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Value of Serial Quantification of Fungal DNA by a Real-Time PCR-Based Technique for Early Diagnosis of Invasive Aspergillosis in Patients with Febrile Neutropenia▽

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A study was designed to assess the reliability of the serial detection of *Aspergillus* sp. DNA to diagnose invasive aspergillosis (IA) in patients with febrile neutropenia. Two blood and two serum samples were taken weekly from 83 patients. A total of 2,244 samples were analyzed by real-time quantitative PCR. Twelve (14.4%) patients were diagnosed with IA. Taking two consecutive positive results as the diagnostic criterion, PCR detected 11 cases, with 4 false positives, giving sensitivity, specificity, positive, and negative predictive values of 91.6%, 94.4%, 73.3%, and 98.5%, respectively. On analyzing in conjunction with high-resolution chest tomography (HRCT) and galactomannan (GM) testing, the combination of serial PCR and GM detected 100% of aspergillosis cases, with a positive predictive value of 75.1%. This diagnostic strategy presented, according to CART analysis, a receiver-operator curve with an area under the curve of 0.97 (95% confidence interval, 0.895 to 1.032; $P < 0.01$), with a relative risk of IA 6.92 times higher than the control population and with predictive success of 95.2%. As regards early diagnosis, the serial detection of *Aspergillus* DNA took on average 21 days less than HRCT and 68 days less than GM. The serial detection of *Aspergillus* DNA using real-time quantitative PCR has great diagnostic applicability, which increases when combined with GM quantification.

The diagnosis of invasive aspergillosis (IA) continues to be one of the greatest challenges with some groups of patients. In recent years, several advances have been made to improve the prognosis of this mycosis. First, patients at greatest risk of suffering from the infection have been defined so that we now know in which situations we should apply therapy strategies, such as prophylaxis, empirical treatment, or preemptive therapy (12, 15, 18, 19). In addition, the more extensive use of diagnostic imaging techniques such as high-resolution chest tomography (HRCT) and microbiological methods such as galactomannan (GM) detection have increased the possibility of the early detection of aspergillosis (13, 16, 22).

Nevertheless, mortality figures continue to be more than 50% in almost all studies published recently (19, 24). Serial imaging techniques are capable of detecting the infection only when lesions are very advanced, and moreover, these techniques may cause serious side effects associated with radiation exposure (14). Determining GM levels has proved extremely useful for oncohematological patients at high risk of aspergillosis, but it has limitations, such as false positives associated with the use of some drugs and decreased sensitivity in patients under antifungal prophylaxis (17, 22).

An increased survival rate for this type of infection appears to be associated with an early diagnosis of the infection (7, 16). 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) revised edition of the diagnostic criteria of invasive fungal infection (IFI) (5) indicates that early diagnosis of the infection may be improved if several different diagnostic techniques are combined together. With this approach, the quantification of another fungal component, the β-glucan (21), has been included as a diagnostic criterion for probable invasive fungal infections.

However, new, more-sensitive, and more-specific diagnostic techniques (6) are needed. The detection of nucleic acids appears to be an option, although to date, there are many doubts as to the profitability of this type of method (2, 9, 25).

Furthermore, the benefit of detecting the DNA of *Aspergillus fumigatus* and of other fungal species depends on the clinical sample used. PCR techniques have shown high diagnostic reliability in tissue biopsies, for instance (13, 23). In respiratory samples, they have good sensitivity, high negative predictive values, and discreet positive predictive values (PPV) (11). Nevertheless, in blood and serum samples, lower diagnostic reliability has been obtained (25, 26).

The early diagnosis of IFI requires detecting the fungus before the infection spreads. For this purpose, serial detection techniques have been developed for fungal components and used with blood or serum samples from patients at risk of mycosis (5, 7). This strategy has been little used in the detection of fungal DNA, and only some studies have analyzed serial samples of serum/blood to detect *Candida* or *Aspergillus* (1, 3, 8, 10). In general, the serial detection of *Aspergillus* DNA has

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had a low PPV (17 to 66%), which rose when two consecutive positive samples were used as the diagnostic criterion. The negative predictive value was always high, which would help to rule out the infection.

