

# Analytical Comparison of *In Vitro*-Spiked Human Serum and Plasma for PCR-Based Detection of *Aspergillus fumigatus* DNA: a Study by the European *Aspergillus* PCR Initiative

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The use of serum or plasma for *Aspergillus* PCR testing facilitates automated and standardized technology. Recommendations for serum testing are available, and while serum and plasma are regularly considered interchangeable for use in fungal diagnostics, differences in galactomannan enzyme immunoassay (GM-EIA) performance have been reported and are attributed to clot formation. Therefore, it is important to assess plasma PCR testing to determine if previous recommendations for serum are applicable and also to compare analytical performance with that of serum PCR. Molecular methods testing serum and plasma were compared through multicenter distribution of quality control panels, with additional studies to investigate the effect of clot formation and blood fractionation on DNA availability. Analytical sensitivity and time to positivity (TTP) were compared, and a regression analysis was performed to identify variables that enhanced plasma PCR performance. When testing plasma, sample volume, preextraction-to-postextraction volume ratio, PCR volume, duplicate testing, and the use of an internal control for PCR were positively associated with performance. When whole-blood samples were spiked and then fractionated, the analytical sensitivity and TTP were superior when testing plasma. Centrifugation had no effect on DNA availability, whereas the presence of clot material significantly lowered the concentration ( $P = 0.028$ ). Technically, there are no major differences in the molecular processing of serum and plasma, but the formation of clot material potentially reduces available DNA in serum. During disease, *Aspergillus* DNA burdens in blood are often at the limits of PCR performance. Using plasma might improve performance while maintaining the methodological simplicity of serum testing.

Invasive aspergillosis (IA) represents a serious health problem for the immunocompromised patient, especially those undergoing cancer chemotherapy or receiving corticosteroid therapy or immunosuppression to avoid allograft rejection or solid organ rejection. *Aspergillus fumigatus* is a ubiquitous mold and the most frequent cause of aspergillosis in humans, causing a wide spectrum of diseases ranging from allergies to severe life-threatening invasive manifestations. Spores of *A. fumigatus* enter the body through inhalation, and infection primarily occurs in the lungs. Accurate diagnosis of IA remains difficult. Given the limitations of clinical signs and the difficulty in obtaining appropriate specimens for diagnosis, a significant proportion of cases remain undetected, resulting in late or inappropriate therapy and increased mortality rates as high as 90% with cerebral disease (1). Hence, there is an urgent need for an accurate and standardized approach for diagnosing IA.

The performance of mycological diagnostics when testing serum or plasma samples is assumed to be similar. The revised 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) definitions for invasive fungal infections include galactomannan (GM)-enzyme immunoassay (EIA) for testing of both serum and plasma (2), although the testing of plasma with this assay has not been validated by the manufacturer. A study

comparing GM-EIA performance in serum and plasma confirmed that the testing of plasma using the same thresholds was appropriate, but interestingly, the mean index generated by testing plasma was significantly higher than that for serum (0.315 versus 0.279;  $P = 0.0398$  [3]). Moreover, four possible IA cases would have been classified as probable IA had plasma been tested. The authors hy-

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pothesized that differences in indices could be attributed to the formation of the blood clot potentially ensnaring some of the GM within the sample taken for serum testing. It is conceivable that the same might be true for extracellular DNA, the *Aspergillus* target in the cell-free fraction of host blood, which would thereby affect PCR performance.

This paper describes further efforts of the European *Aspergillus* PCR Initiative (EAPCRI) to evaluate the analytical performance of plasma and serum samples through the blinded distribution of simulated panels, as described previously for whole blood and plasma (4, 5). We report data on the differences in analytical performance associated with different sample types (plasma versus serum) and on how differences in the initial formation of the sample types (clot versus no clot) and sample processing (whole-blood centrifugation) affected the availability of DNA within the cell-free sample itself.

In a companion paper (6) to this analytical *in vitro* study, a further multicenter clinical study, which compares the performance of plasma and serum, was performed in parallel (6).

## MATERIALS AND METHODS

**DNA source material.** DNA was obtained from a sporulating culture of *A. fumigatus* (strain ATCC 1022). Conidia were harvested, and DNA was extracted as described before (5).

**Participants.** The participants comprised 8 laboratories representing the EAPCRI core facilities and an extended group of an additional 15 laboratories. To maintain impartiality throughout the analytical process, all centers were given a numerical code to allow a blinded review of individual methodological procedures, determination of performance, and statistical analysis.

