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Occurrence and Kinetics of False-Positive *Aspergillus* Galactomannan Test Results following Treatment with β-Lactam Antibiotics in Patients with Hematological Disorders

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Several reports have described a high rate of false-positive *Aspergillus* galactomannan (GM) test results for patients treated with piperacillin-tazobactam. In this retrospective study, we first examined the relationships between intravenous administration of three β-lactam antibiotics and the occurrence of false-positive GM test results in hematology patients. We then estimated the kinetics of clearance of GM after the cessation of treatment. Sequential serum samples from 69 patients that had received β-lactams were analyzed by using a Platelia *Aspergillus* test. A significant association was found between GM positivity (≥0.5) and the administration of β-lactams ($P < 0.0001$). The direct role of β-lactams in patients' serum positivity was assessed by testing 39 batches of β-lactams, of which 27 were positive for GM. None of the latter were positive according to a fungus- and *Aspergillus*-specific PCR. The kinetics of the decrease of GM was analyzed on sequential serum samples obtained after treatment. By use of a nonlinear regression model, the average time to negative antigen was assessed to be 5.5 days (95% confidence interval [CI], 4.1 to [7.0]), with a half-life of elimination of GM of 2.4 days (95% CI, 1.8 to 3.0). This study confirms that the administration of β-lactams containing GM is responsible for false-positive diagnostic results, even up to 5 days after the cessation of treatment.

Invasive aspergillosis (IA) remains a significant cause of morbidity and mortality in immunocompromised patients (6). Despite the availability of new antifungal drugs, the mortality rate for IA remains high (10), due partly to the difficulty and delay of diagnosis based on clinical, radiological, and mycological methods (8, 20, 26). In recent years, the development of a sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of galactomannan (GM) at a threshold of 0.5 to 1 ng/ml was an important advance in the diagnosis of aspergillosis (27), especially in neutropenic patients and allogeneic transplant recipients (4, 11, 12, 28). In these patient populations, a sensitivity of 67 to 100% and a specificity of 86 to 98.9% have been documented (14, 29). Despite this variability in sensitivity and specificity, the performance characteristics of this assay using a cutoff GM index of 0.5 made it a potentially promising tool for the early diagnosis of invasive aspergillosis in high-risk patients (15). This test, named Platelia *Aspergillus*, is marketed by Bio-Rad (Marnes-la Coquette, France) in European countries and was recently approved by the U.S. Food and Drug Administration. Presently, it is routinely used for monitoring patients at risk for aspergillosis. A positive test confirmed on two sequential samples is considered to be contributive for a diagnosis of IA (3).

However, transient false-positive reactions have been reported with the use of Pastorex *Aspergillus* latex agglutination and Platelia *Aspergillus* assays in relation to the possible interactions with antibiotics or in relation to the passage of GM of food origin in the circulation of patients (2, 9, 17).

Between May and July 2003, we were faced in our laboratory with a sudden increase in the number of positive GM antigenemia results, which was in contrast to the absence of clinical or radiological signs for invasive aspergillosis in the tested patients. A chart review showed that most patients positive for antigenemia were receiving a combination of piperacillin and tazobactam, in contrast to patients with persistently negative tests. In the treated patients, GM antigen levels abruptly increased to high positive values in consecutive samples and became undetectable 1 to 6 days after cessation of treatment with the antibiotic (23). Recently, several reports from Europe and the United States confirmed that these false-positive tests were related to the use of piperacillin-tazobactam in hematology patients and stem cell transplant recipients (1, 16, 25, 31, 32).

In the present retrospective study, we aimed to investigate the relationships between intravenous administration of three β-lactams and the occurrence of positive GM tests in hematology patients to search for GM and fungal DNA in several commercial preparations of β-lactam antibiotics and to estimate the kinetics of clearance of GM after the cessation of treatment.

