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Molecular Diagnosis of Invasive Aspergillosis and Detection of Azole Resistance by a Newly Commercialized PCR Kit

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ABSTRACT *Aspergillus fumigatus* is the main species responsible for aspergillosis in humans. The diagnosis of aspergillosis remains difficult, and the rapid emergence of azole resistance in *A. fumigatus* is worrisome. The aim of this study was to validate the new MycoGENIE *A. fumigatus* real-time PCR kit and to evaluate its performance on clinical samples for the detection of *A. fumigatus* and its azole resistance. This multiplex assay detects DNA from the *A. fumigatus* species complex by targeting the multicopy 28S rRNA gene and specific TR_{34} and L98H mutations in the single-copy-number *cyp51A* gene of *A. fumigatus*. The specificity of *cyp51A* mutation detection was assessed by testing DNA samples from 25 wild-type or mutated clinical *A. fumigatus* isolates. Clinical validation was performed on 88 respiratory samples obtained from 62 patients and on 69 serum samples obtained from 16 patients with proven or probable aspergillosis and 13 patients without aspergillosis. The limit of detection was <1 copy for the *Aspergillus* 28S rRNA gene and 6 copies for the *cyp51A* gene harboring the TR_{34} and L98H alterations. No cross-reactivity was detected with various fungi and bacteria. All isolates harboring the TR_{34} and L98H mutations were accurately detected by quantitative PCR (qPCR) analysis. With respiratory samples, qPCR results showed a sensitivity and specificity of 92.9% and 90.1%, respectively, while with serum samples, the sensitivity and specificity were 100% and 84.6%, respectively. Our study demonstrated that this new real-time PCR kit enables sensitive and rapid detection of *A. fumigatus* DNA and azole resistance due to TR_{34} and L98H mutations in clinical samples.

KEYWORDS *Aspergillus fumigatus*, molecular diagnosis, azole resistance, *cyp51A*, TR_{34} L98H

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Accurate diagnosis of invasive pulmonary aspergillosis (IPA) in patients at high risk of invasive fungal infection remains challenging (1, 2) due to difficulties in differentiating IPA from pulmonary infections caused by other molds or bacteria on clinical and radiological grounds. Therefore, culture-based microbiological diagnosis is of primary importance but requires semi-invasive or invasive procedures, such as bronchoalveolar lavage (BAL) or computed-tomography (CT)-guided needle biopsy (3, 4). Alternate diagnostic methods include the detection of biomarkers, such as fungal antigens (*Aspergillus* galactomannan [GM]) or DNA released by *Aspergillus* hyphae in host tissues (3, 4). These biomarkers are well recognized as early IPA predictors (5–7).

Recently, several clinical evaluations to detect *Aspergillus* DNA, either in respiratory or in blood-based samples, have clearly shown the diagnostic value of this biomarker (4–9). In addition, methodological recommendations have been established for PCR protocols (10), and different standardized *Aspergillus* quantitative PCR (qPCR) kits have been commercialized (11, 12). These recent advances show that PCR is now mature for routine use in clinical settings.

Another issue is the emergence of aspergillosis due to azole-resistant isolates. Acquired azole resistance in *Aspergillus fumigatus* has been reported since 1997 (13–16) and has emerged in many countries, particularly in Europe (17–22), as well as on other continents (23–26). In some instances, acquired resistance may be driven by antifungal selection in patients receiving long-term therapy (27). Nevertheless, it seems that many azole-resistant strains originated in the environment due to selection by azole fungicides used in agriculture (28). Azole resistance in *A. fumigatus* is associated mainly with mutations in the *cyp51A* gene, and among several mutations described, the most frequent is the mutation comprising a 34-bp tandem repeat (TR_{34}) and the L98H alteration.

Since azoles are the recommended first-line treatment for IPA (29), the emergence of azole resistance is worrisome and has been shown to be associated with an increased rate of clinical failure (30). For these reasons, routine antifungal susceptibility testing (AFST) of clinical isolates has been recommended recently (31). Nevertheless, isolates are not always retrieved in culture, particularly for patients with hematological malignancies (1). Therefore, molecular detection of resistance may be a major advance for the management of patients with invasive aspergillosis (IA).