**Panels.** All EDTA whole-blood and clotted-blood samples were obtained from consenting healthy volunteers and screened for the presence of infectious agents, as per protocol of the Institute of Transfusion Medicine, Würzburg University Hospital, (Würzburg, Germany), and approved by the local ethics review board. Serum and plasma were fractionated by centrifugation and pooled, respectively, before being distributed to Public Health Wales, Microbiology Cardiff, Cardiff, United Kingdom, to develop the panel. To avoid airborne contamination, all processing of material took place in a category 2 laminar flow cabinet. The panel was validated through testing with an in-house *Aspergillus*-specific real-time PCR (7). The panels were distributed on dry ice by a courier. The participating centers were asked to confirm receipt, comment on the state of the panel (frozen or thawed), and keep specimens frozen at  $-80^{\circ}\text{C}$  until testing.

Analytical testing was performed by using a four-part process, explained below.

**(i) Assessment of analytical performance of *Aspergillus* PCR methods when testing serum.** Initially, serum was divided into  $3 \times 35$ -ml aliquots; one aliquot was retained to provide a negative-control sample, while two aliquots were spiked with 100 and 10 genome equivalents (ge) of *A. fumigatus* DNA per milliliter of serum. The 35-ml serum batches were further divided into 1-ml aliquots and frozen at  $-80^{\circ}\text{C}$  until distribution on dry ice to the 23 centers. This panel was a quality control (QC) exercise designed to confirm the findings of the previous research investigating serum, in doing so providing a reference for a comparison with the plasma results but also determining centers with optimal results for the analyses of further panels, including the analysis of plasma samples (5).

**(ii) Assessment of analytical performance of *Aspergillus* PCR methods when testing plasma.** Plasma was divided in eight 12-ml aliquots; two aliquots were retained to provide negative-control samples, while six aliquots were spiked with various concentrations of *A. fumigatus* genomic DNA (1,000, 100, 50, 10, 5, 1, and 0 ge/ml) and divided into 1-ml aliquots. The panels were distributed on dry ice to the 8 EAPCRI core centers for

testing. In keeping with previous studies, the threshold of detection, based on 95% variance, was set at 10 ge/ml (5).

**(iii) Assessment of DNA availability in serum/plasma with whole-blood spiking.** DNA was spiked into blood collected into an EDTA Vacutainer and into a Vacutainer without any anticoagulant immediately after sampling but before centrifugation. Blood was spiked with various concentrations of genomic *A. fumigatus* DNA (100, 50, 10, and 0 ge/ml; Fig. 1). Both the EDTA and clotted-blood Vacutinners were left for a minimum of 30 min at room temperature to allow the blood without anticoagulants to clot. The Vacutinners were then centrifuged ( $3,500 \times g$  for 5 min) to separate the cell-free fractions, which were pooled according to sample type and initial concentration of fungal burden. Each sample was then divided into 0.5-ml aliquots (potential target burdens, 50, 25, 5, and 0 ge) and frozen at  $-80^{\circ}\text{C}$  prior to distribution on dry ice. Serum and plasma samples were shipped on dry ice to the 8 EAPCRI core centers.

**(iv) Investigating the effects of clot formation and blood fractionation on the availability of *A. fumigatus* genomic DNA.** The following experiments were performed in a two-center study in Cardiff and Würzburg using identical spiking protocols, described below.

**Impact of centrifugation.** Two 4-ml aliquots of serum and plasma were spiked with 100 ge of *A. fumigatus* DNA. An aliquot of plasma and an aliquot of serum were centrifuged at  $3,500 \times g$  for 5 min, mimicking the centrifugation process necessary to fractionate blood samples and potentially identifying any losses associated with this process. The other aliquot of plasma and serum, equivalent to a direct spike control, remained untouched at ambient temperature until further processing by extraction. DNA was extracted from a minimum of three replicates of 0.5 ml of serum and 0.5 ml of plasma that were spiked but not centrifuged and a minimum of three replicates of 0.5 ml of serum and 0.5 ml of plasma that were both spiked and centrifuged. DNA was extracted using the in-house protocols of both centers (8, 9). Briefly, in Cardiff, *Aspergillus* DNA was extracted using the Qiagen EZ1 DSP virus kit, as per the manufacturer's instructions, with DNA eluted in 60  $\mu\text{l}$ ; in Würzburg, the QIAamp UltraSens virus kit (Qiagen) was used, as described by the manufacturer, and DNA was eluted in 70  $\mu\text{l}$ . Both protocols comply with the EAPCRI recommendations for testing serum (5). PCR was performed in duplicate and included an internal control to measure PCR inhibition.

