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Comparison of Galactomannan Detection, PCR–Enzyme-Linked Immunosorbent Assay, and Real-Time PCR for Diagnosis of Invasive Aspergillosis in a Neutropenic Rat Model and Effect of Caspofungin Acetate

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The performance of different in vitro diagnostic tests for the diagnosis of invasive aspergillosis (IA) was investigated in a transiently neutropenic rat model. Rats were immunosuppressed with cyclophosphamide and then inoculated intravenously with 1.5×10^4 CFU *Aspergillus fumigatus* spores. Animals were then either treated with caspofungin acetate, 1 mg/kg/day for 7 days, or not treated. PCR–enzyme-linked immunosorbent assay (ELISA), real-time PCR, and galactomannan (GM) detection were performed on postmortem blood samples, along with culture of liver, lung, and kidney homogenate. Caspofungin-treated animals showed a decrease in residual tissue burden of *A. fumigatus* from organ homogenate compared to untreated animals ($P < 0.002$). PCR-ELISA returned positive results for 11/17 animals treated with antifungal agents and for 10/17 untreated animals. Galactomannan was positive in 8/17 caspofungin-treated animals and 4/17 untreated animals. Real-time PCR was positive in 2/17 treated and 3/17 untreated animals. This study demonstrates that PCR-ELISA is a more sensitive test than either GM detection ($P = 0.052$) or real-time PCR ($P < 0.01$) for diagnosis of IA but that any of the three tests may return false-negative results in cases of histologically proven disease. Galactomannan indices from animals treated with antifungal agents showed a trend ($P = 0.1$) towards higher levels than those of untreated animals, but no effect was observed with PCR-ELISA indices ($P = 0.29$). GM detection, as previously described, may be enhanced by the administration of caspofungin, but PCR-ELISA appears not to be affected in the same way. We conclude that PCR-ELISA is a more sensitive and reliable method for laboratory diagnosis of IA.

Invasive aspergillosis (IA) is a leading cause of death in neutropenic bone marrow transplant patients, patients receiving cytotoxic chemotherapy for leukemia, and solid organ transplant patients (3, 17, 22). The incidence has been estimated to be 5 to 25% in patients with acute leukemia, with mortality approaching 100% (8, 10, 18, 29).

Early and accurate diagnosis of IA is a worthy goal, as it has the potential to improve outcomes by targeting antifungal therapy more effectively. A number of laboratory strategies to improve the early diagnosis of IA have been published, including PCR amplification of *Aspergillus* DNA sequences from blood and other body fluids (6, 9, 14, 16, 28, 32, 36, 40) and detection of cell wall components such as galactomannan (GM) (13, 21, 34, 35, 37).

While studies have generally reported very promising results for new, non-culture-based strategies to diagnose IA in the laboratory, problems surrounding clinical validation of the assays has delayed the inclusion of these strategies in routine clinical and laboratory use. A number of parameters remain to be investigated, including the kinetics of fungal cell elements in the blood and the effect that administration of systemic antifungal agents may have on the detection of these elements. As a result, uncertainty surrounding the optimal use of these diagnostic strategies remains.

The aim of the present study was to study the effect of the administration of caspofungin acetate on the performance of GM and PCR testing and to determine if the phenomenon of intermittent positive results in a subject with heavy fungal burden is a true phenomenon or a function of concomitant antifungal treatment.

To this end, we heavily infected transiently neutropenic rats with *Aspergillus* spores, treated one group with 1 mg/kg/day caspofungin acetate, and left the other group untreated. PCR–enzyme-linked immunosorbent assay (ELISA), real-time PCR, and galactomannan testing were performed at defined time points during the study.

MATERIALS AND METHODS

In summary, animals were immunosuppressed and inoculated with *Aspergillus fumigatus* spores. The study had two arms: the arm treated with antifungal therapy (arm A) and the untreated arm (arm B). Each arm consisted of 21 animals, including 3 control animals. Animals were housed in seven groups of three, according to the projected date of euthanasia. Animals in antifungal treatment arm A were treated with 1 mg/kg caspofungin acetate daily for 7 days, beginning 24 h after inoculation. One group of three animals was sacrificed every second day, beginning 2 days postinoculation. One group of three animals in each arm was used as a control group. These animals were dosed with cyclophosphamide but were not treated with antibacterial or antifungal agents and were not inoculated with fungal spores.

