

Detection of *Aspergillus fumigatus* in a Rat Model of Invasive Pulmonary Aspergillosis by Real-Time Nucleic Acid Sequence-Based Amplification[∇]

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Rapid and sensitive detection of *Aspergillus* from clinical samples may facilitate the early diagnosis of invasive pulmonary aspergillosis (IPA). A real-time nucleic acid sequence-based amplification (NASBA) method was investigated by use of an inhalational rat model of IPA. Immunosuppressed male Sprague-Dawley rats were exposed to *Aspergillus fumigatus* spores for an hour in an aerosol chamber. Bronchoalveolar lavage (BAL) fluid, lung tissues, and whole blood were collected from five infected rats at 1, 24, 48, 72, and 96 h postinfection and five uninfected rats at the end of the experiment. Total nucleic acid (TNA) was extracted on an easyMAG instrument. A primer-molecular beacon set targeting 28S rRNA was designed to detect *Aspergillus* spp. The results were compared to those of quantitative PCR (qPCR) (18S rDNA) and quantitative culture. The analytical sensitivity of the real-time NASBA assay was <1 CFU/assay. A linear range of detection was demonstrated over 5 log units of conidia (10 to 10⁵ spores). Both NASBA and qPCR showed a progressive increase in lung tissue burdens, while the CFU counts were stable over time. The fungal burdens in BAL fluid were more variable and not indicative of a progressive infection. The results of both real-time assays correlated well for both sample types ($r = 0.869$ and $P < 0.0001$ for lung tissue, $r = 0.887$ and $P < 0.0001$ for BAL fluid). For all whole-blood specimens, NASBA identified *Aspergillus*-positive samples in the group from which samples were collected at 72 h postinfection (three of five samples) and the group from which samples were collected at 96 h postinfection (five of five samples), but no positive results were obtained by culture or PCR. Real-time NASBA is highly sensitive and useful for the detection of *Aspergillus* in an experimental model of IPA.

Invasive pulmonary aspergillosis (IPA) is a major cause of morbidity and mortality in immunocompromised patients. Therapeutic success depends on an early diagnosis and the early initiation of antifungal therapy (5). The rapid and sensitive detection of *Aspergillus* from clinical samples may facilitate the early diagnosis of IPA. Molecular diagnostics have emerged as promising methods for the diagnosis of a variety of infectious diseases, including fungal infections (22). Given the advantages of detection sensitivity and speed, real-time quantitative PCR (qPCR) has been extensively studied and explored as a tool for use for the detection and identification of *Aspergillus* and other pathogenic fungi in clinical samples (9–12, 31). However, a lack of standardization often makes it difficult to compare results between laboratories, which is critical for the ultimate approval of a test as a standard diagnostic test for IPA (2).

Nucleic acid sequence-based amplification (NASBA) is an isothermal amplification process that specifically amplifies RNA even in the presence of genomic DNA (30). The amplification is highly robust, yielding >10¹² amplicons in 30 min (4). The amplified single-stranded RNA can easily be detected in real time by the use of molecular beacon (MB) probes, which are self-reporting, hairpin-structured, single-stranded

nucleic acid (NA) probes that brightly fluoresce when they are bound to their targets (33). NASBA-based assays for the detection of *Aspergillus* have been described by a few study groups (14, 36). However, to our knowledge, real-time NASBA has not been applied to the detection of *Aspergillus fumigatus* from an animal model of IPA.

The object of the study described here was to evaluate a highly sensitive real-time NASBA assay for its ability to detect *Aspergillus* burdens in an inhalational rat model of IPA at selected time points in the course of infection and to compare the diagnostic yield with the yields obtained by culture and qPCR.