It should be pointed out that these studies collected a limited number of samples from each patient and only shortly before the diagnosis of IA, which therefore limits their analysis as an early diagnostic technique. In general, the authors gave complementary value to the detection of fungal DNA, since this could assist the early detection of the infection in conjunction with detection by GM and HRCT (8, 10).

In this study, quantitative serial determinations of *A. fumigatus* DNA were undertaken in patients with febrile neutropenia at risk of IA. Samples of blood and serum were taken weekly. The results were compared with those obtained with radiology techniques and GM quantification.

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MATERIALS AND METHODS

Patients and samples. A total of 83 patients with febrile neutropenia (<500 neutrophils/mm^3 and a temperature of >38°C) considered at risk from IA were studied prospectively between October 2004 and November 2005 at the Hospital Universitario 12 de Octubre in Madrid, Spain. Their demographic and clinical data were collected, as well as results from radiology studies (HRCT), microbiological cultures, and GM testing. Four weekly samples (two blood and two serum) were also taken during episodes of febrile neutropenia. A total of 2,244 samples (1,122 blood and 1,122 serum) were collected and sent to the Mycology Department of the Spanish National Center for Microbiology at the Instituto de Salud Carlos III for real-time PCR.

Quantitative real-time PCR (RT-PCR). A pair of primers and a molecular beacon-type probe specific to *A. fumigatus* were designed and directed at ribosome DNA, specifically internal transcribed spacer region 1 (ITS1). For the design of the primers and the probe, sequences of the ITS regions of ribosome DNA from 24 strains of *A. fumigatus* available from the collection of the Mycology Department were analyzed. The primers and probe were designed with the help of the probe design program Beacon Design 5.0 (Premier Biosoft, Palo Alto, CA). The direct primer was OliAFMB1 5′-TCCCACCCGTGTCTATCG-3′, and the reverse was OliAFMB2 5′-GAACCAAGAGATCCGTTGTTG-3′. The molecular beacon probe was marked at the 5′ end with the 6-carboxyfluorescein fluorophore and at the 3′ end with the BHQ1 quencher, its sequence being 5′-FAM CGCGATCGCCGAAGACCCCAACATGAACGCTGAT CGCG BHQ1-3′.

A study was done to establish the specificity of the sequences chosen for the primers and in the probe. For the specificity study, DNA from strains belonging to other species of the genus *Aspergillus*, as well as strains belonging to 25 other species of filamentous fungi which cause IFIs, was used. Also included in the experiments were human and mouse genomic DNA (Promega, Madrid, Spain). After designing the primers and probes, a BLAST type search was undertaken in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to ensure that the probes designed were not homologous with other microorganisms. The specificity of the probe and primers designed using phylogenetic analysis of their sequences was also verified. For these analyses, we used the sequence database at the Mycology Department, which holds 4,000 strains belonging to 270 different fungal species, and the Fingerprinting II Informatix program, version 3.0 (Bio-Rad, Madrid, Spain). This database has been designed by the Spanish National Center for Microbiology and has restricted access.

DNA was extracted from the samples using the QiampDNA mini kit (Qiagen, Izasa, Madrid, Spain). Two microliters of DNA from each sample was used for each RT-PCR, which contained a final volume of 20 μl with 3 mM of Cl_2Mg, 0.5 μM from each primer, and 0.4 μM of molecular beacon probe. Preincubation was at 95°C, followed by 45 denaturation cycles (15 s at 95°C), annealing (30 s at 56°C), and extension (5 s at 72°C).

Each experiment was run twice and included negative controls, PCR inhibiting controls, and one positive control (27). For the positive control, DNA from the *A. fumigatus* CNM-CM-237 strain (collection of filamentous fungi from the Mycology Department of the Spanish National Center for Microbiology) was used.

The results were considered positive when an exponential increase in fluorescence was detected compared with that of the negative controls before cycle 40 of amplification. The detection limit was 10 fg of DNA per μl of sample (cycle 42 of amplification).

In cases where a positive result was obtained, the presence of a 185-bp amplicon was verified using electrophoresis with a 2% agarose gel. The amplified fragments were sequenced in the ABI Prism 377 DNA sequencer system (Applied Biosystems, Madrid, Spain), and their sequences were compared with databases available in the lab to ensure that they were *A. fumigatus*.