In this context, some assays have been developed to enable the detection of both *A. fumigatus* DNA and *cyp51A* mutations associated with azole resistance in clinical samples (32, 33). Only one kit has been evaluated recently with either respiratory (32, 34) or serum (33) samples.

In the present study, a new CE-IVD (In Vitro Diagnostics)-compliant multiplex real-time qPCR assay has been designed, optimized, and validated.

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RESULTS

Analytical performance of the MycoGENIE *A. fumigatus* real-time PCR kit. (i) Sensitivity and specificity. The limit of blank (LoB) for the MycoGENIE *A. fumigatus* real-time PCR kit was fixed at 40 cycles. The limit of detection (LoD) for the *cyp51A* gene harboring the TR_{34} and L98H mutations (a single-copy-number gene) was determined at 6 copies, while the LoD for the *Aspergillus* 28S rRNA gene (a highly repetitive gene) was <1 copy (verified with five clinically mutated isolates and one wild-type isolate). As shown in Fig. 1, the detection of both mutations in the single-copy-number *cyp51A* gene was not affected by the amplification of the *A. fumigatus*-specific 28S rRNA multicopy gene.

No cross-reactivity was detected when qPCR was performed with DNA from *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus versicolor*, *Aspergillus terreus*, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida lusitaniae*, *Penicillium* spp., *Fusarium* spp., *Staphylococcus epidermidis*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa*.

The specificity of *cyp51A* mutation detection was assessed by testing DNA samples from 25 wild-type or mutated (TR_{34} L98H, TR_{46} Y121F T289A) clinical *A. fumigatus* isolates (Table 1). All isolates harboring the TR_{34} L98H mutations (10/25) were accurately detected by qPCR.

The performance of the MycoGENIE *A. fumigatus* real-time PCR kit was also validated using the *Aspergillus* DNA Calibrator (35), which was purified from strain AF293 using limiting-dilution analysis (35). Starting from the concentrated calibration solution

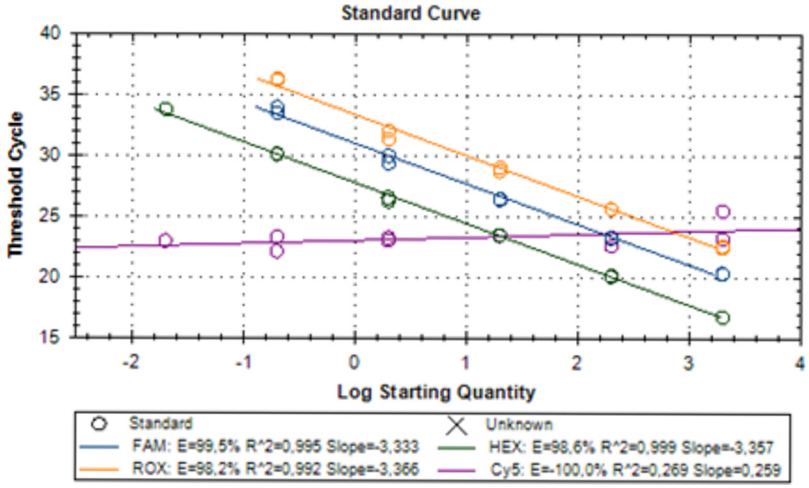

FIG 1 Analytical performance of the MycoGENIE real-time PCR. The efficiency and dynamic range of the PCR are shown.

(1.73E+6 U/ml), 10 replicates of 10-fold serial dilutions were quantified using the MycoGENIE *A. fumigatus* real-time PCR kit, and an LoD of 65 U/ml was reported.

(ii) Repeatability and reproducibility. Reproducibility was determined by four different operators testing four-point serial dilutions of synthetic DNA in triplicate. The synthetic DNA contains all the sequences targeted by the MycoGENIE *A. fumigatus* real-time PCR kit. Experiments were performed using a single batch from the qPCR kit on a CFX96 qPCR instrument. The mean standard deviation and coefficient of variation (CV) were 0.25 and 0.9%, respectively.