Animals. Forty-two adult male Sprague-Dawley rats (mean body weight, 365 g) were used in these experiments. Animals were housed in groups of three in sterile microisolator cages. Animals were allowed ad libitum access to sterile

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drinking water and a pathogen-free rat diet. All handling and manipulations were performed in a laminar flow cabinet.

Ethical approval for the experiments was obtained from the local animal ethics committee, and animals were monitored and maintained in accordance with local animal ethics guidelines.

Immunosuppression/antibacterial prophylaxis. All animals were immunosuppressed 24 h prior to inoculation, with a single dose of cyclophosphamide (60 mg/kg intraperitoneally). Previous studies have proven that this regimen induces a transient neutropenia (33) ($<0.5 \times 10^9$ neutrophils/liter) from day 0 to day 4 without undue loss of condition (our unpublished data). Antibacterial prophylaxis commenced 4 days prior to fungal spore inoculation and consisted of ciprofloxacin (1 mg/kg/day) and neomycin (5 mg/kg/day) given by oral gavage. Gentamicin (6 mg/kg subcutaneously) and amoxicillin (50 mg/kg orally) were given daily beginning on the day of inoculation. Animals were inoculated into the lateral tail vein with 0.5 ml of a suspension containing 3×10^4 CFU *A. fumigatus* spores. Previous studies have shown that this inoculum size is sufficient to reliably cause invasive disease that allows survival for at least 10 days (our unpublished data). Control animals were "sham inoculated" with 0.5 ml Tween saline. Animals showing signs of dehydration (weight loss, especially if accompanied by diarrhea and loss of skin elasticity) were given 5 ml sterile saline administered by subcutaneous injection.

Antifungal treatment. Animals in the arm treated with an antifungal agent were treated with caspofungin acetate (1 mg/kg/day) administered by intraperitoneal injection for 7 days beginning 24 h after inoculation.

Microbiology: preparation of fungal spore suspension for inoculation. The strain of *A. fumigatus* used in these experiments was isolated from the lung biopsy of a patient with cystic fibrosis who developed IA. Testing for sensitivity to caspofungin was performed by E-test (AB Biodisk, Solna, Sweden) to confirm that the isolate was sensitive to caspofungin (0.25 mg/liter).

Fungal spore suspensions were prepared by growing cultures on Sabouraud's dextrose agar for 48 h at 30°C and were then harvested by adding 5 ml Tween saline (0.15 M NaCl containing 0.025% Tween 20) to the plate and rolling over the surface of the colonies. The suspension was counted in a hemocytometer and adjusted to the appropriate concentration in sterile saline solution. Aliquots (100 μl) were spread onto triplicate sheep blood agar plates and incubated for 24 h at 30°C, and the number of resulting colonies was counted and averaged, as a confirmation of the manual count.

Animal tissues. Euthanasia was achieved by halothane overdose. Following euthanasia, EDTA and clotted blood samples were collected by cardiac puncture, and organs (liver, lungs, and kidneys) were removed by dissection. Organs were kept at 4°C during transit to the laboratory. EDTA blood samples and sera were frozen and stored at −20°C until testing.

Once in the laboratory, organs were weighed and examined macroscopically. Macroscopic fungal lesions were graded visually (0, no visible lesions; 1, 1 to 2 lesions; 2, 3 to 10 lesions; 3, 11 to 20 lesions; 4, >20 lesions). The tissues were then homogenized, 0.1 g of each homogenate was cultured on Sabouraud's dextrose agar at 30°C for 72 h, and the number of resulting colonies were enumerated. Residual fungal tissue burden was expressed as log CFU/gram of tissue.

Molecular methods. PCR-ELISA was performed as described previously (9). Briefly, pelleted fungal cells were recovered from 1 ml EDTA-anticoagulated blood, followed by enzymatic lysis of fungal cell walls and purification of fungal DNA using a standard phenol-chloroform method. PCR amplification and PCR-ELISA (PCR ELISA DIG-Detection kit; Roche Molecular Biochemicals, Germany) were performed using an oligonucleotide probe that binds specifically to DNA of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus versicolor*. Negative controls were included at all steps of the assay to control for environmental or laboratory carryover contamination. The optical density (OD) of each sample was measured on a bench-top spectrophotometer (Elisa II processor; Boehringer GmBH, Germany) at a λ of 405 nm with a reference λ of 492 nm. The negative cutoff value was determined as the sum of the negative controls ($n = 5$) plus 3 standard deviations from the mean. Results were expressed as a positivity index calculated by dividing the OD of the sample by the negative cutoff value. Samples with a positivity index of ≥1.5 were considered positive.