**Impact of clot formation.** Immediately after blood was drawn, 4 ml of EDTA whole blood and 4 ml of clotted blood were spiked with 100 ge of *A. fumigatus* DNA. The clotted blood was left at ambient temperature for 30 min to coagulate, while the EDTA blood remained untouched at room temperature for the same period. The samples were then centrifuged at  $3,500 \times g$  for 5 min. After centrifugation, a minimum of three 0.5-ml aliquots of plasma and serum and the cell pellet from the EDTA whole-blood samples were extracted using local protocols, and DNA was eluted in  $<100 \mu\text{l}$ . The clot was not processed, as no EAPCRI-validated procedures are currently available to do so. PCR was performed in both centers using in-house amplification protocols and an inhibition control, all in accordance with EAPCRI guidelines (5).

**Statistical analysis.** Centers were requested to return both qualitative (positive/negative) and quantitative (quantification cycle [ $C_q$ ]) results within a designated time frame and provide detailed protocols for their DNA extraction and PCR amplification systems. The information required included sample volume used, extraction method, DNA elution volume, PCR method, PCR target, PCR template input volume, PCR total reaction mixture volume, PCR amplification platform, and internal control PCR results.

The correlation between  $C_q$  and genomic load was estimated by linear regression. The  $C_q$  was the dependent variable, whereas the explanatory variables were (i) the genomic load (as  $\log_{10}$  genomic DNA), (ii) the spiked sample type (as a binary variable for serum or plasma), and (iii) the interaction between genomic load and fluid. A multilevel mixed-effects model was performed, using the centers as a grouping variable. This model was comparable to a simplified calibration curve, also comparing

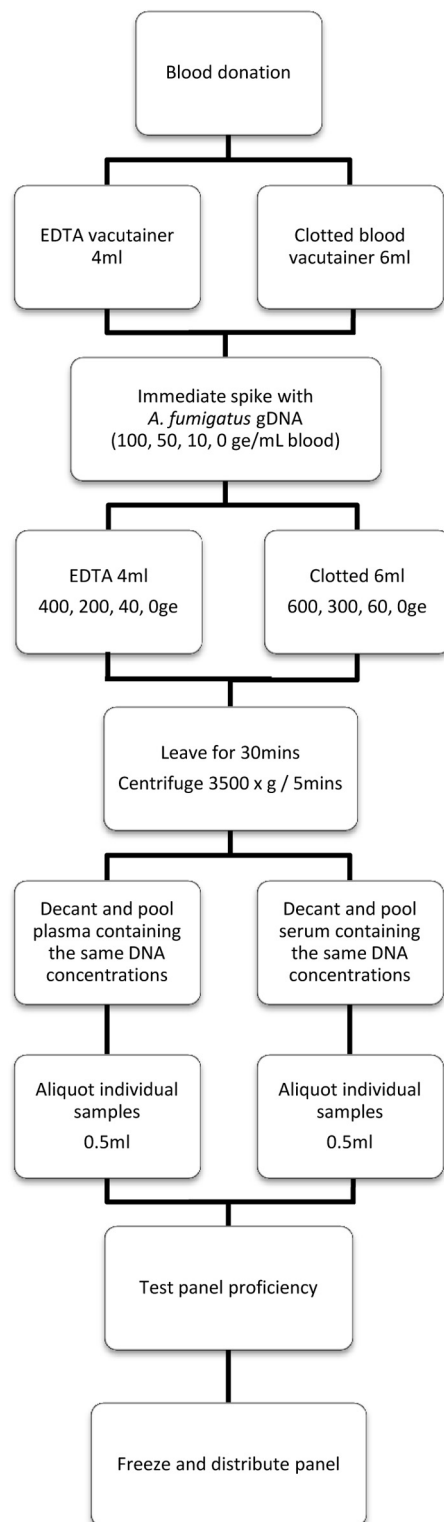


FIG 1 Flow diagram highlighting the process for determining the availability of *Aspergillus* DNA in serum and plasma samples post-blood fractionation.

the calibration lines concerning serum and plasma. The model was graphically reported.

In addition, bivariate linear regression was performed to analyze possible associations between PCR sensitivity and selected covariates (Table 1).

TABLE 1 Bivariate analysis for the sensitivity of *Aspergillus* PCR testing spiked plasma specimens distributed to 8 EAPCRI core centers<sup>a</sup>

Variable	Z score <sup>b</sup>	P value
No. of plasma samples analyzed/mo	-1.61	0.107
Plasma starting vol	-2.50	0.01 <sup>c</sup>
DNA elution vol	-0.65	0.513
Ratio starting plasma vol to elution vol	-2.32	0.02 <sup>c</sup>
Total PCR vol	-2.64	0.01 <sup>c</sup>
Template vol	-1.47	0.141
Use of internal control	-4.90	0.00 <sup>c</sup>
Use of $\geq 2$ replicates	-2.31	0.02 <sup>c</sup>
% of eluate vol used in PCR	-0.95	0.343

<sup>a</sup> All continuous and binary center-specific covariates were included into the basic model.