MATERIALS AND METHODS

Animal model. Thirty male Sprague-Dawley rats (Charles River, Margate, United Kingdom) weighing 200 to 250 g were used in the experiment, after being allowed 7 days to acclimatize. The rats were housed in cages provided with HEPA filters and were allowed free access to food and water. The rats were immunosuppressed with cyclophosphamide (75 mg/kg of body weight intraperitoneally twice) plus prednisolone (Depo-medrone; 16 mg/kg intramuscularly once) 2 days before infection. The animals received enrofloxacin (Baytril; 10 mg/kg subcutaneously daily) from day 2 preinfection to prevent secondary bacterial infections. A frozen glycerol stock of *A. fumigatus* A1163 was cultured into Sabouraud dextrose agar (SAB) for 10 days. The spores were harvested by the use of phosphate-buffered saline (PBS) plus 0.05% Tween 80 and counted with a hemocytometer. The rats were infected with 4×10^8 /ml spores in an inhalation acrylic chamber in a class II hood for an hour (28). The rats ($n = 5$) were checked for symptoms such as body weight loss, dyspnea, and lethargy and were euthanized at 1 h, 24 h, 48 h, 72 h, and 96 h postinfection. Five uninfected rats were euthanized at 96 h after the inoculation. Bronchoalveolar lavage (BAL) fluid and lung tissue were collected from the euthanized rats. Whole-blood samples were

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also collected from the rats at 48 h, 72 h, and 96 h postinfection as well as from uninfected rats.

***Aspergillus fumigatus* burden assessment.** One gram of lung tissue was homogenized with 2 ml of sterile PBS. Tenfold dilutions of lung tissue homogenates were plated onto SAB plates, and the plates were incubated at 37°C for 24 to 36 h, after which the *A. fumigatus* burdens (CFU per gram of lung tissue) were determined. The primary lung tissue homogenates were spun down, and the pellets were resuspended in 1 ml of RNAlater RNA stabilization reagent and stored at -20°C until NA extraction for real-time detection. The fungal burdens in the BAL fluid samples were also determined by CFU counting. One milliliter of BAL fluid was mixed with 2 ml of the RNAlater reagent and stored at -20°C until NA extraction. qPCR and real-time NASBA of lung tissue homogenates, BAL fluid, and blood samples were performed; and the fungal burden in each sample was calculated on the basis of the standard curve included in each run.

TNA extraction. Frozen lung tissue homogenates (1 g lung tissue per sample) in RNAlater reagent were thawed at room temperature before extraction was performed. Following centrifugation at 16,000 × g for 10 min, the supernatant was discarded and the pellet was resuspended in 1 ml of NucliSENS lysis buffer (bioMérieux, Durham, NC) with proteinase K (100 µg/ml). The entire solution was transferred to a tube with lysing matrix D (containing 1.4 mm ceramic spheres) (MP, Biomedicals, Inc., Solon, OH) and processed in a FastPrep instrument (MP, Biomedicals, Inc.) at speed level 6 twice for 45 s each time. After 1 h of incubation at 55°C, the cell lysates were centrifuged at 16,000 × g for 10 min. The supernatant was transferred and mixed with 2 ml of NucliSENS lysis buffer in the NucliSENS easyMAG sample cartridge (bioMérieux) for the automatic total nucleic acid (TNA) extraction. Magnetic silica solution was added, and the extraction was performed according to the easyMAG user manual. The final elution volume was 110 µl. Sterile PBS was processed in parallel as the negative extraction control.

Frozen BAL fluid samples (1 ml BAL fluid plus 2 ml RNAlater reagent) were thawed at room temperature and centrifuged at 16,000 × g for 10 min. A 1-ml mixture of NucliSENS lysis buffer with proteinase K was added to resuspend the pellet, and then the entire resuspension was transferred into the lysing matrix tube and the tube was vigorously vortexed in the FastPrep instrument, as described above. The following TNA extraction process was the same as that for lung tissue samples, and the final elution volume was 60 µl.

One milliliter whole blood was immediately treated with an equal volume of in-house nucleus lysis buffer (containing 1% Triton X-100, 0.01 M Tris, pH 7.6, 0.005 M MgCl₂, and 0.32 M sucrose) and centrifuged at 16,000 × g for 10 min. The supernatant was discarded and another 1 ml of nucleus lysis buffer was added without disturbing the pellet. After the second centrifugation at 16,000 × g for 10 min, the supernatant was discarded and the pellet was resuspended in 1 ml of NucliSENS lysis buffer with proteinase K (100 µg/ml). The specimen was transferred to a lysing matrix tube for mechanical disruption, and automatic TNA extraction was performed as stated above. The extracts were eluted in 50 µl of elution buffer.