MATERIALS AND METHODS

Patients. Sixty-nine patients hospitalized in either the bone marrow transplantation ward or the adult or pediatric hematology ward at Hôpital Saint-Louis were included in this retrospective study. Patient characteristics are presented in Table 1. All patients had been monitored for the presence of galactomannan

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TABLE 1. Characteristics of studied patients

Characteristic	No. of patients with indicated characteristic (%)[a]
Total no. of patients	69
Sex	
Females	34 (49)
Males	35 (51)
Age	46 (3–85)
Stem cell transplantation	28 (41)
Allogeneic	20 (71)
Autologous	8 (29)
Underlying disease[b]	
AML	24 (35)
ALL	17 (25)
MDS	6 (9)
CML	4 (6)
NHL	4 (6)
Other	14 (20)
Antibiotic treatment	
Amoxicillin	19 (27)
Amoxicillin-clavulanic acid	15 (22)
Piperacillin-tazobactam	35 (51)

[a] For age, the mean age in years is shown, with the range given in parentheses. For all other data, percentages are specific to the total number of patients within that characteristic category. The percentage of patients with stem cell transplantation relative to the total number of patients is also shown.

[b] AML, myeloid or nonlymphoblastic leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloblastic leukemia; NHL, non-Hodgkin's lymphoma.

antigen, and all had been treated with intravenous β-lactam antibiotics (Table 1). Seventy-two series of samples corresponding to individual episodes of treatment with β-lactams were collected from 69 patients, with a mean of 6.6 samples per patient. For three patients, two sets of samples were collected a few months apart and were considered as independent episodes. Forty patients had been monitored for GM before the administration of antibiotherapy. Details of collected data are given in Fig. 1, along with information pertaining to the inclusion of the various analyses.

Antigen detection technique. Circulating *Aspergillus fumigatus* GM was detected using a sandwich immunocapture ELISA (Platelia *Aspergillus*; Bio-Rad, Marnes-la-Coquette, France) using a rat anti-GM monoclonal antibody, EB-A2, which recognizes the (1→5)-β-D-galactofuranoside side chain of the GM molecule and which can function as a capture and detector antibody (27). The technique was performed as recommended by the manufacturer. Results were expressed as an index of positivity. A result was considered a true positive when two consecutive samples from one patient had an index of ≥0.5, including the retesting of the first positive sample. Sequential sera were tested in parallel on the same ELISA plate.

In vitro studies of the antibiotics. Nine batches of piperacillin (Piperacilline; Sanofi-Synthélabo, France) (4 g/vial), 10 of piperacillin-tazobactam (Tazocilline; Wyeth Pharmaceuticals, France) (4 g/vial), 10 of amoxicillin (Clamoxyl; Glaxo-Smith Kline, France) (1 g/vial), and 10 of amoxicillin-clavulanic acid (Augmentin; Glaxo-Smith Kline, France) (1 g/vial) were tested for the presence of GM by use of a Platelia *Aspergillus* test. The vials were reconstituted at the concentration used for intravenous administration according to the manufacturer's instructions and tested. For each batch, an aliquot of 300 μl was processed by a procedure the same as that applied to serum. A result was considered positive when the GM index was ≥0.5. In order to estimate the true level of GM in positive batches, serial dilutions of each positive batch were tested. Serial twofold dilutions of batches that yielded GM indexes of >1 when undiluted were prepared in sterile 0.9% NaCl or pyrogen-free water and then tested for GM level as described above.

The dilution that yielded a GM index value close to 1 was retained for an estimation of the true level of GM. This level was estimated in ng/ml by multiplying the GM index at this dilution by the dilution factors.

All tests were performed on the same batch from a Platelia *Aspergillus* kit (batch number 4B2045).

PCR. Antibiotic batches that were found positive by a Platelia *Aspergillus* test were subjected to a panfungal PCR assay. Two hundred microliters of each antibiotic sample was incubated with 10 U of lyticase (Sigma Aldrich, Saint Quentin Fallavier, France). DNA was then extracted by using a High Pure PCR template preparation kit (Roche Applied Science, Meylan, France) according to the manufacturer's instructions. Amplification of a fragment of fungal 18S rRNA gene was performed by using primers 5′-ATTGGAGGGCAAGTCTGGTG and 5′-CCGATCCCTAGTCGGCATAG as previously described (7). Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, Calif.) and reagents from a PCR digoxigenin labeling mix (Roche Applied Science) were used for amplification. Panfungal PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. Digoxigenin-labeled PCR products from the *Aspergillus* sp. 18S rRNA gene were also detected with a PCR ELISA digoxigenin detection kit (Roche Applied Science) and a biotin-labeled oligonucleotide probe (5′-CATGGCCTTCACTGGCTGTGGGGGGAACCA) specific to the amplified fragment of the *Aspergillus* sp. 18S rRNA gene (7). After