Amplified DNA was quantified by linear regression with the results of five repetitions of different dilutions of *A. fumigatus* CNM-CM-237 DNA (from 10 ng to 1 fg DNA/μl). Regression lines were made between the logarithms of DNA concentrations and the PCR cycle in which we began to detect fluorescence (threshold cycle [C_T]). The regression line constructed between the known concentrations of DNA and the C_T had a coefficient of determination (r^2) of 0.99 ($P < 0.01$). The reproducibility of the technique was very high, as the mean coefficient of variation was 2.8%.

Analysis of the results. The information collected on each patient, as well as the PCR results, was entered in a database (SPSS 15.0, SPSS Ibérica S.L., Madrid, Spain). The definitions of proven, probable, and possible IA were set according to the definitions of the EORTC/MSG published in 2008 (5).

We assessed the sensitivity, specificity, and predictive values of the PCR technique first with one blood or serum sample positive result, then with two or more consecutive blood or serum sample positive results in less than 1 week, and finally with three or more consecutive blood or serum sample positive results in less than 12 days. The percentages of true-positive, false-positive, and false-negative results were calculated with each of the above-mentioned criteria. For patients with positive PCR results, the amount of DNA in each positive sample was quantified in order to analyze the kinetics of the nucleic acid and find possible relationships between the C_T and the clinical evolution of the patients.

The general performance of the diagnostic test was assessed by considering the number of correctly and incorrectly classified cases in the PCR testing set. The performance (positive diagnostic likelihood ratio) of the test expressed by its true-positive rate (sensitivity) and false-positive rate (1 − specificity) was plotted in a receiver-operator curve (ROC) space. A P value of <0.01 was considered to have statistical significance.

In order to know the reliability of the diagnostic tools, a data-mining type analysis was run using the software CART for Windows version 6.0 (Correlation and Regression Trees; Salford Systems, San Diego, CA). CART is a robust decision-tree tool for data mining.

Several variables were included in the model. The target variable was the presence or absence of IA with equal priority, including proven, probable, and possible cases. Predictor variables were a PCR positive result one or more times, a PCR positive result two or more times, a PCR positive result three or more times, and the results of the GM test. The method for testing the tree was V cross-validation with 10-fold cross-validation. The selection of the best tree was performed by means of a minimum-cost tree regardless of the size. The splitting method for the tree was the Gini algorithm.

RESULTS

Patients. The underlying diseases of the 83 patients included in the study were non-Hodgkin's lymphoma (28), acute myeloblastic leukemia (21), acute lymphoblastic leukemia (ALL) (10), chronic lymphatic leukemia (7), multiple myeloma (7), Hodgkin's disease (6), myelodysplasic syndrome (3), and medullary aplasia (1). The mean age of these patients was 52 years; 48 were men and 35 were women.

A total of 12 patients, 7 men and 5 women, were diagnosed with IA according to the EORTC/MSG 2008 criteria (5), which gave a prevalence of 14.4%. One IA was classified as proven, nine as probable, and two as possible. Table 1 gives the key clinical data for patients diagnosed with IA, as well as the results of the HRCT and the GM determinations. The most-frequent underlying diseases were acute myeloblastic leukemia and ALL, with six (50%) and three (25%) cases, respectively. Ten patients (83%) received itraconazole prophylaxis. Three

TABLE 1. Demographic, radiological, and microbiological data of patients suffering from aspergillosis

Patient	Gender	Underlying condition[a]	Aspergillosis diagnosis	Prophylaxis	HRCT	GM	PCR result[e]	Days before HRCT[f]	Days before GM[g]
1	Male	ALL	Proven[b]	Fluconazole/itraconazole	Positive	Positive	Positive	67	65
2	Male	NHL	Probable	None	Positive	Positive	Positive	0	0
3	Female	AML	Probable	Itraconazole	Positive	Positive	Positive	−27	−28
4	Female	AML	Probable	Itraconazole	Positive	Positive	Positive	61	199
5	Female	MM	Probable	Fluconazole	Negative[c]	Positive	Positive	0	0
6	Male	AML	Probable	Itraconazole	Positive	Positive	Positive	1	37
7	Male	ALL	Probable	Itraconazole	Positive	Negative[d]	Positive	84	79
8	Female	AML	Probable	Itraconazole	Positive	Positive	Positive	19	107
9	Male	ALL	Probable	Itraconazole	Positive	Positive	Positive	90	154
10	Female	AML	Probable	Itraconazole	Positive	Positive	Negative	NC[h]	NC
11	Male	MDS	Possible	Itraconazole	Positive	Negative	Positive	−69	NC
12	Male	AML	Possible	Itraconazole	Positive	Negative	Positive	8	NC