<sup>b</sup> With a negative Z score, the variable tended to exert a favorable effect on the PCR assay.

<sup>c</sup> Covariates exerting a significant effect ( $P < 0.05$ ) and an absolute Z score of  $> 1.96$ .

## RESULTS

(i) **Assessment of analytical performance of *Aspergillus* PCR methods when testing serum.** The serum analysis in this QC panel compared favorably with the previous EAPCRI serum evaluation (5). All 23 centers were able to reproducibly detect the sample that was spiked with 100 ge and generate negative results when testing the sample not containing *Aspergillus* DNA. Samples spiked with 10 ge/ml generated an overall positivity rate of 74% (17/23 centers) and a positivity rate of 82% (14/17) in centers that were strictly in accordance with EAPCRI recommendations for serum. Five out of the 6 centers that were not in accordance with EAPCRI recommendations were unable to detect 10 ge/ml. Centers that were unable to detect the 10 ge/ml did not routinely process serum samples (6/6) or used serum volumes of  $< 0.5$  ml (5/6; volume range, 0.1 to 0.2 ml of serum). In addition, these centers used only a small portion of their original serum sample volume in the subsequent PCR assay (mean 7.9%, range, 1% to 25%), compared to a mean volume of 24.4% (range, 16% to 82%) for centers that were able to detect 10 ge/ml ( $P = 0.028$ ).

(ii) **Assessment of analytical performance of *Aspergillus* PCR methods when testing plasma.** All centers that had received this plasma panel were requested to adhere to the EAPCRI guidelines for serum testing, and their positivity rates in the related QC serum panel were 100%, 100%, and 0% (100, 10, and 0 ge/ml, respectively).

Table 2 summarizes the results of the 8 centers. No samples

TABLE 2 *Aspergillus* PCR performance using plasma samples spiked with different fungal loads<sup>a</sup>

Sample no.	Burden (ge/ml)	95% sample variance	Positivity rate (%)	Mean $C_q$ (95% CI)	SE
1	1,000	939.6–1,060.4	100	32.4 (30.7–34.0)	0.70
2	100	80.8–119.2	100	36.1 (34.1–38.1)	0.84
3	50	36.4–53.6	100	36.9 (35.1–38.7)	0.75
4	10	4.0–16.0	100	39.00 (37.1–40.9)	0.79
5	5	0.8–9.2	100	39.9 (37.8–42.0)	0.88
6	1	0–2.92	8.3	40.0 <sup>b</sup>	NA <sup>c</sup>
7, 8	0 $\times$ 2	NA	0	No signal	NA

<sup>a</sup> Plasma samples 1 to 8 were spiked with different fungal burdens (genome equivalents [ge]/ml of plasma).

<sup>b</sup> Plasma 6 was positive in one center in a single sample only.

<sup>c</sup> NA, not available.

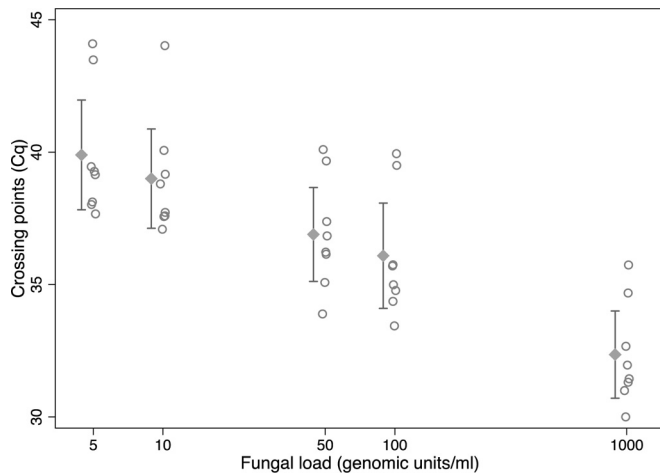


FIG 2 Descriptive statistics for *Aspergillus* real-time PCR crossing points when testing plasma samples containing various concentrations of *A. fumigatus* genomic DNA. Means (closed diamonds), 95% confidence intervals of the means (vertical bars), and single observations (open circles) are shown for each fungal load.

were found to be inhibitory to PCR amplification. All 8 centers achieved 100% positivity for fungal burdens between 5 ge/ml and 1,000 ge/ml, while the sample spiked with 1 ge/ml was positive in a single sample at one center only (Fig. 2). Six out of 8 centers stated that they use either serum or plasma or both in their routine diagnostic procedures (2 centers mainly tested bronchoalveolar lavage fluid).