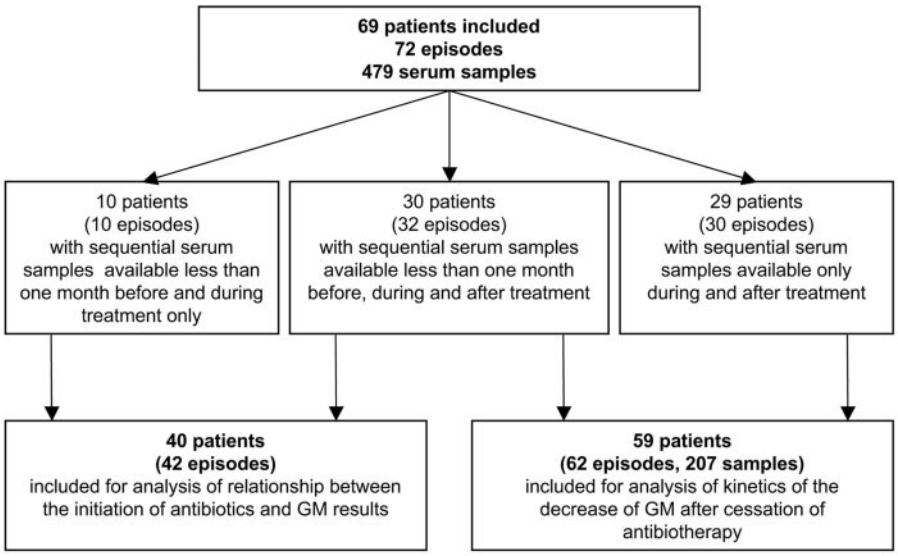

FIG. 1. Flow chart of patients included in the study and in the analyses.

TABLE 2. Number of positive GM tests in 42 episodes monitored before and after initiation of treatment with intravenous β-lactam antibiotics according to the delay after initiation of treatment

Time of test	No. of samples tested	No. (%) of samples with positive GM index[a]
Pretreatment	42	5 (12)
Day 1	6	1 (17)
Day 2	12	9 (75)
Day 3	20	18 (90)
Overall	42	42 (100)[b]

[a] A GM index of ≥0.5 was considered positive.
[b] $P < 0.0001$ as compared to before treatment.

TABLE 3. Number of positive GM tests in 42 episodes monitored before and after initiation of treatment with intravenous β-lactam antibiotics according to the antibiotic

Antibiotic	No. of samples with positive GM index/no. tested[a]	
	Before treatment	During treatment
Amoxicillin-clavulanic acid[b]	0/6	6/6
Amoxicillin[b]	2/16	16/16
Piperacillin-tazobactam	3/20	20/20

[a] A GM index of ≥0.5 was considered positive.
[b] Amoxicillin (sodium salt) and amoxicillin-clavulanic acid (sodium and potassium salts) are available for intravenous use in France and elsewhere in Europe.

immobilization on a streptavidin-coated microtiter plate, the probe-PCR product hybrids were visualized with a peroxidase-conjugated antidigoxigenin antibody and a colorimetric substrate. Serial 10-fold dilutions of *A. fumigatus* DNA extracted from cultures and measured spectrophotometrically at 260 nm were used as external standards in each run. The diluted standards corresponded to 0.2, 2, 20, 200, 2,000, and 20,000 pg/μl, respectively, with the limit of the detection of the assay being <0.2 pg/μl. The presence of PCR inhibitors was looked for in the drug solutions. This was performed by spiking various drug solutions with 2 pg of *A. fumigatus* DNA.

Statistical analysis. The aims of the analysis were both to quantify the impact of the administration of β-lactams on the results of GM tests (i.e., the positivity of the GM index) and to model the kinetics of the evolution of the GM index after the cessation of therapy.

Categorical data are presented as counts (percent) and continuous variables as medians (ranges). Parameters estimated in regression models are presented as estimates with 95% confidence intervals.

The association between β-lactams and GM results was analyzed by a comparison of the rates of positive GM indexes before and after the introduction of treatment by use of the McNemar test. Patients were considered as positive before antibiotherapy if they presented a positive index (at the 0.5 threshold) at any time during the 30 days preceding β-lactam introduction and were considered negative otherwise. To evaluate the kinetics of GM under antibiotherapy, the analysis was repeated at days 1, 2, and 3.