[a] ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin's lymphoma; AML, acute myeloblastic leukemia; MM, multiple myeloma; MDS, myelodysplasic syndrome.
[b] Proven aspergillosis by positive culture from tissue biopsies.
[c] Patient was diagnosed with sinonasal aspergillosis after imaging showing sinusitis.
[d] Patient was diagnosed by sputum culture with *A. fumigatus*.
[e] The table includes analysis taking into account only two or more positive PCR results.
[f] Time in days taken by the PCR-based technique to detect *Aspergillus* DNA before signs appeared on HRCT; a minus sign indicates that fungal DNA was detected later than signs on the HRCT.
[g] Time in days taken by the PCR-based technique to detect *Aspergillus* DNA before the GM test was positive; a minus sign indicates that fungal DNA was detected after the GM test was positive. For patient 7, the calculation was made using the date the sputum culture was positive.
[h] NC, not calculated. Values were not calculated because the test was negative.

patients (25%) underwent an allogeneic hematopoietic stem cell transplant. Eleven patients presented radiological signs suggestive of fungal infection in the thorax HRCT and one had radiological signs of sinonasal infection. Nine individuals (75%) had positive GM tests.

In addition, two more patients were diagnosed with IFI; one patient suffered from disseminated candidiasis, and another was diagnosed with zygomycosis.

Results of the PCR technique. Table 2 shows the results of the PCR technique. The analysis was done considering 1, ≥2, and ≥3 positive results. With both one and two positive results, PCR detected 11 out of the 12 cases of IA (91.6%). With two positive results, four false positives were obtained, but with one single positive PCR result, 14 false positives were observed, giving a PPV of 43.9%. It is of note that two out of the four false positives with two positive PCR results also tested positive for GM. These two patients presented multiple myeloma and ALL as underlying diseases and did not show any symptoms or clinical signs compatible with IFI, so they were thus considered false positives.

The analysis with three or more positive PCR results showed a drop in sensitivity, since it detected only 75% of cases. With three positive results, the number of false positives dropped to two. One of these cases was one of the two patients commented on above who was positive for GM.

It should be noted that all the samples analyzed from the patient with candidiasis and the patient with zygomycosis were negative for the *Aspergillus* PCR technique.

The study of correlation between DNA quantification and patients' clinical symptoms found no significant relationships between the C_T and the appearance of signs of IA. That is to say, no cutoff was found in the RT-PCR cycles which would enable us to diagnose the infection with greater reliability or distinguish between false and true positives.

On analyzing the results per type of sample (blood or serum), no significant differences were found, and the two types proved to be of similar diagnostic usefulness.

CART analysis. A CART analysis was done, including the most-relevant clinical and microbiological variables, in order to establish the predictive ability of the PCR technique with 1, ≥2, and ≥3 positive results.

To assess the diagnostic capability of the PCR technique, ROC curves were produced, representing true- and false-positive results and calculating the area under the curve (AUC). Table 3 and Figure 1 show the AUCs and 95% confidence intervals (CI) for each of the diagnostic criteria. With one, two,

TABLE 2. Results of the PCR-based technique

No. of positive PCR results	No. of IA cases detected/ total no. with indicated result		Sensitivity (%)	NPV (%)[a]	No. of IA cases detected/ total no. with indicated result		Specificity (%)	PPV (%)
	True positive	False negative			True negative	False positive		
1	11/12	1/12	91.6	98.3	57/71	14/71	80.3	43.9
2	11/12	1/12	91.6	98.5	67/71	4/71	94.4	73.3
3	9/12	3/12	75.0	95.8	69/71	2/71	97.2	81.8

[a] NPV, negative predictive value.