For external standardization, TNAs from titrated *A. fumigatus* conidia (5 × 10⁶ to 5 × 10⁹ spores per ml) were extracted in parallel. A 200-µl aliquot of each dilution was mixed with 800 µl of lysis buffer (with 100 µg/ml of proteinase K) for the extraction, and the final elution volume was 50 µl.

Quantitative real-time NASBA assay. A real-time NASBA assay targeting 28S rRNA, the so-called pan-*Aspergillus* NASBA, was developed for the detection of *Aspergillus* spp. The primers and MB probe were designed as described in a previously published report (37). The P1 primer was 5'-AATTCTAATACGACTCACTATAGGGGAGAATCCACATCCAGGTGC-3', and the P2 primer was 5'-CAGCAGTTGGACATGGGTTA-3'. The MB probe was 5'-CGCGAGTGC GCCGTGTGCCGAAATCGCG-3' and was labeled with 5-carbofluorescein (FAM) fluorophores at the 5' end and 4-(4-dimethylaminophenyl)diazenylbenzoic acid (DABCYL) quencher at the 3' end.

The NASBA reactions were performed with a NucliSENS basic kit (version 2; bioMérieux). Each reaction mixture consisted of 0.2 µM each primer, 0.1 µM MB probe, 5 µl of TNA template, and 5 µl of NASBA enzyme mixture (T7 RNA polymerase, avian myeloblastosis virus reverse transcriptase, RNase H, bovine serum albumin) in a total volume of 20 µl. The enzyme mixture was added after the rest of the reaction mixture was incubated at 65°C for 2 min and 41°C for another 2 min, and then the reaction was run at 41°C for 90 min on a NucliSENS EasyQ analyzer (bioMérieux). The fluorescence signal was measured at time intervals of 30 s for each independent reaction at two wavelengths by using the accompanying NucliSENS EasyQ Director software (version 2).

The specificity of the real-time NASBA assay was tested as described before (37). Briefly, total nucleic acids from *Aspergillus* spp. (*A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*) as well as other fungal species (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, *Candida guil-*

liermundii, *Candida dubliniensis*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Fusarium* spp.) and bacteria (*Escherichia coli*, *Staphylococcus aureus*) were tested in parallel for cross-reactivity by the pan-*Aspergillus* NASBA assay. Nucleic acid-free water was also included as the no-template control (NTC). The extraction of TNA from the culture was performed as described previously (37).

The analytical sensitivity of the pan-*Aspergillus* NASBA assay was assessed by using triplicate TNA samples extracted from 10-fold serially titrated *A. fumigatus* conidia (10⁶ to 10⁹ spores). The time to positivity (TTP), which is the time at which the fluorescence signal rises above the cutoff value (20% higher than the end-point signal from NTC), was determined, as well as linearity between TTP and the log input of conidia. These TNA samples were also used as external standards in each run. The conidial equivalents (CEs) in the rat samples were calculated by comparing the TTP of the sample with that of the external standards.

Real-time PCR. An *Aspergillus* genus-specific real-time PCR assay was performed in this study so that the results could be compared with those of the NASBA assay. The primers and probe were modified from those used in a previous study (14) and targeted the 18S rRNA gene. The sequences were as follows: forward primer, 5'-GCCGCGGTAATCCAGCTCCAATA-3'; reverse primer, 5'-GAGCAAAGGCCTGCTTTGAACA-3'; MB probe, 5'-CGCACGG GTCCGCCTCACCGGAGTACTGCGTGCG-3'. The PCR master mixture contained 0.2 µM each primer, 0.2 µM MB probe, 2 µl of TNA, and 12.5 µl of 2× Brilliant II qPCR master mixture (Stratagene) in a total reaction volume of 25 µl. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The same standard TNAs used in the NASBA assay were run in conjunction with each set of samples to quantify the *Aspergillus* burden.

Statistical analysis. The *Aspergillus* burdens quantified by the three different methods were analyzed by the use of SPSS (version 11.0) software (SPSS Inc., Chicago, IL). The multiple comparison among samples collected at different times postinfection was performed by two-way analysis of variance on the basis of the sample types. The relationship between the results of qPCR and those of the real-time NASBA assay were analyzed by using Spearman's correlation coefficient. The level of statistical significance was set at a *P* value of <0.05.