The kinetics of GM decrease during the two months after treatment discontinuation was analyzed using nonlinear mixed models (5). Several models with exponential decrease were postulated. Their parametric form, as well as the random-effects and residual variance-covariance structure, was selected by minimization of the Bayesian information criterion (24). Models were fitted using restricted maximum likelihood, as is usually favored (5). However, comparisons of models involving different fixed effects were based on maximum likelihood estimates, as likelihood comparisons of restricted maximum likelihood fits are not valid (22). *P* values were then obtained using likelihood ratio tests.

Confidence intervals for the average decrease curve, the average time to negative GM levels, and the average half-life time were obtained by the delta method (19).

All tests were two-sided at a significance threshold of 0.05. Analyses were performed with R 2.0.1 statistical software (The R Development Core Team).

RESULTS

Detection and kinetics of serum GM in patients treated with β-lactams. During and after the sampling periods, no patient presented clinical or radiological symptoms suspicious for invasive aspergillosis, according to the EORTC criteria of diagnosis (3), and no fungal culturing of blood or respiratory specimens was performed to isolate *Aspergillus*.

Among 40 patients (42 treatment episodes) with sera available 1 month before treatment, 5 were positive on one serum sample before treatment. According to our definition of positivity (i.e., two sequential positive results), they cannot be considered as true positives. None had signs of IA or presented any other identified cause of a false-positive antigenemia result, such as concurrent mucositis. During treatment, all patients presented positive GM indices, resulting in a significant association between the introduction of β-lactam therapy and the positivity of the test ($P < 0.0001$). Overall, calculated odds ratios for patients tested at days +1, +2, and +3 indicate a gradual increase of positive GM indices during these first three days of treatment, with a median GM index value of 3.65 (range, 0.47 to 6.0) at day 3 (Table 2). Among patients with samples available at day 1, one had also a sample at day 2 and three had samples at both day 2 and day 3. Eleven patients had samples at days 2 and 3. The association of positive GM indexes with β-lactams seemed to be of the same magnitude, whatever the type of antibiotics used (Table 3). For one patient, the GM index rose from 0 to 2.93 as early as 1 hour after the intravenous infusion of piperacillin-tazobactam was started.

The kinetics of the decrease of GM after the cessation of antibiotherapy was analyzed for 59 patients (Fig. 2), with a median follow-up period of 11 days (range, 0 to 62 days) after the discontinuation of β-lactams. All the GM index values

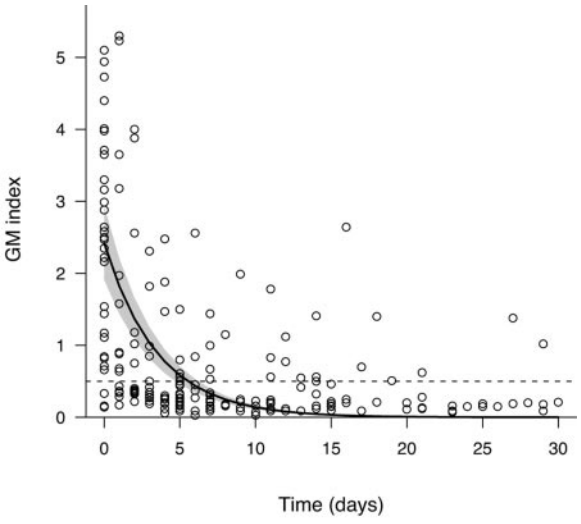

FIG. 2. GM index decrease during the month after the cessation of antibiotherapy. The solid line represents the population average kinetics as estimated by use of a nonlinear mixed regression model with a pointwise 95% confidence band (shaded region). Circles represent individual GM index values measured during this period, and the dashed line represents the 0.5 threshold for positive indices.