The centers used between 0.1 ml and 1 ml (mean, 0.5 ml) of plasma for subsequent DNA extraction, 50 to 100  $\mu$ l (mean, 60  $\mu$ l) for the elution volume, and 2 to 15  $\mu$ l (mean, 8.7  $\mu$ l) for the template volume in their PCR assays. In bivariate analyses, we observed significant positive correlations between PCR sensitivity and the volume of plasma used for DNA extraction, the ratio between the volume of plasma used for extraction and the subsequent elution volume, the reaction mixture volume used for PCR, the analysis of  $\geq 2$  replicates, and the use of an internal control (Table 1).

To provide complete  $C_q$  values for a range of different plasma volumes, a linear mixed model was used, which predicts  $C_q$  values for hypothetical plasma volumes (0.1 ml to 0.5 ml) and various *A. fumigatus* DNA concentrations (5 to 1,000 ge/ml). The model shows that both plasma volume and DNA load influence  $C_q$  values (e.g., predicted  $C_q$  values for 5 ge/ml ranged depending on the plasma volume between 37.6 and 40.7 [Table 3]).

TABLE 3 Influence of sample volume (0.1 to 0.5 ml) on *Aspergillus* real-time PCR crossing points when testing plasma samples containing a range of *A. fumigatus* genomic DNA concentrations (5 to 1,000 ge/ml)<sup>a</sup>

Plasma vol (ml)	Fungal load (ge/ml of plasma)				
	5	10	50	100	1,000
0.1	40.68	39.70	37.43	36.45	33.21
0.2	39.90	38.92	36.65	35.67	32.42
0.3	39.11	38.14	35.86	34.89	31.64
0.4	38.33	37.35	35.08	34.11	30.86
0.5	37.55	36.57	34.30	33.32	30.07

<sup>a</sup> The prediction of  $C_q$  values was calculated using a linear mixed model.

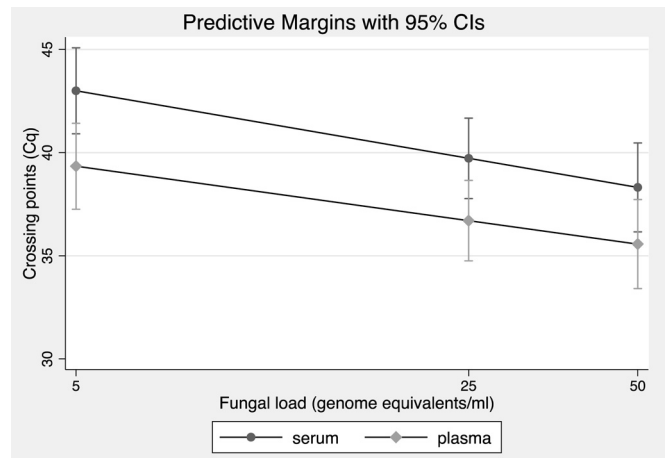


FIG 3 Comparison of *Aspergillus* real-time PCR when testing serum and plasma samples containing various fungal loads using a linear mixed model. Data are shown after the regression of the  $C_q$  value versus the fungal genomic burden, with serum or plasma as a binary covariate.

(iii) **Assessment of DNA availability in serum/plasma with whole-blood spiking.** In order to measure the availability of DNA in the cell-free fraction post-whole-blood processing, an additional panel, consisting of 4 serum samples and 4 plasma samples, was shipped to the 8 core EAPCRI centers. Our linear model showed that while the slope for PCR from plasma and serum did not differ significantly ( $P = 0.381$ ), the plasma intercept was 4.3 cycles lower (standard error, 1.38; 95% confidence interval  $-6.996$  to  $-1.585$ ) than the serum intercept (Z score =  $-3.11$ ,  $P = 0.002$ ). Interestingly, this shift in  $C_q$  values was achieved at all DNA concentrations tested (50, 25, and 5 ge/ml; Fig. 3). No inhibition was observed. Center-specific methodological details are shown in Table 4. The individual performance of each center in this panel was significantly associated with the volume of sample used for DNA extraction. While both centers using 0.2 ml of serum or plasma achieved suboptimal sensitivity, as the serum sample spiked with 5 ge/ml was not detected, the centers using  $\geq 0.5$  ml of plasma and serum were able to detect this sample as positive ( $P < 0.001$ ) (Table 4). In addition, we observed a trend that laboratories that routinely test serum or plasma by *Aspergillus* PCR achieved higher sensitivity ( $P = 0.07$ ).

(iv) **Investigating the effects of clot formation and blood fractionation on the availability of *A. fumigatus* genomic DNA.** In order to identify any potential loss of DNA during centrifugation necessary to fractionate blood samples, the recovery of DNA from spiked serum and plasma specimens that were centrifuged at  $3,500 \times g$  was compared to that of spiked samples that were not centrifuged (Fig. 4). In both centers, there were no significant differences in  $C_q$  values between centrifuged or noncentrifuged samples for either plasma or serum (Table 5). These results demonstrate that centrifugation at  $3,500 \times g$  is not sufficient to sediment free DNA from serum and plasma.