Real-time PCR was performed as described previously (21). The same aliquot of fungal DNA that was extracted for PCR-ELISA was subjected to real-time PCR using the LightCycler instrument (Roche diagnostics, Mannheim, Germany) and LightCycler software, version 3.5. Reagents were supplied in the LightCycler Hybridization Probes Master Mix kit (Roche diagnostics, Mannheim, Germany). Two internal hybridization probes specific to *A. fumigatus* were used; the first was labeled at the 5′ end with LC Red 640 and at the 3′ end with phosphorous and the second labeled at the 3′ end with fluorescein as has been previously described (20).

Galactomannan detection was performed using a commercially available assay kit (Platelia *Aspergillus*; Sanofi Diagnostics, Marnes-La Coquette, France) according to the manufacturer's instructions. Three hundred microliters of serum was used in each test. Results were expressed as a positivity index calculated by dividing the OD of the sample by the mean of the two threshold sera (supplied by the manufacturer). Samples with a positivity index of ≥1.5 were considered positive, with an equivocal range of <1.5 to ≥1, (according to the manufacturer's instructions).

Statistical methods. Data were initially entered into a Microsoft Excel spreadsheet. Comparisons within and between groups were made using SPSS version 11 (SPSS Inc. Chicago, Ill.).

RESULTS

Mortality. One animal in each arm of the study required euthanasia prior to the planned date, on ethical grounds. No samples were obtained from these animals.

Macroscopic fungal disease and culture. Macroscopic lesions were easily identifiable, and the majority of lesions were seen in the livers. The findings are shown in Tables 1 and 2.

Significantly fewer *A. fumigatus* colonies were isolated from the liver, spleen, and lung homogenates from caspofungin-treated animals than from those of untreated animals (52 versus 123; $P < 0.001$ [Mann-Whitney U test]). *Aspergillus* cultures of the liver showed that more caspofungin-untreated than treated animals had positive results (17 versus 11; $P = 0.019$ [Fisher's exact test]), higher numbers of colonies (29 versus 98; $P > 0.01$ [Mann-Whitney U test], and residual tissue burden (log CFU/gram) in the livers (1.665 versus 0.878; $P = 0.002$ [Mann-Whitney U test]).

There were comparatively few positive cultures from kidneys (treated, 16 colonies; untreated, 17 colonies) and lungs (treated, 1 colony; untreated, 8 colonies) of infected animals.

Diagnostic tests. Galactomannan assay, PCR-ELISA, and real-time PCR were performed on 20 animals (including control animals) from each arm of the study. No positive results were returned by control animals by any test. PCR-ELISA returned positive results from 11/17 animals treated with an antifungal agent and 10/17 untreated animals. Galactomannan was positive in 8/17 treated animals and 4/17 untreated animals. Real-time PCR was positive in 2/17 treated and 3/17 untreated animals (Tables 1 and 2). The PCR-ELISA test was a more sensitive test than either the GM assay ($P = 0.052$) or real-time PCR ($P < 0.01$) for the diagnosis of IA. (Tables 1 and 2).

The overall values for sensitivity, specificity, positive predictive value, and negative predictive value for each test for diagnosis of IA are shown in Table 3.

Indices. Median GM indices from animals treated with an antifungal agent showed a trend ($P = 0.1$) towards higher levels than those of untreated animals, but little effect was observed with PCR-ELISA indices ($P = 0.29$) (Mann-Whitney U test).

DISCUSSION

We have established a neutropenic rat model of IA using intravenous sublethal inoculation of *A. fumigatus* spores. This was chosen to ensure a high fungal load but also high survival to the endpoint of the experiment. Tests were done on blood samples taken only at death so that there was sufficient sample to perform all tests in an optimal manner and to avoid com-

TABLE 1. Macroscopic findings and GM, PCR-ELISA, and real-time PCR results for animals in the treated arm of the study

