analysis: 8 of 24 specimens from cases (33 %) proved to be true positive (TP) with both assays and 3 of 24 (8 %) found to be negative (FN) by both of them. There was only one specimen (4 %) of 24 that was missed by facC-PCR but not by GM-EIA. 50 % of specimens (12 of 24) however were detected only by PCR but not with GM-EIA, b 51 of 53 (96 %) and 44 of 53 (83 %) serum specimens from controls (unclassified patients with no evidence of IA) proved to be negative (TN) thus 2 of 53 (3.8 %) and 9 of 53 (17 %) were undetected. There were 9 of 53 (17 %) specimens that proved to be false-positive with PCR but real-negative with GM and only 2 of 53 (4 %) that proved to be false-positive with GM-EIA but true-negative with facC-PCR. Section analysis: 83 % of specimens (44 of 53) remained consistently negative with both of the assays but there were none that proved to be false-positive when testing with both methods.
EIAs and biological samples, organized

case-presenting participants, who were evaluated and treated for at least one


duration of the postmortem histology, (IA) the presence of hyphae representative of invasive fungal infection was confirmed by PAS or H&E staining, (IA) the presence of hyphae could not be confirmed by PAS or H&E staining. Black symbols represent controls while red symbols represent cases. Contoured symbols represent episodes where fever ceased, while shaded symbols represent episodes with present or recurrent fever refractory to broad spectrum antibiotic treatment. Small sized symbols represent children. Numbers intend to denote patient ID numbers.

Additional file 3: Figure S3. Postmortem histological findings from case ID 4 showing congestion and focal atelectasis of the lung (3A, x10 magnification, H&E staining). Detailed evaluation underlined the Aspergillus specific PCR assay based assumption of the presence of invasive Aspergillus hyphae (3B). This was also supported by PAS staining (3C, x10 magnification) although definite identification is not possible on a morphological basis therefore the presence of other filamentous fungi cannot be excluded.

Additional file 4: Figure S4. Gross view (4A, x10 magnification) and high-power image (4B, x40 magnification) of the postmortem lung specimens in case ID 24. Atelectasis with fibrohemorrhagic exudate and alveolar damage is clearly visible but evidence of microscopic fungal manifestation is absent.

Conclusion

In this pilot-study we have shown the potential of combination testing to diagnose IPA more accurately in FNEs with different hematological malignancies. CBT should combine the gold-standard serology (GM-EIA) with sensitive and specific nucleic acid based techniques (e.g. facC-PCR) from the onset of febrile neutropenia. This study is relatively small and carried out over a single year but the observed disease prevalence proved to be high (25.9%). The sensitivity of GM-EIA was found to be only 36%, which is lagging behind the average results found in other studies. Although the low number of patients presents the major obstacle to drawing strong conclusions we intend to alert clinicians and researchers in this field to the discriminatory power of combined biomarker testing. The CBT approach considers the results of PCR assays together with the results of GM-serology and radiology in clinical decision making thus complementing the relatively low sensitivity of GM-EIA by the more efficient nucleic acid based methods [25]. In doing so we concluded that our patient management strategy has the potential to be accurate and prompt enough, possibly decreasing hospital expenditures and supporting evidence-based diagnosis.

Additional files

Author details

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References

1. Xiao PL, Huang LM, Huweh PR. Recent advances and challenges in the treatment of invasive fungal infections. Int J Antimicrob Agents. 2007;30:487-95.

2. White PL, Parr C, Thornton C, Barnes RA. Evaluation of real-time PCR, galactomannan enzyme-linked immunosorbent assay (ELISA), and a novel lateral-flow device for diagnosis of invasive aspergillosis. J Clin Microbiol. 2013;51:1510-6.

竟争利益

The authors declare that they have no competing interest

作者的贡献

MP conceived the nucleic acid based biomarker screening, participated in the design of the study plan and coordination of the study protocol, review and interpretation of results, development of statistical analyses plan and performing basic statistical analysis and drafted the manuscript. GF conceived in nucleic acid based biomarker screening and performing of the statistical analyses. JK and EB participated in the development of study protocol, coordination of GM-EIA serology analyses and participated in interpretation of data with critical revision. LR and GM recruited study participants and biological samples, organized standard clinical examinations e.g. CT, BAL and PMH, categorized the patients according EORTC/MSG recommendations. JL reviewed manuscript and conducted critical revision of the manuscript for important intellectual content. SB participated in the design of the study, manuscript writing, reviewing and interpreting results. All authors read and approved the final manuscript.

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Abstract

The purpose of this pilot study was to evaluate a combination of serological and molecular diagnostic tools in patients with hematological malignancies and febrile neutropenia (FNE) while comparing our results to previously published studies. We collected serum samples from 27 consecutive patients with hematological malignancies and FNE during the study period. Patients were serologically tested for the presence of galactomannan (GM), using latex agglutination (Platelia<sup>®</sup>-GM) and in-house GM enzyme-linked immunosorbent assay (ELISA). GM assays were considered positive if the optical density (OD) ratio was ≥ 0.5 or if the assay result was positive in two out of three independent testing runs. GM-positive samples were further analyzed by using PCR to detect the specific nucleic acid sequences of Aspergillus (facC). RT-qPCR was performed using the LightCycler<sup>®</sup>480 system and the Doris<sup>®</sup>-PCR kit. The average Cq-value for FNE was 28.76 (± 1.4), with a range of 23.40-31.77. In all positive samples PCR analysis confirmed the invasive fungal infection (IFI) diagnosis. Positive RT-qPCR for Aspergillus facC (Cq values) showed a high correlation with the results of GM testing (Cq values). Using Plattelia<sup>®</sup>-PCR and GM-EIA (DOR) we observed a sensitivity of 41.2% (95% confidence interval [CI]: 21.5, 61.9). The specificity was 95% (95% CI: 85.3, 99.3). The negative and positive predictive values were 78% (95% CI: 51.3, 93.1) and 85% (95% CI: 69.8, 93.2), respectively.