RESULTS

Specificity, sensitivity, and linear range of real-time NASBA assay. The pan-*Aspergillus* real-time NASBA assay was designed to detect *Aspergillus* spp. and showed a high degree of specificity (>95%). However, because *Penicillium chrysogenum* is 99% homologous to *Aspergillus* spp. in the amplification region, the cross-reaction from this species would be expected. Other species, such as the AIDS pathogen *Penicillium marneffei* (35), have the potential to cross-react. The NASBA assay did not cross-react with the other fungal species or the bacterial species tested in this study. The lowest detection limit of 0.1 CFU per reaction, corresponding to 1 CFU/ml of conidial suspension, was observed in 6 of 10 runs, and the level of 1 CFU per reaction was consistently detected by the pan-*Aspergillus* NASBA assay. The linear range of detection was shown to be from 10 to 10⁵ *Aspergillus* conidia (Fig. 1A). Given that only 5 µl of TNAs was used for the NASBA assay, the detection limits were 22 CFU per assay for lung tissue, 12 CFU for BAL fluid, and 10 CFU for whole blood. Similarly, the lower quantification limits were 220 CFU for lung tissue, 120 CFU for BAL fluid, and 100 CFU for whole blood.

Reproducibility of real-time NASBA assay. To assess the reproducibility of the pan-*Aspergillus* NASBA assay, 5 log units of TNAs (10 to 10⁵ conidia) were tested in triplicate. The coefficients of variation (CVs) of the TTP values for 10⁵ to 10 conidia were 2.54, 1.96, 0.90, 1.43, and 2.80%, respectively.

Linearity of qPCR assay. Because the qPCR assay was modified from the *Aspergillus* NASBA detection assay reported by Loeffler et al. (14), the analytical sensitivity and the linear detection range were reassessed by using TNA extracts from 1

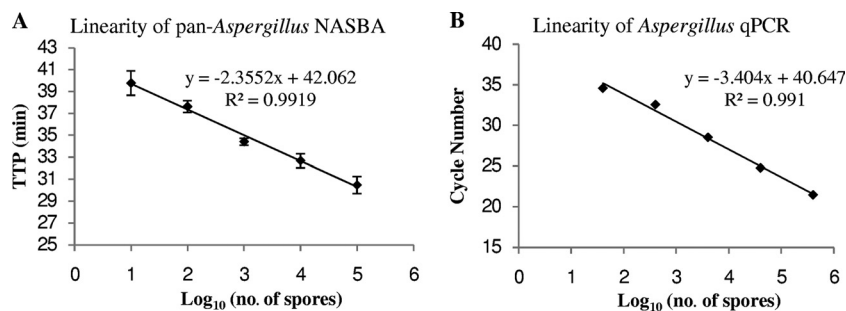


FIG. 1. (A) Linearity of the pan-*Aspergillus* NASBA assay determined by triplicate amplification of total nucleic acids extracted from serially diluted *A. fumigatus* conidia with 10^5 to 10^1 spores. (B) Linearity of *Aspergillus* qPCR assay. The same TNA series used for the experiment whose results are presented in panel A were used to generate the standard curve.

to 10^6 spores of *A. fumigatus* conidia. The lower detection limit of 4 CFU was achieved in 5 of 10 repeated runs, while 40 CFU was consistently achievable in all runs. The linear range was from 40 to 4×10^5 CFU (Fig. 1B). The calculated PCR efficiency was 1.96. Again, considering that the portion of TNAs taken for the test varied, we calculated the lower limit of quantification to be 2,200 CFU for lung tissue, 1,200 CFU for BAL fluid, and 1,000 CFU for whole blood.