TABLE 3. ROC spaces and statistical data produced by results obtained with the PCR-based technique and the GM test

Diagnostic criterion	AUC	95% CI	*P* value
1 positive PCR result	0.860	0.752–0.967	<0.01
2 or more positive PCR results	0.930	0.834–1.026	<0.01
3 or more positive PCR results	0.861	0.712–1.010	<0.01
Positive GM test	0.861	0.712–1.010	<0.01

and three positive PCR results and with the GM technique, the ROC curves showed very good results, with AUCs close to 1 and statistically significant. The best AUC value was obtained in the case of the PCR technique with two or more positive results, with an AUC of 0.930 (95% CI, 0.834 to 1.026; $P < 0.01$).

After obtaining the ROC curves, a data-mining analysis was done with the CART program. In this analysis, the variable which predicted the presence of IA with greatest probability was the PCR diagnostic technique when two or more positive results in less than 1 week were used as the diagnostic criterion. With this criterion, the relative risk of IA increased 5.04 times and the predictive success of the resulting algorithm stood at 93.98%. The relative risks and predictive successes of the other three criteria (one and three positive PCR and GM results) were also high, although lower than those found with two positive results.

A CART analysis was also undertaken with the aim of discovering which combination of diagnostic tests could be the most efficient in detecting aspergillosis. Figure 2 shows the algorithm which best predicts the presence of IA in patients included in the study, which was the combination of GM and two positive PCR results. This algorithm presented an AUC of 0.97 (95% CI, 0.895 to 1.032; $P < 0.01$), with a relative risk of aspergillosis 6.92 times higher than that of the control population and a predictive success of 95.2%. The combination of two positive PCR results plus GM quantification detected 100% of cases of aspergillosis, with a PPV of 75.1%.

Analysis of early diagnosis capability. Excluding patient 10, for whom the PCR result was negative, it was possible to calculate the time gain in diagnosis for the PCR technique compared to those for HRCT and GM for the other 11 patients with IA (Table 1). In two patients, fungal DNA detection occurred at the same time as the observation of disease in HRCT images. In another two cases, HRCT detected the infection before the PCR technique. In the other seven cases, the detection of fungal DNA came earlier than that seen with HRCT, with a mean diagnosis time gain of 21 days (1 to 84 days).

In the case of GM detection, the early diagnosis study was run on nine patients (Table 1). For two patients, fungal DNA and GM detection occurred at the same time. These two cases were the ones in which disease was detected at the same time in the HRCT images. In one case, the quantification of GM preceded the detection of *Aspergillus* DNA, but in six patients, DNA detection preceded that of GM detection, with a mean advance diagnosis time of 68 days (37 to 199 days).

DISCUSSION

The diagnosis of IA and other IFIs should be based on the use of several different techniques which would enable early detection of the infection. The combination of HRCT and the quantification of fungal antigens has improved the prognosis of these patients, although we are still far from resolving the problem (7, 16).