TABLE 4. Estimated concentrations of GM in different batches of amoxicillin, amoxicillin-clavunate, and piperacillin-tazobactam delivered at Saint-Louis Hospital between November 2003 and May 2004

β-Lactam antibiotic or other constituent	No. of batches	Mean (SD) concn (ng/ml) of GM	Median concn (ng/ml) of GM	Range (ng/ml)
Amoxicillin	10	840 (1,297)	640	8–4,320
Amoxicillin-clavulanic acid	10	376 (1,106)	13.6	2–3,500
Piperacillin-tazobactam	10	44 (51)	24.8	0.4–141
Diluents		0.00	0.00	

during the first 30 days of the follow-up periods are plotted in Fig. 2 and show an overall trend to decrease and become negative after cessation of treatment. Among the several models tested to summarize this decrease, the one best fitting the data was $a \times \exp(-b \times t)$, where parameter a represents the average index at the time of the cessation of β-lactam treatment, parameter b represents the kinetics of decrease, exp represents the exponential function, and t represents time. Parameter a was estimated at 2.42 (95% confidence interval [CI], 1.91 to 2.93) and parameter b at 0.29 per day (95% CI, 0.21 to 0.36 per day). This yielded the average decrease curve represented in Fig. 2. Note that such a model has a null asymptote, which shows that the GM index tends to negative values (<0.5) during follow-up. A model with a residual GM index did not fit the data better ($P = 0.53$). The duration of treatment was not significantly associated with the pattern of decrease of GM ($P = 0.11$), but the times to negative antigen tended to be longer for treatments of longer duration.

From the estimated decrease model, the average time to negative antigen (at the 0.5 GM index threshold) was estimated as 5.5 days (95% CI, 4.1 to 7.0), and the average half-life of GM was estimated as 2.4 days (95% CI, 1.8 to 3.0).

Determination of GM levels and fungal DNA in antibiotic preparations. First, each antibiotic batch was tested after reconstitution at the concentration used for intravenous administration, i.e., piperacillin at 200 mg/ml, amoxicillin at 50 mg/ml, amoxicillin-clavulanic acid at 20 mg/ml, and piperacillin-tazobactam at 200 mg/ml. Sterile pyrogen-free water and 0.9% NaCl, used as diluents, were GM free.

No GM was detected in the piperacillin batches, whereas it was detected in all 10 batches of amoxicillin (median index of GM, 6.58 [4.34 to 8.11]) and amoxicillin-clavunate (median index of GM, 5.0 [1.89 to 8.09]). Seven of 10 batches of piperacillin-tazobactam tested positive, with a median GM index of 2.55 [0.67 to 7.76] (Table 4).

A calibration curve provided by Bio-Rad (France) showed that the ratio between the GM index and the purified GM levels expressed in ng/ml was close to 1 in the GM index value range of 0.5 to 2. For GM index values of up to 4, this ratio increased to 2, and for higher values it could not be reliably determined. This is probably due to the fact that Platelia *Aspergillus*, like any semiquantitative immunoassay, is subjected to saturation with coated anti-GM antibodies when large amounts of GM are present in the sample, causing a prozone effect.

Therefore, positive antibiotic batches were tested at serial twofold dilutions until a GM index of 1 ± 0.2 was obtained. Galactomannan indices remained high despite sequential dilutions of up to 1/128 and were sometimes higher on the first dilution than on the undiluted batch, possibly indicating a prozone effect at high concentration. Based on values for the dilution that resulted in the GM index closest to 1, the GM levels were estimated in ng/ml (Table 4). For amoxicillin, GM levels ranged between 8 and 4,320 ng/ml, whereas they ranged between 2 and 3,520 ng/ml for amoxicillin-clavulanic acid. Seven piperacillin-tazobactam batches that were positive when tested undiluted were positive on serial dilutions, with GM levels between 0.4 and 140.8 ng/ml. Note that one batch (A9GG/11) was close to positivity when tested undiluted (GM index = 0.45) and was found positive at 5 ng/ml when tested at a twofold dilution.

All batches (either negative or positive for GM) tested by PCR for fungal and *Aspergillus* DNA were negative. Spiking experiments with *A. fumigatus* DNA confirmed that drug solutions were not inhibitory for PCR.

DISCUSSION

The detection of serum GM using a Platelia *Aspergillus* test is now routinely done to screen neutropenic patients and allogeneic transplant recipients at risk for aspergillosis, and, when positive, results from this test are considered contributive for early diagnosis of invasive aspergillosis. In this context, the occurrence of false-positive results with the *Aspergillus* GM detection test is a major drawback of this technique, as such results may lead to unjustified invasive investigation or antifungal therapy.