Repeatability was assessed with serial dilutions of DNA from mutated clinical isolates. Each point was quantified 20 times. The LoD was determined at 6 copies for

TABLE 1 Detection of TR_{34} L98H *cyp51A* mutations in *Aspergillus fumigatus* isolates by the MycoGENIE *A. fumigatus* qPCR assay

Isolate	Mutations (detected by PCR/sequencing)	C_T by qPCR[a]		
		TR_{34}	*A. fumigatus*	L98H
1			23.47	
2			20.72	
3	TR_{34} L98H	23.56	20.89	24.49
4	TR_{46} Y121F T289A		20.64	
5			20.57	
6	TR_{46} Y121F T289A		20.61	
7	TR_{34} L98H	22.93	20.39	24.06
8			21.41	
9	TR_{34} L98H	23.74	21.35	24.80
10	TR_{34} L98H	23.45	20.61	24.43
11	TR_{46} Y121F T289A		20.87	
12			21.22	
13			20.87	
14	TR_{34} L98H	23.06	20.46	24.21
15	TR_{34} L98H	23.02	20.59	24.15
16			21.74	
17			21.36	
18	TR_{34} L98H	23.77	21.18	24.92
19	TR_{46} Y121F T289A		20.97	
20			21.08	
21	TR_{34} L98H	23.35	20.37	24.13
22			20.15	
23	TR_{34} L98H	22.67	20.28	23.73
24	TR_{34} L98H	23.21	20.63	24.36
25	TR_{46} Y121F T289A		21.18	

[a]C_T, threshold cycle.

TABLE 2 Concordance between qPCR and classical mycology for respiratory samples

Mycology for *A. fumigatus*[a]	No. of samples with the following qPCR[b] result:		Total
	Positive	Negative	
Positive	52	4	56
Negative	3	29	32
Total	55	33	88

[a]Includes positive culture and/or positive GM by BAL.
[b]Sensitivity, 92.9% (52/56); specificity, 90.1% (29/32).

the TR_{34} and L98H mutations, with a mean standard deviation and CV of 0.75 and 2.18%, respectively.

Clinical evaluation. (i) Respiratory samples. Among the 88 respiratory samples (from 62 patients), 59 were culture positive for *Aspergillus* species (51 with *A. fumigatus* and 8 with *Aspergillus* species other than *A. fumigatus*), 11 grew other fungi, and 18 were culture negative (see Table S1 in the supplemental material). Five BAL fluid samples found negative for *A. fumigatus* by culture were positive for GM. Therefore, 56 samples were considered positive for *A. fumigatus*, and 32 samples were considered negative. qPCR results were positive for 55 samples and negative for 33 samples, indicating an overall sensitivity and specificity of 92.9% and 90.1%, respectively (Table 2).

(ii) Serum samples. For the 16 patients with proven or probable IA, all 16 serum samples obtained at the time of diagnosis ($\pm$2 days) were qPCR positive (Table 3). Among the 13 individuals without IA, there were 10 healthy individuals and 3 patients with hematological malignancies. Of these three patients, two had fungal infections other than aspergillosis (hepatosplenic candidiasis; fungemia due to *Magnusiomyces capitatus*) and one had no fungal infection. Eleven of the 13 serum samples tested were qPCR negative, while 2 serum samples from 2 patients without IA tested qPCR positive, corresponding to the 2 false-positive DNA detection cases. Overall, the sensitivity and specificity of *A. fumigatus* qPCR were 100% and 84.6%, respectively.

Comparison between the MycoGENIE procedure and the reference procedure. For 30 samples (19 serum and 11 respiratory samples), the MycoGENIE procedure (extraction from 200 μl of the sample and qPCR) was compared to a validated in-house procedure (a large extraction volume [1 ml of the sample] and qPCR) (7), considered as a reference procedure, using the same DNA target (Table 4). Among the 30 samples, 18 were positive and 12 were negative by the reference qPCR, and in all cases, the MycoGENIE qPCR gave identical results, indicating 100% concordance between the two qPCR techniques.

DNA kinetic study. For four patients with IA, serial serum samples (3 to 17 serum samples per patient) were available, and these were tested for both DNA and GM detection. For two patients, DNA was detected in the serum 5 days earlier than GM. For one patient, DNA and GM were detected concomitantly in the same sample, and for one patient, only positive samples for the two markers were available, precluding kinetic analysis.