Detection of *A. fumigatus* in lung tissue. At 1 h postinfection, the pulmonary fungal burden counts were very high by quantitative culture ($5.17 \pm 0.25 \log_{10}$ CFU/g), reflecting the presence of conidia, whereas the burden obtained by qPCR was lower ($3.07 \pm 0.25 \log_{10}$ CE/g) and no positive signal was obtained by the NASBA assay (Table 1). At 24 h postinfection, the qPCR-based *Aspergillus* burden counts were close to those obtained by quantitative culture ($3.50 \pm 0.39 \log_{10}$ CE/g versus $3.80 \pm 0.44 \log_{10}$ CFU/g), while the NASBA-based burden counts ($2.87 \pm 0.61 \log_{10}$ CE/g) were still lower than the measurements obtained by the other two assays. After 24 h postinfection, the CFU counts stayed relatively stable throughout the experiment. In contrast, both qPCR and the real-time NASBA assays demonstrated progressive increases in the *Aspergillus* burden over the time of infection (by linear regression analysis, $F = 40.66$ and $P < 0.0001$ for qPCR measurements

and $F = 141.10$ and $P < 0.0001$ for real-time NASBA measurements) (Fig. 2), and the results from both real-time platforms correlated well ($r = 0.869$, Spearman's correlation; $P < 0.0001$) (Fig. 3A). All lung tissue samples from the uninfected control group were negative by the qPCR and NASBA assay and by CFU counting.

Detection of *A. fumigatus* in BAL fluid. Real-time NASBA was more sensitive than culture in detecting the *Aspergillus* burden in BAL fluid. It detected *A. fumigatus* in 22 of 25 (88%) BAL fluid specimens from rats with experimental IPA, which was significantly more than the number detected by culture (13 of 25 [52%]) (Fisher's exact test, $P = 0.0121$). The PCR assay detected *Aspergillus* in 16 of 25 (64%) positive BAL fluid samples, but there was no significant difference in the rate of detection compared to the rates by the other two methods, possibly due to the small sample size. At 1 h postinfection, quantitative culture found all five samples to be positive and to have mean counts of $3.20 \pm 0.51 \log_{10}$ CFU/ml; in the meantime, only two and three of five samples were positive for *Aspergillus* by PCR ($2.66 \pm 0.47 \log_{10}$ CE/ml) and NASBA ($2.48 \pm 0.17 \log_{10}$ CE/ml), respectively. With the progression of the infection, the real-time assays became more sensitive than culture (Table 1), especially at 72 h postinfection, when all five BAL fluid samples were culture negative, while three of

TABLE 1. *Aspergillus* burden in lung tissue and BAL fluid measured by CFU, qPCR, and real-time NASBA assays

Sample type	Time (h) postinfection (no. of rats)	<i>Aspergillus</i> burden determined by ^a :					
		Culture		qPCR		NASBA	
		No. of positive samples	Mean \log_{10} CFU/g (ml) ^b	No. of positive samples	Mean \log_{10} CE/g (ml) ^b	No. of positive samples	Mean \log_{10} CE/g (ml) ^b
Lung tissue	1 (5)	5	5.17 ± 0.25	5	3.07 ± 0.25	0	
	24 (5)	5	3.80 ± 0.44	5	3.50 ± 0.39	5	2.87 ± 0.61
	48 (5)	5	3.70 ± 0.50	5	3.66 ± 0.67	4	3.92 ± 0.88
	72 (5)	5	4.00 ± 0.64	5	4.23 ± 0.66	5	5.10 ± 0.77^c
	96 (5)	5	3.75 ± 0.62	5	5.46 ± 0.76^c	5	6.00 ± 0.78^c
BAL fluid	1 (5)	5	3.20 ± 0.51	2	2.66 ± 0.47	3	2.48 ± 0.17
	24 (5)	3	2.44 ± 0.28	5	4.01 ± 0.97^c	5	4.37 ± 0.53^c
	48 (5)	2	2.24 ± 0.09	4	4.55 ± 1.54^c	5	4.13 ± 1.16^c
	72 (5)	0		3	3.40 ± 0.38	4	3.99 ± 0.45^c
	96 (5)	3	1.70 ± 0.00	2	3.20 ± 1.37	5	3.37 ± 1.01^c

^a Mean $\log_{10} \pm$ standard deviation was used to describe the *Aspergillus* burden.

^b Mean \log_{10} CFU/g and mean \log_{10} CE/g are units for lung tissue burden measurement, and mean \log_{10} CFU/ml and mean \log_{10} CE/ml are units for BAL fluid burden assessment.

^c Statistically significant difference ($P < 0.05$) compared to culture CFU counts.