Several possible causes of false-positive reactions have been suspected (2, 23, 30), among which the direct interaction of some antibiotics with a Platelia *Aspergillus* test has been recently highlighted (1, 18, 25, 28, 31). Such cross-reactivities have been found mainly for some β-lactams (2) and not for other commonly used antibiotics of fungal origin (penicillins and cephalosporins), nonfungal origin (erythromycin, gentamicin, and vancomycin), and synthetic origin (quinolones) (25, 32). For these reasons, our study focused on the two β-lactams that are the most widely prescribed for hematology patients, i.e., amoxicillin and piperacillin, either alone or combined with β-lactamase inhibitors (tazobactam and clavulanic acid). We monitored only patients treated intravenously with β-lactams, since we never observed false-positive GM test results in patients receiving oral prophylactic amoxicillin (data not shown). Although there were differences in the administered dosages of amoxicillin (3 to 6 g/day for 7 days) and piperacillin (usually 12 g/day for 7 to 14 days), we were able to analyze the kinetics of GM in a retrospective study of sequential sera from 69 patients treated intravenously with these antibiotics. We confirmed the strong and significant relationships between the administration of these drugs and the occurrence of a positive Platelia *Aspergillus* test result. Follow-up for GM after the initiation of treatment indicated that GM became detectable within 3 days in 90% of patients but could occasionally be detected very early after the antibiotic infusion. Galactomannan reached a median index of 3.65 at day 3, remained detectable throughout treatment for some patients, and then persisted after the cessation of treatment with the antibiotics. It should be underlined that none of the patients, even those with persisting

positive GM indices, developed clinical or radiological signs of IA. From our data, we were able to model the kinetics of decrease of GM and to estimate the average half-life of elimination of GM as 2.4 days until treatment with antibiotics is interrupted. The average time to negative antigen (i.e., a GM index of <0.5) was estimated to be 5.5 days. This kinetic markedly differs from the pharmacokinetics of the administered β-lactam drugs, for which the half-life does not exceed 1 h after intravenous infusion; this difference strongly suggests that the drug itself is not responsible for the false-positive reaction. These results are also in agreement with those obtained experimentally. In rabbits receiving a single 10-min intravenous infusion of piperacillin-tazobactam, GM levels rapidly increased in serum within 0.5 hour, and repeated administration over 7 days resulted in an accumulation of circulating GM, reaching a steady state after the third day (32).

Although the β-lactam molecules are probably not responsible for the false-positive reactions, our results showed that the manufactured drug suspensions do contain GM. Indeed, these semisynthetic drugs are derived from natural compounds produced by molds of the genus *Penicillium* that contain in the cell wall galactofuran-bearing molecules (30). Galactomannan or similar moieties bearing an epitope reacting with monoclonal antibody EB-A2 might be carried through the production process of these antibiotics and be preserved for months due to the high resistance of GM molecules (11).

Almost all batches of amoxicillin (alone or combined with clavulanic acid) provided to our institution between November 2003 and May 2004 were positive by the GM test. Using a limiting dilution method, the estimated amounts of GM in the preparations were found to be over a very wide range of concentrations (2 to 4,320 ng/ml). On the other hand, none of the 10 piperacillin batches were positive and only 7/10 of piperacillin-tazobactam batches were positive at low concentrations. Such marked variations between antibiotics and between batches might explain why false-positive β-lactam-related results were not reported for one center (21) during a time period in which such positivities were frequent in other countries.

The fact that all batches, either negative or positive for GM, were negative by PCR (with no PCR inhibitors in drug solutions) and the stringent microbiological tests done by drug companies can probably rule out the presence of *Aspergillus* sp. in the manufactured drugs.

Based on these results, we recommend that until a clear explanation is provided by drug companies as to the origin of GM and until proof of their clearance from marketed compounds is provided, every amoxicillin-based antibiotic or piperacillin-tazobactam batch which is intended for use in immunocompromised patients should be tested for the presence or absence of GM.

In conclusion, in the treatment of patients at risk for aspergillosis, (i) GM levels should be monitored before administration of the above-mentioned β-lactam antibiotics and (ii) results of GM determinations should be examined with extreme caution for patients treated with intravenous amoxicillin (alone or combined) or piperacillin-tazobactam. Any positive test in a patient without any sign of IA must be confirmed by a biweekly follow-up and at least 5 days after the cessation of therapy.

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