TABLE 3 Performance of the MycoGENIE qPCR assay for the diagnosis of invasive aspergillosis from serum samples

Result by MycoGENIE qPCR[a]	No. of patients[b]		Total
	IA	Non-IA	
Positive	16	2	18
Negative	0	11	11
Total	16	13	29

[a]At the time of diagnosis. MycoGENIE qPCR had a sensitivity of 100% (16/16) and a specificity of 84.6% (11/13).
[b]One serum sample per patient.

TABLE 4 Comparison between MycoGENIE and an in-house reference procedure[a]

Sample ID	Sample type	qPCR result (C_T)	
		In-house	MycoGENIE
N1	Serum	−	−
N2	Serum	−	−
N3	Serum	−	−
N4	Serum	−	−
N5	Serum	−	−
N6	Serum	−	−
N7	Serum	−	−
N8	Serum	−	−
N9	Serum	−	−
N10	Serum	−	−
S1	Serum	+ (34.9)	+ (38.5)
S2	Serum	+ (29)	+ (32.9)
S3	Serum	+ (34.6)	+ (36)
S4	Serum	+ (32.2)	+ (34.6)
S5	Serum	+ (36.7)	+ (36.5)
S6	Serum	+ (34.1)	+ (35.4)
S7	Serum	+ (38.8)	+ (38.3)
S8	Serum	+ (38.2)	+ (37.7)
S9	Serum	+ (31.6)	+ (34.4)
P11	Respiratory	+ (28.3)	+ (26.2)
P12	Respiratory	+ (31.3)	+ (29.1)
P13	Respiratory	+ (26.6)	+ (27.4)
P15	Respiratory	+ (33.2)	+ (35.6)
P16	Respiratory	+ (29.2)	+ (30.7)
P18	Respiratory	+ (30.1)	+ (28.2)
P19	Respiratory	−	−
P20	Respiratory	+ (29.3)	+ (29.6)
P21	Respiratory	+ (26.4)	+ (29.1)
P22	Respiratory	+ (34.7)	+ (33.5)
P23	Respiratory	−	−

[a]The in-house reference procedure included a large extraction volume (1 ml) using a MagnaPure kit, and qPCR was performed as published previously (7). The MycoGENIE procedure included a 200-μl extraction volume, and qPCR was performed as described in Materials and Methods.

DISCUSSION

It is well known, on the basis of several clinical studies, that detection of *Aspergillus* DNA in serum and/or BAL fluid is of great importance for the diagnosis of IA in immunocompromised patients (5). Nevertheless, the most recent guidelines have recommended that PCR should be used carefully for the management of patients, owing to the lack of validation of commercially available kits (36).

In this study, we present the results of the technical evaluation of a new standardized multiplex real-time *A. fumigatus* PCR kit, which allows detection of both *A. fumigatus* DNA, for IA diagnosis, and *cyp51* mutations responsible for azole resistance. According to our results, this PCR kit showed good performance, including a low LoD for DNA detection and a lack of cross-reactivity with other pathogenic fungi. In addition, for the detection of the TR_{34} L98H mutation, azole-resistant isolates harboring different *cyp51* mutations as well as wild-type isolates were tested, and this PCR kit showed very good specificity with no loss of efficiency.

Furthermore, the kit was evaluated using a large panel of clinical samples, either serum samples from patients with proven aspergillosis or BAL fluid specimens, which were previously characterized according to the gold-standard mycological methods.

With serum samples, both sensitivity and specificity were high (100% and 84.6%, respectively). This high sensitivity was reached by use of an optimized extraction volume of 200 μl, lower than the extraction volume (1 ml) of the reference method. By processing 5 times more sample volume and analyzing 15 μl of the eluate, the reference method should lead to better detection of A. *fumigatus* DNA (with a ΔC_T close to 2.9) than the MycoGENIE procedure. Table 4 shows that in most cases (16/18), the MycoGENIE kit led to detection earlier than expected, with only a 200-μl sample,

highlighting its more-efficient DNA extraction procedure. Indeed, the DNA extraction protocol was specifically optimized to process serum samples efficiently. The high yield of the extraction relies mainly on the magnetic particles used, which offer a very large specific area and enhanced binding kinetics thanks to their uniform surface and size. Thus, they can capture very small amounts of DNA from complex samples (37). Their performance is already well known in very demanding sample preparation applications, such as forensic science, where they are used in commercial kits designed for the recovery and purification of trace DNA from crime scenes (38). This remarkable performance is combined with a very low elution volume ($<$100 μl) to increase the DNA concentration. In addition, the 10-μl eluate used for qPCR analysis also contributes to improving the limit of detection of the assay.