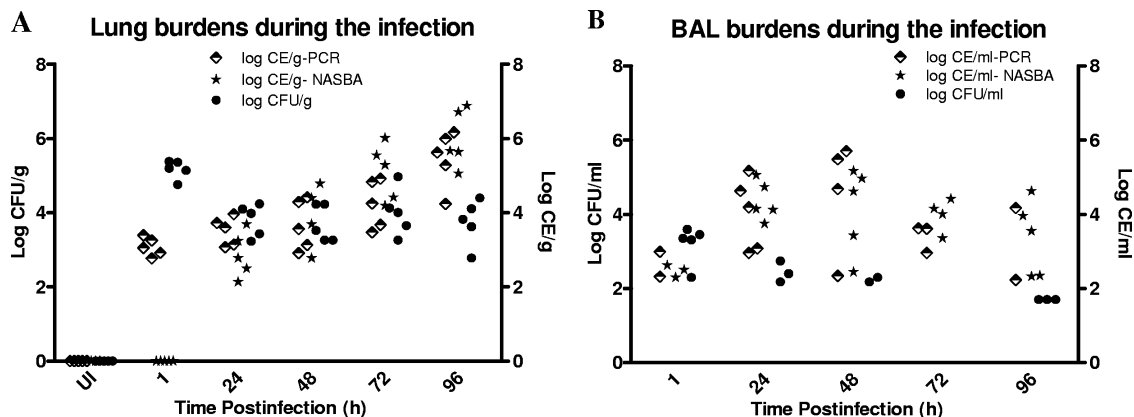


FIG. 2. *Aspergillus* burdens in lung tissues (A) and BAL fluids (B) in terms of CFU counting, qPCR, and real-time NASBA measurements. UI, uninfected group. A linear increase in the *Aspergillus* burden in lung tissues was observed by PCR ($F = 40.66, P < 0.0001$) and NASBA ($F = 141.10, P < 0.0001$).

five specimens were PCR positive and four of five specimens were NASBA positive, with the mean counts by the two assays being 3.40 ± 0.38 and $3.99 \pm 0.45 \log_{10}$ CE/ml, respectively. Although the burdens in BAL fluid varied during the infection and no obvious trend was observed by any measurement method (Fig. 2B), the PCR measurements correlated ($r = 0.887, P < 0.0001$) well with the NASBA assay results (Fig. 3B), consistent with the results observed for lung tissue.

Detection of *A. fumigatus* in whole blood. Apart from lung tissue and BAL fluid, whole-blood samples from rats with experimental IPA failed to demonstrate *Aspergillus* infection by culture or PCR, although at 72 h postinfection, three of five samples elicited positive signals by the real-time NASBA assay, with the TTPs ranging from 42 min to 47 min. At 96 h postinfection, all samples were NASBA positive, with the average TTP being 40 min, which is on the borderline of the lowest linear quantification range of the pan-*Aspergillus* NASBA assay. The estimated fungal burdens were about or below 100 CFU per ml of blood, although the extrapolation was not applied to obtain the exact load in blood because the TTPs were out of the lowest linear quantification limit. Meanwhile,

the result of the PCR assay and culture remained negative throughout the experiment.

DISCUSSION

The early diagnosis of IPA continues to be problematic for immunocompromised patients due to the lack of rapid, sensitive, and specific diagnostic methods. Use of the combination of galactomannan (GM) detection and standard clinical, radiological, and histological examinations has been an important advance in the detection of IPA. However, the fact that testing for GM frequently has false-positive results and decreased sensitivity for patients receiving antifungal prophylaxis (17, 23, 32) highlights the critical need for more sensitive, specific, and reliable diagnostic techniques. Nucleic acid-based detection is a viable option, although the PCR technique still has not been approved for use for defining IA because of a lack of standardization and the absence of a commercially available system (1, 2).