Abbreviations

± SD: Standard deviation; AUC: Area under the ROC curve; BAL: Bronchoscopy with lavage; CBT: Combined biomarker testing; CI: Confidence interval; CT: Thoracic computed tomography; DOR: Diagnostic odds ratio; EORTC/MSG: European Organization for Research and Treatment of Cancer/Mycosis Study Group; facC-PCR: Factor C PCR; FN: False-negative; FNE: Febrile neutropenic episode; FP: False-positive; GM: Galactomannan; GM-EIA: GM enzyme-immunoassay; H&E: Hematoxylin and Eosin; IA: Invasive aspergillosis; IPA: Invasive pulmonary aspergillosis; LR<sup>+</sup>: Likelihood ratio positive; LR<sup>−</sup>: Likelihood ratio negative; PV<sup>−</sup>: Predictive value negative; PV<sup>+</sup>: Predictive value positive; ROC: Receiver operator curve; TN: True-negative; TP: True-positive; IFI: Invasive fungal infection.

Competing interest

The authors declare that they have no competing interest

Additional files

Additional file 1: Figure S1. Dot histogram (1/A) visualizing the distribution of the Platelia Aspergillus GM-EIA hits (OD<sub>450nm</sub>) with the regard to patient status. Dot histogram (1/B) visualizing the distribution of the facC-PCR hits (Cq values) with the regard to patient status. Group-control Cq-values correspond to outcomes by testing specimens from possible episodes while group-case Cq-values correspond to outcomes by testing specimens from proven/probable cases.

Additional file 2: Figure S2. Representation of the data on postmortem histology, (IA) the presence of hyphae representative of invasive fungal infection was confirmed by PAS or H&E staining, (IA) the presence of hyphae could not be confirmed by PAS or H&E staining. Black symbols represent controls while red symbols represent cases. Contoured symbols represent episodes where fever ceased, while shaded symbols represent episodes with present or recurrent fever refractory to broad spectrum antibiotic treatment. Small sized symbols represent children. Numbers intend to denote patient ID numbers.

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3. Girme C, Miccozi A, Gentile G, Santilli S, Arleo E, Cardarelli L, et al. Clinically driven diagnostic antifungal approach in neutropenic patients: a retrospective feasibility study. J Clin Oncol. 2010;28:667–74.

4. White PL, Bretagne S, Klingspor L, Melchers WJ, McCulloch E, Schulz B, et al. Aspergillus PCR: one step closer to standardization. J Clin Microbiol. 2010;48:1231–40.

5. Maertens J, Theunissen K, Verhoef G, Verschakelen J, Lagrou K, Verbeke E, et al. Galactomannan and computed tomography-based preemptive antifungal therapy in neutropenic patients at high risk for invasive fungal infection: a prospective feasibility study. Clin Infect Dis. 2005;41:1242–50.

6. Mikolajewska A, Schwartz S, Ruhinke M. Antifungal treatment strategies in patients with haematological diseases or cancer: from prophylaxis to empirical, pre-emptive and targeted therapy. Mycoses. 2012;55:2–16.

7. Maertens J, Groll AH, Cordonnier C, de la Camara R, Rolledge M, Marchetti O. Treatment and timing in invasive mould disease. J Antimicrob Chemother. 2011;66 Suppl 1:337–43.

8. Cuenca-Estrella M, Bassetti M, Lass-Flörl C, Nosari A, Van Lint MT, Candoni A, Offidani M, et al. BMC Infectious Diseases (2015) 15:251.

9. Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drigo L, Bilijevens N. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. J Antimicrob Chemother. 2011;66 Suppl 1:15–14.

10. Springer J, Morton CO, Perry M, Heinz WJ, Paholcsek M, Alzheimer M, et al. Critical stages of extracting DNA from lymphocytes: the SEIFEM-2004 study. Haematologica. 2006;91:1068–75.

11. Pagano L, Caira M, Nosari A, Van Lint MT, Candoni A, Offidani M, et al. Fungal infections in recipients of hematopoietic stem cell transplants: results of the SEIFEM B-2004 study. Clin Infect Dis. 2007;45:1161–70.

12. Paholcsek M, Leiter E, Markovics A, Biro S. Novel and sensitive qPCR assays for the detection and identification of aspergillosis causing species. Acta Microbiol Immunol Hung. 2014;61(3):273–84.

13. Fitzpatrick DA. Horizontal gene transfer in fungi. FEMS Microbiol Lett. 2012;329(1):1–8.

14. Freifeld AG, Bow EJ, Sepkowitz KA, Boeckh MJ, Ito JI, Mullen CA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the infectious diseases society of America. Infectious Diseases Society of America. Clin Infect Dis. 2011;52:e56–93.

15. De Pauw B1, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis. 2008;46:1813–21.

16. Millon L, Fierro C, Millon C, Paholcsek M, Bretagne S, Lass-Flörl C, et al. Antifungal pre-emptive strategy for high-risk neutropenic patients: why the story is still ongoing. Clin Microbiol Infect. 2014;20 Suppl 6:27–33.

17. Cornillet A, Camus C, Nimubona S, Ganderner V, Tattevin P, Bellequic C, et al. Comparison of epidemiological, clinical, and biological features of invasive aspergillosis in neutropenic and nonneutropenic patients: a 6-year survey. Clin Infect Dis. 2008;46:577–84.