Interestingly, in both centers, plasma obtained after centrifugation of spiked EDTA blood samples showed earlier  $C_q$  values than those with serum obtained from the corresponding clotted-blood samples (difference of mean  $C_q$  values between plasma and serum, 2.26; standard error, 0.45;  $P = 0.0002$ ). The concentration of genomic DNA added to the PCR was significantly lower ( $P =$

TABLE 4 Details of the molecular procedures used by the EAPCRI centers investigating the performance of *Aspergillus* PCR when testing serum and plasma

EAPCRI center no.	No. of serum samples/mo	DNA extraction vol (ml)	DNA extraction system	Vol ( $\mu$ l) of:			PCR target gene	Sensitivity (%) in:	
				Elution	Template	PCR		Serum	Plasma
1	0	0.2	MagNA Pure	50	10	20	18S	66.7	100
2	80	1	QIAamp UltraSens virus	70	10	21	ITS	100	100
3	40	1	QIAamp UltraSens virus	38	10	21	ITS	100	100
4	15	0.2	QIAamp DNA mini	50	2	20	ITS	0	100
5	70	1	MagNA Pure total NA large volume	50	10	20	18S	100	100
6	240	0.5	EZ1 DSP virus	60	15	50	28S	100	100
7	50	0.5	MagNA Pure total NA large volume	100	5	20	28S	100	100
8	10	0.5	MagNA Pure viral NA large volume	50	10	50	28S	100	100

0.028) in serum samples post-clot formation (mean, 1.9 copies; standard deviation [SD], 0.8) compared to DNA extracted from serum not influenced by clot formation (mean, 4.2 copies; SD, 1.3). Although it is likely that a substantial amount of *A. fumigatus* DNA is bound during blood clot formation and consequently is unavailable for DNA extractions using serum, it was not possible to test the clot material, as optimized protocols are not available, and technical limitations prevent the processing of the entire clot material, which in this scenario was  $>2$  ml in volume. However, whole-blood cell pellets from centrifuged EDTA Vacutainers containing peripheral blood mononuclear cells were tested. Relatively small amounts of *A. fumigatus* DNA (mean, 0.4 input copies) were detectable in samples at both centers (center 1, 1/4 samples positive [threshold cycle  $\{C_T\}$  value, 51.9 cycles], center 2, 2/5 samples positive [ $C_T$  values, 40.2 and 42 cycles]), although it appears that the majority of the DNA remains in the plasma fraction (mean, 3.3 input copies).

## DISCUSSION

The European *Aspergillus* PCR Initiative (EAPCRI) has published standards for *Aspergillus* PCR and protocols for detecting *Aspergillus* DNA in whole blood (4) and serum (5). The identification of the critical stages of *Aspergillus* DNA extraction from both specimen types allowed the EAPCRI to propose a protocol that helps ensure optimal performance of *Aspergillus* PCR across laboratories. However, no such data exist for plasma, and no direct comparison between plasma and serum specimens has been performed. Thus, EAPCRI continued its efforts to evaluate the analytical performance of plasma compared with serum samples through the blinded distribution of simulated panels. In parallel, a multicenter clinical study was performed to compare the detec-

tion of *A. fumigatus* DNA isolated from plasma and serum obtained from hematological patients (6).

Plasma specimens were spiked with *A. fumigatus* genomic DNA. The group of 8 EAPCRI core laboratories was able to demonstrate that the  $C_q$  values obtained from plasma were significantly lower ( $P = 0.002$ , Fig. 3) than  $C_q$  values from serum (50, 25, and 5 ge).

Almost 15 years ago, Loeffler et al. (10) compared the sensitivities of *A. fumigatus* DNA detection from plasma and whole blood. Although plasma and whole-blood samples spiked with *Aspergillus* conidia showed an identical lower detection limit (10 CFU), the sensitivity of plasma PCR was inferior to that of PCR performed on whole-blood samples obtained from patients with proven IA. However, both DNA extraction and PCR amplification technology have advanced over the past decade. In addition, the quality of molecular diagnostics has advanced through numerous external quality assessment initiatives, including the EAPCRI, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (11), and the Quality Control for Molecular Diagnostics (QCMD) (<http://www.qcmd.org>). In a recent study comparing EAPCRI methods for the testing of serum and whole blood, there was a trend toward superior sensitivity when testing whole blood, but this did not reach statistical significance, and any benefit was outweighed by the simplicity of testing serum (8).