As an alternative to PCR for molecular detection, real-time NASBA has been widely used for the detection of RNA vi-

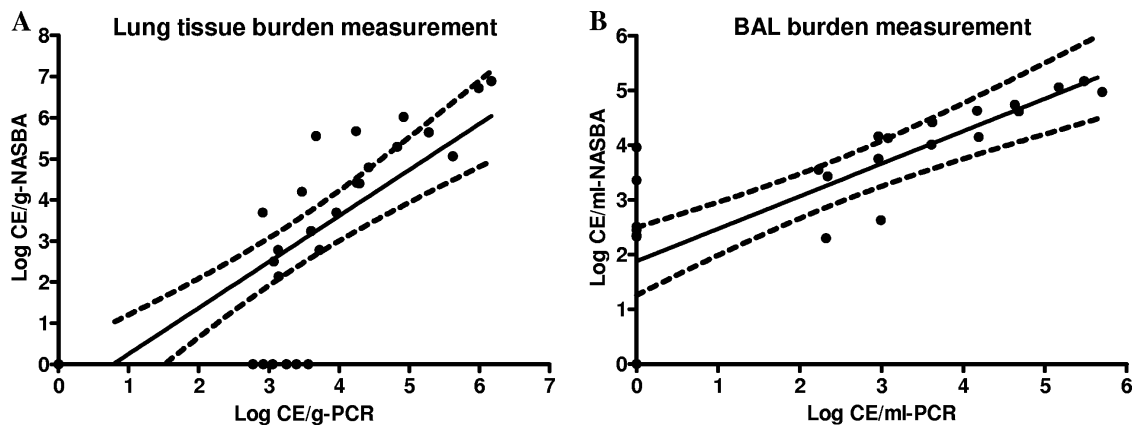


FIG. 3. The correlation between the results of the real-time NASBA and qPCR assays for the detection of the *Aspergillus* burden in lung tissue (A) and BAL fluids (B) from rats with IPA was statistically significant ($P < 0.0001$). Spearman's correlation coefficients (r) were 0.869 (A) and 0.887 (B). The black lines represent the linear regression, and the dotted lines represent the 95% confidence intervals.

ruses, such as enterovirus and HIV (13, 18, 25); certain microbial pathogens, including *Legionella* species and *Vibrio cholerae* (7, 16); and pathogens from food and environmental samples (3, 20). However, only studies of small scope have been reported on the use of NASBA to detect *Aspergillus* species (14, 36); nevertheless, the good diagnostic value of NASBA was demonstrated by those studies. To extend the applicability of the real-time NASBA assay to the quantification of the fungal load in order to monitor the progression of *Aspergillus* infection, we established a pan-*Aspergillus* real-time NASBA assay and used it in tests with an experimented model of IPA. We chose to develop a pan-*Aspergillus* assay to gain the complete capture of *Aspergillus* species, even though *A. fumigatus* remains the most abundant invasive species. The results were compared to those obtained by quantitative culture and qPCR. To our knowledge, this is the first report of a study that has used the real-time NASBA assay to assess the *Aspergillus* burden in an animal model of IPA.

A rapid real-time NASBA assay aimed at the detection of medically important *Aspergillus* spp. was developed by using the multicopy 28S rRNA target. The pan-*Aspergillus* NASBA assay demonstrated a high sensitivity, with the detection limit being 1 CFU of conidia or even lower (66% of the runs detected 0.1 CFU), which is comparable to the detection limit of the qPCR used in this and other studies (6, 27, 31). Good linearity was also observed by this assay when TNAs extracted from serially diluted conidial suspensions were used. This good linearity provides a reasonable basis for quantification of the *Aspergillus* burden by NASBA.

We measured the lung tissue burden by three different methods. As expected, throughout the course of the infection, the lung tissue burden assessed by NASBA or qPCR showed distinctly similar trends of consistent increases. The quantitative culture, instead, showed an initial drop, followed by a relatively stable lung tissue burden. This is in line with the findings of studies described in previous reports (29, 34). The mean value of the \log_{10} CE per gram of lung tissue obtained by NASBA was almost 1 \log_{10} unit lower than those assessed by quantitative culture at 24 h postinfection. However, the NASBA-based burdens increased more than 1 \log_{10} unit 1 day later and climbed to 6.00 \log_{10} CE per gram of lung tissue at 96 h postinfection, which was 2.25 \log_{10} units more than the burdens obtained by CFU counting. In addition, we noticed that at 1 h postinfection, the NASBA assay failed to detect lung tissue infection and the qPCR obtained lung tissue burden measurements with mean values of $3.07 \pm 0.25 \log_{10}$ CE per gram of lung tissue, which is just between the limit of detection and the lower linear quantification range of the PCR assay (220 to 2,200 \log_{10} CE/gram lung tissue). In contrast, the CFU counts for the rats at this time point yielded an average of 5.17 \log_{10} CFU per gram of lung tissue. This paradox could partly be explained by the low efficiency of extraction of conidia from tissue samples, especially for the recovery of RNA. It was estimated that less than 0.1% of the fungal DNA was yielded when the *A. fumigatus* conidia in lung tissue and BAL fluid samples from the rabbits were subjected to the extraction (21). Similar difficulties were also seen in other studies (8, 19). This result may be accounted for by the fact that RNA is less stable than DNA and is easily digested by the endogenous nuclease released from the tissue during homogenization.