Day	Rat ID	Disease[a]	Tissue burden[b]	GM		PCR-ELISA		Real-time PCR
				Index	Result[d]	Index	Result	Result
+2	1	0	0	1.250	NEG	0.207	NEG	NEG
	2	0	0	0.589	NEG	0.221	NEG	NEG
	3	0	1	1.255	NEG	0.221	NEG	NEG
+4	4	0	1.3	8.270	POS	10.329	POS	POS
	5	0	1	0.454	NEG	0.225	NEG	NEG
	6	0	0	1.648	POS	5.141	POS	NEG
+6	10	2	0	0.639	NEG	0.230	NEG	NEG
	11	1	1	7.906	POS	2.343	POS	NEG
+8	7	4	1.5	>MC[c]	POS	4.282	POS	NEG
	8	3	1	2.774	POS	3.906	POS	POS
	9	1	1	0.589	NEG	0.033	NEG	NEG
+10	16	4	1.9	>MC	POS	5.235	POS	NEG
	17	3	1	0.818	NEG	1.690	POS	NEG
	18	1	1	0.343	NEG	5.418	POS	NEG
+12	13	4	1.7	8.183	POS	3.455	POS	NEG
	14	3	1.45	0.220	NEG	2.235	POS	NEG
	15	2	0	>MC	POS	2.418	POS	NEG
+12	19	0	0	0.581	NEG	0.146	NEG	NEG
	20	0	0	0.427	NEG	0.244	NEG	NEG
	21	0	0	0.253	NEG	0.582	NEG	NEG
No. of infected animals with positive results/total no.					8/17		11/17	2/17

[a] Disease indicates the macroscopic extent of disease in livers, graded from 0 to 4.
[b] Tissue burden indicates log CFU/gram of tissue cultured.
[c] >MC OD was greater than the measurement capacity of the instrument. For the purposes of statistical analysis, these cases were assigned an index of 10.0. Rats 19, 20, and 21 are negative control animals in both groups.
[d] NEG, negative; POS, positive.

promising animal survival by repeated blood sampling or influencing bloodstream dissemination by repeated handling of the animals.

There was obvious macroscopic disease in untreated animals from day 4 and in caspofungin-treated animals from day 6. Despite this, 6 of 25 animals (24%) with obvious macroscopic aspergillosis were negative by all tests. Over the whole population from day 2, the results were more disappointing. The PCR-ELISA test was the most sensitive test for diagnosis of IA in this model (sensitivity, 62%), and by comparison, the other tests performed poorly. There is only one previously published report of the use of real-time PCR testing of blood samples in an animal model (intravenous inoculation) of IA (20). This study found that only 25% of samples from infected animals were positive. Similarly, in a neutropenic mouse model infected by the tracheal route, 62% of samples were positive by PCR-ELISA (4). These results are all in agreement with the observation from small numbers of human cases of IA that GM and PCR-ELISA may be negative or intermittently positive (12, 16).

These results are explained in part by the differences in the lower detection limits of the assays. Our own comparisons of these tests in vitro have shown that the PCR-ELISA is 100-fold more sensitive (10 CFU) than either the real-time PCR (100 to 1,000 CFU) or the GM test. Given that the detection limit of the real-time PCR assay used in the present study was at best 10^2 CFU, this suggests that the fungal DNA load seldom reaches this level even with a large burden of disease. This is in keeping with previous estimates of fungal load in blood samples (19).

The large number of blood samples containing *Aspergillus* DNA and GM at levels below the detection limit in the face of obvious invasive disease suggests that fungal DNA reaches the bloodstream in an irregular manner, and it is likely that host lysis of hyphae is poor. It is possible that the nature of tissue invasion by *A. fumigatus* contributes to this phenomenon. It has been shown that platelets and fibrinogen adhere to hyphae of *A. fumigatus* (7) and participate in fungal killing. Histological sections typically show angioinvasion with regions of associated infarction, indicating that at least partial occlusion of the vessel has occurred. Thus, fungal elements (antigen and DNA) could be contained within this region and hence could be unavailable for detection in the peripheral blood.