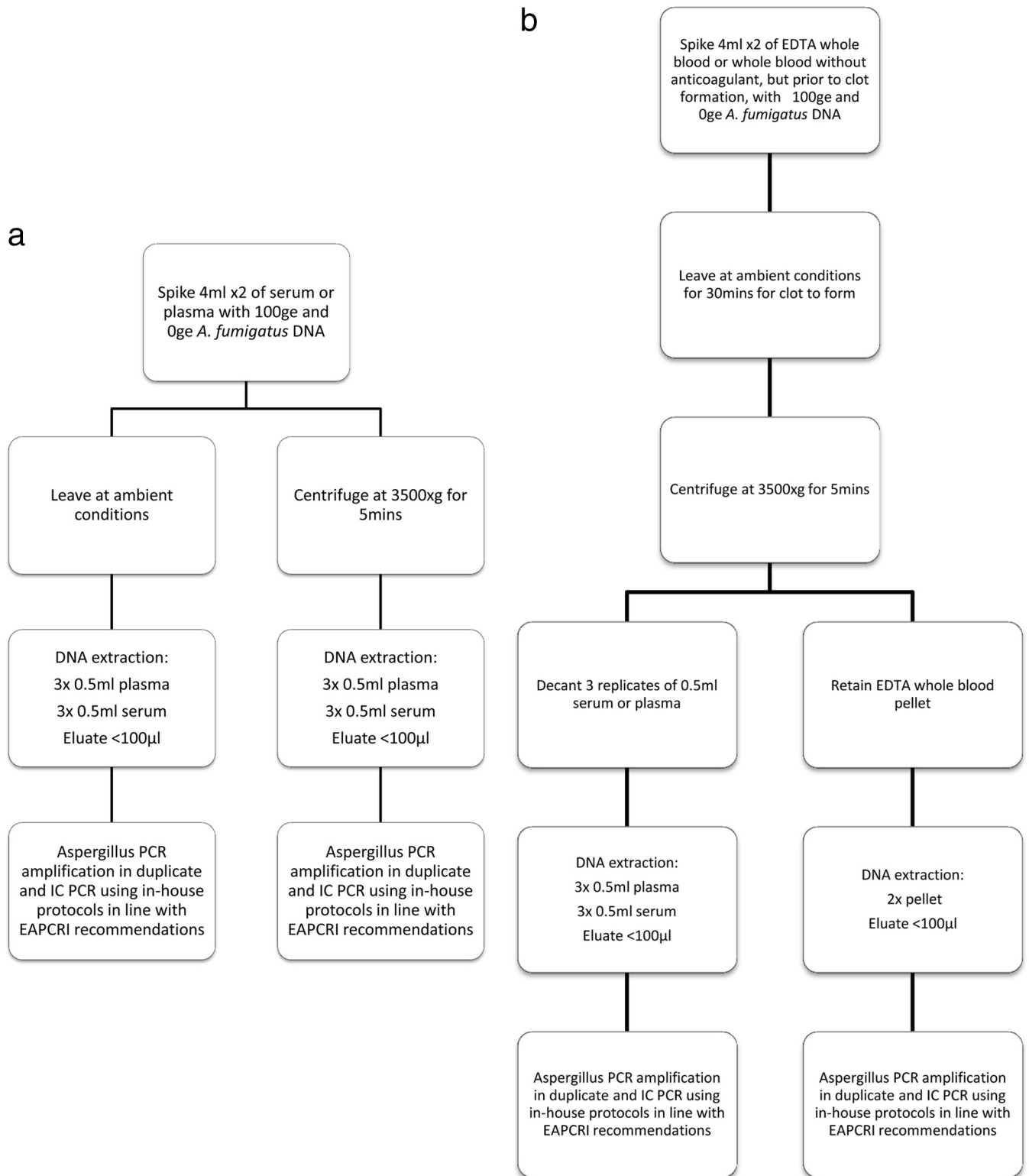
The current study demonstrated that after spiking whole blood, the PCR testing of plasma samples showed superior sensitivity to that of serum ( $P = 0.002$ , Fig. 3). To our knowledge, no data exist comparing the detection of *A. fumigatus* DNA detection in both serum and plasma. Lau et al. (12) showed that *Candida* DNA was detected more often in serum (71%) and plasma (75%) than in whole blood (54%) in a study of 109 patients with candidemia. However, there was no significant difference between plasma and serum, possibly because of the relatively small numbers of serum and plasma specimens that were tested ( $n = 29$  and 24, respectively) or the differences in fungal disease manifestation. With respect to IA, the concentration of galactomannan as determined by EIA was shown in another study to be significantly higher in plasma than in serum ( $P: 0.0398$ ) and may have been associated with clot formation (3).