The fungal burden in BAL fluid specimens did not show clear trends over time by either measurement method. Although the burden counts varied from time to time, the counts obtained by the two real-time methods still correlated very well ($r = 0.887$) and both were more sensitive than CFU counting from 24 h postinfection throughout the experiment. It is interesting to point out that a similar paradox was encountered in assessment of the BAL fluid burden at 1 h postinfection. Quantitative culture found that at this time point all five rats were infected with *A. fumigatus* with a mean value of 3.20 \log_{10} CFU per ml of BAL fluid, while both nucleic acid-based methods obtained positive results for only two or three of the five rats and the mean \log_{10} CE/ml counts for positive samples were also lower than the values obtained by CFU counting. However, NASBA detected *A. fumigatus* in almost all the samples at later time points, and the burden counts were also higher than those obtained by culture. The lack of sensitivity of using BAL fluid to detect *A. fumigatus* in IPA cases has been well documented (26); thus, the need to improve the methodology of detection of *A. fumigatus* in BAL fluid was proposed (21). Our study demonstrated that the real-time NASBA assay can be an option for the detection of *A. fumigatus* in BAL fluid, because the sensitivity of the NASBA assay is comparable to or even greater than that of qPCR. NASBA consistently detected the infection throughout the study and provided higher estimates of the fungal burden.

Although PCR was unable to detect *Aspergillus* in whole blood (and the culture results for all whole-blood samples were negative), NASBA showed positive results when the infection was getting severe and rats started showing some early clinical symptoms, such as slight weight loss but no breathing problems at 72 h postinfection. Although PCR has been proved to be able to detect fungal DNA in whole-blood specimens (15, 24), there are a couple of possible reasons for the negative PCR results in this study. First, a relatively small volume of blood (1 ml) was used for detection, whereas previous studies used 3 to 5 ml (15, 24) and the use of a small volume decreased the sensitivity. Second, according to Loeffler and colleagues, only 25% of the blood samples were PCR positive even after 6 days of infection with *A. fumigatus* and the fungal load in blood was low (10 to 100 CFU/ml) (15). Thus, the relatively small sample size (25 infected rats) of our study and the relatively short postinfection time (4 days) could also have led to the negative PCR results. Nevertheless, the positive NASBA results with whole blood were encouraging because they demonstrated that it is possible that the NASBA assay may be used to detect fungal RNA in whole blood. Exact measurement of the fungal burden in blood was difficult, because the TTPs of positive samples were about 40 min or later, which could be translated as being the time when the burden was close to or below the lower quantification limit (100 CFU). This is consistent with the findings of a study described in a previous report (15).

There are several limitations of our study. First, the nucleic acid extraction method remains inefficient, especially for blood samples and when conidia are the majority of fungal cells in the samples. Our blood spiking experiment showed that the rate of recovery of RNA was below 0.03% when less than 1 ng of *A. fumigatus* RNA was spiked in 2.5 ml of sheep blood (data not shown). More efficient extraction methods are urgently needed, and such studies are ongoing. Second, for exact mea-

surements, the TNAs extracted from the titrated conidia spiked in uninfected lung tissues or BAL fluids should be used as the quantification standards.

In conclusion, the real-time NASBA assay developed in the present study is highly sensitive and useful for the detection of *Aspergillus* from lung tissue and BAL fluid in an experimental model of IPA.

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