With respiratory samples, sensitivity and specificity were 92.9% and 90%, respectively. The presence of free-circulating DNA in serum may explain the better efficiency of the extraction procedure in serum samples than in respiratory samples. Indeed, in respiratory samples, *Aspergillus* is present mainly in the hyphal form, for which DNA extraction is known to be more labor-intensive and less efficient.

Until now, only one other *Aspergillus* PCR kit (AsperGenius) combining the detection of *Aspergillus* DNA with the detection of *cyp51* mutations associated with azole resistance has been evaluated (32–34).

The performance of the MycoGENIE kit appeared to be similar to that of the AsperGenius kit. With BAL fluid specimens from hematological and intensive-care-unit patients, the AsperGenius kit showed sensitivities of 88.9% and 80.0% and specificities of 89.3% and 93.3%, respectively (32). The sensitivity and specificity of the AsperGenius kit for serum samples were 78.6% and 100%, respectively.

In our study, no sample positive for the TR_{34} L98H mutations was detected. However, azole resistance has emerged during the past 15 years (39) and now seems to be a worldwide problem (22). For these reasons, we designed an *Aspergillus* qPCR assay to enable the detection of the TR_{34} L98H alterations in the *cyp51* gene. These alterations constitute the most frequent mechanism of azole resistance in either clinical or environmental *A. fumigatus* isolates (22, 39). Although resistance is generally detected phenotypically, by AFST of isolates obtained in culture, molecular detection of resistance markers offers many advantages. First, detection of resistance may be obtained at the same time as the results of diagnostic PCR and may be more rapid than standard AFST when the strain is available. Second, in many clinical settings of aspergillosis, strains are not available, so direct detection of DNA-based resistance markers in tissues or in serum may be valuable. Overall, the MycoGENIE *A. fumigatus* kit could be a useful tool for the management of different populations of patients (oncohematology, lung transplantation, and cystic fibrosis).

To date, only two kits combine the detection of *Aspergillus* DNA with the detection of resistance markers. These kits allow the detection of only a restricted number of Cyp51A alterations (TR_{34} and L98H for the MycoGENIE *A. fumigatus* kit; TR_{34}, L98H, Y121F, and T289A for the AsperGenius kit). Although several other Cyp51A mutations or mechanisms of azole resistance have been described, the TR_{34} L98H mutations have been reported as the most frequent (50%), according to a recent multicenter international study (22). Due to the limitations of real-time multiplex qPCR technology, only a restricted number of mutations can be efficiently detected simultaneously.

Our study is the first evaluation of the MycoGENIE *A. fumigatus* kit. This kit has been validated for both serum and respiratory samples. These results demonstrate the good performance of the kit. The MycoGENIE *A. fumigatus* kit enables sensitive and rapid detection of *A. fumigatus* DNA and azole resistance due to TR_{34} L98H alterations in clinical samples. Larger prospective studies with different groups of patients at high risk of IA are warranted.

MATERIALS AND METHODS

Study design. The study was divided into two steps. The first step was performed to determine the analytical performance of the MycoGENIE *A. fumigatus* real-time PCR kit, including the limit of detection,

cross-reactivity, sensitivity, specificity, repeatability, and reproducibility. The second step was clinical evaluation, consisting of kit validation using clinical serum and respiratory samples.