The presence of compounds that may interfere with molecular assays is always a concern when processing clinical samples. Compared to serum, plasma samples contain various coagulation factors that lead to the conversion of fibrinogen to fibrin and clot

TABLE 5 Effect of centrifugation at  $3,500 \times g$  on DNA recovery (as indicated by mean  $C_q$  values) from spiked serum and plasma specimens<sup>a</sup>

Material	Mean $C_q$ value (cycles) at center:	
	1	2
Plasma centrifuged	47.1	37.8
Plasma noncentrifuged	46.9	37.6
Serum centrifuged	46.1	37.2
Serum noncentrifuged	47.2	37.0

<sup>a</sup> The two centers revealed no significant differences in  $C_q$  values between centrifuged and noncentrifuged plasma and serum samples, respectively ( $P > 0.05$ ).



**FIG 4** (a) Flow diagram highlighting the process for determining the effect of centrifugation on the availability of *Aspergillus* DNA in serum and plasma samples. (b) Flow diagram highlighting the process for determining the effect of clot formation on the availability of *Aspergillus* DNA in serum compared to the processing of plasma samples. IC, internal control.

formation. Fibrinogen is a soluble protein, which is exclusively found in plasma (normal range, 150 to 400 mg/dl) but not in serum, and it has been shown to interact with magnesium, which is also an essential component of the PCR (13). If this interaction results in lowering the magnesium concentration, it might result in PCR failure. All DNA extraction protocols used by the 8 EAPCRI study centers (Table 4) provided plasma DNA eluates that yielded superior sensitivity and lower  $C_q$  values than those of serum, indicating that the additional components in plasma had no effect on PCR efficiency. In addition, the source of anticoagulant is also important; García et al. (14) showed in rat blood that when comparing sodium citrate, heparin, and tripotassium-EDTA as anticoagulants, only tripotassium-EDTA did not interfere with PCR used to diagnose IA.

The EAPCRI recommendations for *Aspergillus* PCR in serum included the use of a minimum volume of 0.5 ml of serum as starting material, DNA elution in a volume of <100  $\mu$ l ( $P = 0.003$ ), and the use of an internal control (5). For plasma, bivariate linear regression was used to evaluate similar covariates, finding that there was a statistically significant positive association between PCR sensitivity and larger sample volumes ( $\geq 0.5$  ml of plasma), the use of  $\geq 2$  replicates (from a single eluate), and an internal control. While the DNA elution volume itself is not significantly associated with PCR sensitivity, the ratio of the initial plasma sample volume to the subsequent elution volume was significantly associated ( $Z$  score =  $-2.32$ ,  $P = 0.02$ , Table 1). All 8 core centers complied with EAPCRI serum recommendations on elution volume (<100  $\mu$ l [5]), so a correlation with PCR sensitivity is to be expected. However, the use of an internal control was correlated and found to be statistically significant. Although this parameter is only indirectly associated with the detection of *A. fumigatus* DNA, it reflects the degree of diligence and accuracy of an individual laboratory.

Interestingly, *A. fumigatus* genomic DNA, spiked into EDTA whole blood and left untouched for 30 min, was detectable in small amounts in some leukocyte pellets and in the cell-free fraction. This suggests that circulating extracellular DNA reaches the cell pellet either by gravitational force or due to binding to leukocyte surface receptors. Indeed, Bennett et al. (15) showed that there is a common binding site for DNA on white blood cells. However, in a comparison to the detection of *A. fumigatus* DNA in serum clots, only 3/9 leukocyte pellets showed weak PCR-positive results, whereas McCulloch et al. (16) reported significantly more *Aspergillus* DNA detectable in serum clots than that in the cell-free fraction ( $P < 0.001$ ; 95% CI, 2.24 to 6.48). These authors found that the average  $C_q$  value for the clot sample was 2.38 cycles lower than that for EDTA blood and 3.69 cycles lower than that for serum. While this equates to 7.9- and 12-fold increases in DNA yield from the clot in EDTA blood and serum, respectively, difficulties in processing these samples limit their use in routine practice. This observation might partially explain our observation that  $C_q$  values from native serum (used before clotting) developed consistently later than  $C_q$  values obtained from native plasma samples, which were spiked with identical number of genome equivalents.

In conclusion, the analytical sensitivity of plasma, as determined by a multicenter evaluation of PCR-based detection of *A. fumigatus* DNA, is superior to that with serum. Recommendations published by EAPCRI for serum (5), including sample volume, a minimum of duplicate PCR testing of each DNA extract, and an internal control, can be applied to plasma.

To confirm the analytical findings described here, a parallel multicenter clinical study (6) was performed. The retrospective case-control study supports the findings of this analytical paper and comes to the conclusion that there is a trend toward increased sensitivity of plasma, although this did not reach significance ( $P = 0.0897$ ), and positivity was most frequently earliest when testing plasma by PCR.

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