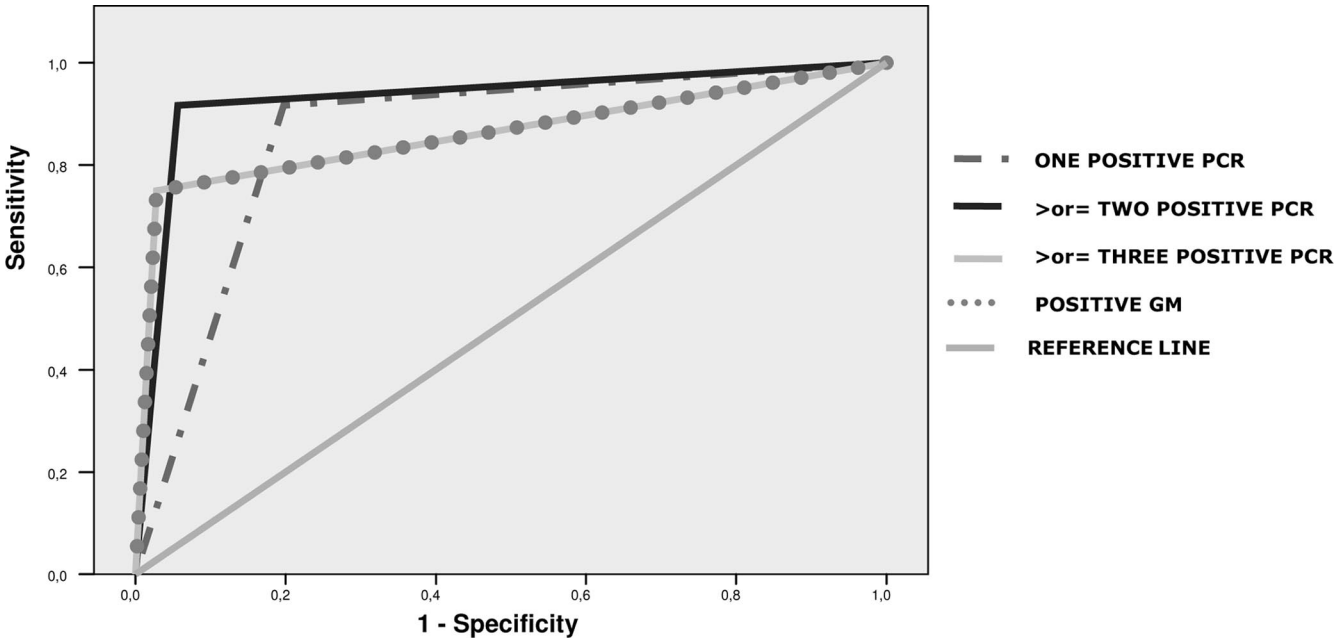

FIG. 1. ROC spaces produced by the results obtained with the PCR-based technique and the GM test. ROC spaces for three or more positive PCR results and GM results were of equal area.

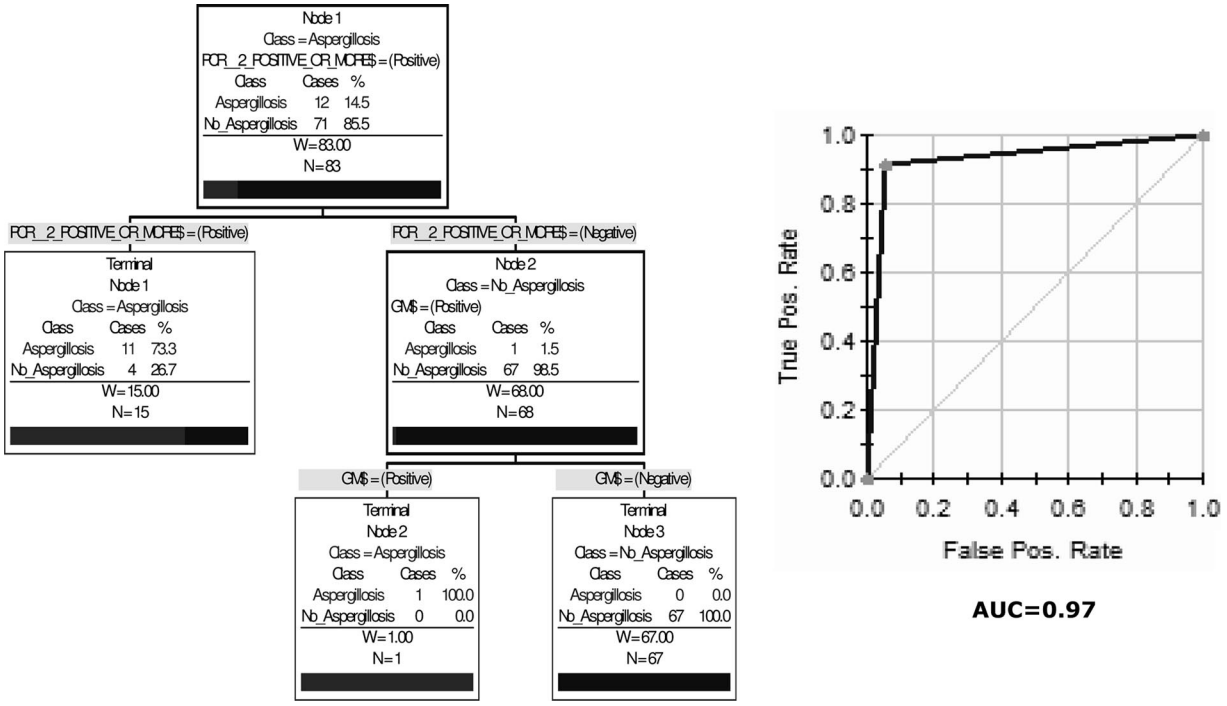

FIG. 2. Model obtained by means of CART analysis with two or more positive PCR results and GM results.