MycoGENIE procedure. (i) DNA extraction. In all experiments, DNA was extracted from *A. fumigatus* isolates, sera, and respiratory samples according to the manufacturer's protocol, using the MycoGENIE DNA extraction kit for AutoMag solution (Ademtech, Pessac, France). The AutoMag instrument was used as an automated magnetic particle processor for DNA purification kits. It processes as many as 12 samples per run in a DNA extraction plate (a ready-to-use sealed 96-well plate) and includes reagents for lysis/capture, washing, and elution. For each extraction, 200 μl of sample and 10 μl of an internal control (IC) were added to the capture well. The IC was used to monitor the DNA extraction procedure and to detect PCR-inhibitory substances. After lysis/capture for 5 min, the DNA attached to the magnetic particles in the well was washed by three successive washing steps to remove unbound substances (proteins, cell debris, and PCR inhibitors). Following this, the elution step was performed in the heating well filled with 60 μl of elution buffer. DNA solutions were transferred to microtubes, directly processed for real-time PCR, and stored at −20°C for further analysis.

(ii) Real-time PCR. Real-time PCR for the detection of *A. fumigatus* DNA was performed using the MycoGENIE *A. fumigatus* real-time PCR kit (Ademtech, Pessac, France) on a CFX96 instrument (Bio-Rad, USA). This multiplex assay specifically detects DNA from the *A. fumigatus* species complex by targeting the 28S rRNA multicopy gene and specific TR_{34} and L98H mutations in the *A. fumigatus* single-copy-number gene *cyp51A*.

Multiplex amplification reactions were carried out in a 25-μl reaction volume containing 12.5 μl of a UTP-containing master mix (2×), 10 μl of previously extracted DNA, and 2.5 μl of DNase- and RNase-free water. To avoid carryover contamination between PCRs, the MycoGENIE *A. fumigatus* mixture contains UDG (uracil-DNA glycosylase). After preincubation at 37°C for 10 min for UDG activation and at 95°C for 10 min for *Taq* activation, amplification was performed over 45 cycles of denaturation at 95°C for 30 s and annealing/extension at 62°C for 45 s. Negative and positive controls were included in each assay. A positive control, with all targeted sequences, is provided in the MycoGENIE PCR kit to assess the integrity of the nucleic acid amplification assay. The IC reaction was considered inhibited for Cy5 signal results higher than 35 cycles.

Clinical samples. (i) Respiratory samples. A total of 88 respiratory samples were obtained from 62 patients in two centers (Pellegrin University Hospital, Bordeaux, France, and Georges Pompidou European University Hospital, Paris, France). These comprised 51 BAL fluid, 23 sputum, and 14 bronchial fluid samples. Direct examination and culture of samples were routinely performed according to standard mycological methods (40). Filamentous fungi were identified phenotypically by macroscopic and microscopic observations (41). GM was detected in BAL fluid by the Platelia *Aspergillus* enzyme immunoassay (EIA) (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's instructions, and samples were considered positive at an index value of ≥0.5. GM detection was not performed for respiratory samples other than BAL fluid. Samples were stored frozen at −20°C until further molecular analysis. qPCR results were compared to the results of culture and/or GM detection. A respiratory sample was considered positive for *A. fumigatus* if the culture yielded *A. fumigatus* and/or if the sample was positive for GM (for BAL fluid). A sample was considered negative for *A. fumigatus* if the culture was negative or if another mold was detected.

(ii) Serum samples. A total of 55 serum samples from 16 patients with proven or probable aspergillosis (according to EORTC criteria) (42) in two centers (Pellegrin University Hospital, Bordeaux, France, and Necker University Hospital, Paris, France) were included. For five patients, serial serum samples were available (3 to 17 per patient) and were used to evaluate the kinetics of both GM and DNA detection. GM was detected in serum by the Platelia *Aspergillus* EIA (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's instructions. Fourteen serum samples from 13 patients without IA were included as negative controls.

The sensitivity and specificity of qPCR for IA diagnosis were evaluated using results obtained from serum samples at the time of IA diagnosis, defined as the day of initiation of curative antifungal treatment (±2 days), and serum samples from negative controls.

Comparison with a reference technique. The performance of the MycoGENIE procedure (extraction and qPCR) was also evaluated by testing 19 serum samples (from 9 IA patients and 10 controls) and 12 respiratory samples concomitantly with a previously clinically validated in-house procedure (a large extraction volume for serum samples, 200 μl for respiratory samples, and qPCR) (7).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01032-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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