Treatment with caspofungin acetate produced a different pattern of results from the untreated group. There were strikingly fewer colonies of *A. fumigatus* from organ homogenate (29 versus 98), but the sensitivity of the PCR-ELISA assay was similar for the untreated group, and there was a high rate of

TABLE 2. Macroscopic findings and GM, PCR-ELISA, and real-time PCR results for animals in the untreated arm of the study

Day	Rat ID[c]	Disease[a]	Tissue burden[b]	GM		PCR-ELISA		Real-time PCR
				Index	Result[d]	Index	Result	Result
+2	16	0	1.3	0.711	NEG	3.083	POS	NEG
	17	0	1.6	1.095	NEG	3.083	POS	NEG
	18	0	1.7	0.251	NEG	0.343	NEG	NEG
+4	13	2	1.3	8.751	POS	0.281	NEG	POS
	14	2	1.6	0.911	NEG	0.394	NEG	NEG
+6	10	1	1.9	0.495	NEG	0.511	NEG	NEG
	11	1	1.9	0.719	NEG	1.958	POS	NEG
	12	2	1.5	0.803	NEG	1.288	POS	NEG
+8	7	2	2	2.030	POS	1.784	POS	POS
	8	2	1.6	4.665	POS	0.946	NEG	NEG
	9	1	1	7.462	POS	1.575	POS	POS
+10	4	1	1.6	0.338	NEG	1.646	POS	NEG
	5	4	1.7	0.403	NEG	1.544	POS	NEG
	6	2	2.1	0.676	NEG	0.859	NEG	NEG
+12	1	3	2	0.503	NEG	0.762	NEG	NEG
	2	2	1.7	0.549	NEG	2.510	POS	NEG
	3	1	1.6	0.219	NEG	2.352	POS	NEG
+12	19	0	0	0.678	NEG	0.644	NEG	NEG
	20	0	0	0.200	NEG	0.752	NEG	NEG
	21	0	0	0.254	NEG	0.726	NEG	NEG
No. of infected animals with positive results/total no.					4/17		10/17	3/17

[a] Disease indicates the macroscopic extent of disease in livers, graded from 0 to 4.
[b] Tissue burden indicates log CFU/gram of tissue cultured.
[c] Rats 19, 20, and 21 are negative control animals in both groups.
[d] NEG, negative, POS, positive.

detection of galactomannan, in terms of both the number of animals returning positive results and the mean or median GM positivity index of treated animals. The paradoxical relationship between caspofungin administration and clinical improvement but stable or increasing GM indices was previously reported by Petraitiene et al., who described the same effect in an experimental rabbit model of IA (26). These observations are also in keeping with reports of clinical improvement following echinocandin therapy but stable or increasing tissue burden and circulating GM compared to untreated controls. Echinocandins inhibit apical-tip growth and may promote fragmentation that releases more GM and possibly DNA into the circulation (15). This pattern differs from that of azoles (with the exception of itraconazole) and deoxycholate amphotericin B at therapeutic dosages, all of which significantly decrease tissue *Aspergillus* burden, improve survival and decrease morbidity, and decrease or eliminate GM from the serum (5, 11, 23–25, 27, 30).

These studies clearly demonstrate that the relationship between tissue burden and fungal GM or DNA levels in the circulating blood is complex, especially when an antifungal agent is being used. In our study, there was no correlation between tissue burden and GM or PCR-ELISA positivity. The nature of antifungal treatment needs to be taken into account when considering the value of both GM and PCR-ELISA as diagnostic tests or when monitoring the response to therapy as well as other factors that predispose to variable sensitivity or false-positive results (1, 2, 31, 37–39). Few studies have been designed to specifically study the effect of the administration of antifungal agents on the performance of the test. The results of

TABLE 3. Evaluations of the diagnostic tests in this study for treated and untreated rats and overall results[a]

Method	Sensitivity (%)			Specificity (%)			PPV			NPV		
	Trt	Untrt	Total	Trt	Untrt	Total	Trt	Untrt	Total	Trt	Untrt	Total
PCR-ELISA	64.7	58.8	61.7	100	100	100	1	1	1	0.333	0.3	0.28
GM	47	23.5	35.2	100	100	100	1	1	1	0.25	0.187	0.19
Real-time PCR	11.7	17.6	14.7	100	100	100	1	1	1	0.157	0.176	0.16

[a] Trt, treated rats; Untr, untreated rats; PPV, positive predictive value; NPV, negative predictive value.

this and other studies suggest that the use of GM levels in serum to guide treatment in neutropenic hosts should be treated with caution, especially if the patient is receiving antifungal therapy with an echinocandin.

In summary, the results of this study show that GM assay and PCR-ELISA used for the diagnosis of IA may return negative results even in cases of invasive disease. While much has been made of the various causes of false-positive results for these tests, the nature of the invasive process of *A. fumigatus* may cause false-negative results, and the use of antifungal therapy may influence the performance of these tests. The value of these tests needs to be better defined and investigated.

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