All these diagnostic methods are based on serial results which provide a dynamic view of patient evolution and, in theory, detect the infection in its very early stages. Nevertheless, these methods present some limitations which must be noted. Serial HRCTs produce levels of radiation which could be considered unacceptable (14). Moreover, they show low specificity, since other pulmonary diseases may develop with the same type of lesion. Lastly, they do not identify the fungus which causes the infection (4). Antigen quantification has also helped to improve the diagnosis of IA; however, false negatives observed in patients under treatment and false positives due to contamination or drugs have undermined diagnostic reliability (17, 22).

The serial detection of fungal DNA is a diagnostic strategy which has been assessed with somewhat irregular results, as indicated earlier (3, 8, 10). This is one of the most extensive studies on serial detection of *A. fumigatus* DNA. The mean number of samples available per patient was 27, some of which were obtained months before the development of IA.

With this amount of samples, several different analyses could be done. First, taking two consecutive positive samples as the criterion for defining a positive PCR result, 11 out of the 12 cases of aspergillosis (91.6%) were detected, producing only four false positives. With these figures, the PPV was 73.3%, enabling us to establish that the serial detection of fungal DNA should be considered as a reliable diagnostic technique, with accuracy percentages comparable to those for HRCT or fungal antigen detection (7, 13, 16, 21).

The quantification of fungal DNA using quantitative RT-PCR did not serve to establish cutoffs which would distinguish between patients colonized or infected or between false- or true-positive results. This is due to the fact that both in serum and blood, the quantity of fungal DNA was small and the positive samples had C_T between 35 and 40 cycles, with mean fungal DNA levels of 10 to 500 fg per μl of sample. This low quantity of DNA would lead us to speculate over the need to develop more-sensitive quantitative PCR techniques and over the fact that the low sensitivity of a number of previous studies which evaluated these techniques may be due to the small quantity of fungal DNA in blood samples (3).

Another aspect to emphasize is the confirmation that quantitative PCR as a technique may play an additional role to that of radiology and GM testing. The CART analysis confirmed that with two consecutive positive PCR results, plus positive GM quantification, the relative risk of IA was 6.92 times higher than that of the control population and that the predictive success of the algorithm diagnosis was 95.2%. These figures show that it is recommendable that both techniques be used for patients with a high risk of developing IA.

To conclude, one of the most significant results was the earliness of diagnosis. Due to the availability of such a large number of samples, in some cases taken several weeks before the appearance of IA, we were able to analyze the time to diagnose the infection. It should be noted that fungal DNA was detected much earlier than GM in patients with aspergillosis and that HRCT detected the lesions compatible with IFI. The mean time gain was 21 days compared with HRCT and 68 compared with GM.

It is difficult to find an explanation for this result, since little is known of the kinetics of fungal components. A study published in 2006 (20) analyzed the kinetics of the markers used in vitro for the diagnosis of IA. The authors showed that GM and other fungal antigens are released when *Aspergillus* is found in exponential growth phase, while fungal DNA is released when the hyphae break up, a phenomenon which occurs naturally by

autolysis when the amount of nutrients is limited or when antifungal agents are present.

Bearing these data in mind, our study appears to indicate that the majority of patients who develop IA could have been colonized earlier by *Aspergillus*. Prophylaxis with itraconazole may have limited the exponential growth of the fungus, decreasing the amount of GM (17) until the infection had spread sufficiently to render the prophylactic treatment ineffective. Up to that moment, the only data on colonization were DNAemia detected by PCR, presumably caused by the periodic fragmentation of the hyphae. Moreover, the two patients for which the infection was detected by PCR at the same time as radiology or GM testing were the only two patients who were not on itraconazole prophylaxis. This result could strengthen our hypothesis that increased autolysis after the introduction of the antifungal agent leads to increased DNA release.

This fact needs be confirmed by a study with a greater number of patients. In any case, the serial detection of *Aspergillus* DNA has shown itself to be of great diagnostic use, which further increases when used in combination with HRCT and GM quantification. The real clinical usefulness of this diagnostic strategy should be analyzed in a new study to show that early diagnosis and treatment at an earlier stage are associated with a significant increase in the survival of these patients.

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