Clinical Performance of *Aspergillus* PCR for Testing Serum and Plasma: a Study by the European *Aspergillus* PCR Initiative

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Aspergillus PCR testing of serum provides technical simplicity but with potentially reduced sensitivity compared to whole-blood testing. With diseases for which screening to exclude disease represents an optimal strategy, sensitivity is paramount. The associated analytical study confirmed that DNA concentrations were greater in plasma than those in serum. The aim of the current investigation was to confirm analytical findings by comparing the performance of *Aspergillus* PCR testing of plasma and serum in the clinical setting. Standardized *Aspergillus* PCR was performed on plasma and serum samples concurrently obtained from hematology patients in a multicenter retrospective anonymous case-control study, with cases diagnosed according to 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) consensus definitions (19 proven/probable cases and 42 controls). Clinical performance and clinical utility (time to positivity) were calculated for both kinds of samples. The sensitivity and specificity for *Aspergillus* PCR when testing serum were 68.4% and 76.2%, respectively, and for plasma, they were 94.7% and 83.3%, respectively. Eighty-five percent of serum and plasma PCR results were concordant. On average, plasma PCR was positive 16.8 days before diagnosis and was the earliest indicator of infection in 13 cases, combined with other biomarkers in five cases. On average, serum PCR was positive 10.8 days before diagnosis and was the earliest indicator of infection in six cases, combined with other biomarkers in three cases. These results confirm the analytical finding that the sensitivity of *Aspergillus* PCR using plasma is superior to that using serum. PCR positivity occurs earlier when testing plasma and provides sufficient sensitivity for the screening of invasive aspergillosis while maintaining methodological simplicity.

Attempts to standardize PCR to aid in the diagnosis of invasive aspergillosis (IA) have provided an optimal methodology for the testing of serum and whole blood (WB) (1, 2). A multicenter comparison of the clinical performance of PCR showed a trend toward improved sensitivity (85% versus 79%) and earlier positivity when testing WB compared to serum (3). Even in very high-risk patient groups, the incidence of IA rarely exceeds 15%, and diagnostic tests are best used as a screening tool to exclude disease, reducing the need for empirical antifungal therapy through their excellent negative predictive value (4–6). Any reduction in sensitivity, albeit even if statistically insignificant, might result in an increase in false-negative results, a lack of confidence in the assay, and continued reliance on empirical therapy.

Testing plasma may improve sensitivity compared to PCR detection of *Aspergillus* DNA in serum. A study comparing the performance of a galactomannan (GM)-enzyme immunoassay (EIA) in serum and plasma showed that the mean index generated by testing plasma was significantly greater than that for serum (0.315 versus 0.279, respectively; $P = 0.0398$), and more cases were diagnosed (7). It was proposed that the blood clot had reduced the available GM within serum samples. In an animal model study evaluating *Aspergillus* PCR testing on different blood fractions, the clot was found to contain the greatest concentration of *Aspergillus* DNA (8). Testing the clot material in routine clinical diagnostics is feasible but has practical limitations. A solution is to simply avoid clot formation by testing plasma.

The accompanying paper (9) describes a multicenter analytical evaluation of *Aspergillus* PCR testing of serum and plasma that confirmed this difference in performance. When whole blood was spiked with clinically expected quantities of *A. fumigatus* genomic DNA and subsequently fractionated into serum and plasma, it was found that positivity, as determined by *Aspergillus* PCR crossing points (quantification cycle [C_q]) were significantly earlier for plasma compared to those for serum, indicating that there was a greater fungal burden available in plasma than in serum, as PCR efficiency appeared to be comparable for the two samples (9).

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TABLE 1 Patient demographics with EORTC-MSG diagnosis of IA[a]

Demographic[b]	Proven/probable IA (n = 19)	NEF (n = 42)[c]
No. of males/no. of females	13/6	25/17
Age (mean [range]) (yr)	52.2 (24–73)	52.5 (21–78)
Underlying condition (no.)		
AML/MDS	12	22
Lymphoma	4	5
Myeloma		6
ALL	1	2
AA	1	1
CML/CLL	1	4
Other		2[d]
IPA (no.)	15	None
IPA/sinusitis (no.)	4	

[a] IA, invasive aspergillosis.
[b] AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; AA, aplastic anemia; CLL, chronic lymphoblastic leukemia; CML, chronic myeloid leukemia; IPA, invasive pulmonary aspergillosis. Lymphoma comprises Hodgkin, non-Hodgkin lymphoma and diffuse large B-cell lymphoma.
[c] NEF, no evidence of fungal disease (control population).
[d] Includes one case of myelofibrosis and one case of combined immunodeficiency with lymphoproliferation.

To further investigate the difference in available *Aspergillus* DNA load in serum and plasma, a retrospective case-control study was undertaken in two centers using DNA extraction and *Aspergillus* PCR methodology, in accordance with the recent European *Aspergillus* PCR Initiative (EAPCRI) recommendations (2).

MATERIALS AND METHODS

Study design and patient population. The clinical evaluation was performed over 12 months by two centers experienced in *Aspergillus* PCR testing based in Public Health Wales, Microbiology Cardiff (PHW), Cardiff, United Kingdom, and the University of Würzburg (UKW), Würzburg, Germany. Initially, EDTA whole-blood and clotted-blood samples were concurrently sent at the request of a consulting clinician for routine *Aspergillus* PCR and GM-EIA diagnostic investigations as part of local screening strategies. On completion of routine testing, excess plasma and serum were stored at −80°C for quality control and performance assessment purposes. Retrospective testing on excess serum and plasma was performed as an anonymous case-control study to assess the performance of *Aspergillus* PCR assays, and it did not impact patient management. All clinical information was gathered as part of routine diagnostic care, and no further information specific to this study was required. The study was approved by local ethical and research and development boards.

Cases were selected on the basis of a diagnosis of proven/probable IA, categorized using 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, relying on GM-EIA as the mycological criterion for defining probable cases (10). The PCR result was not used in defining disease. Cases of probable IA in each center were confirmed by the other center reviewing the radiological evidence. Controls were selected on the basis of risk factors, but possible IA cases were not included in the study, due to diagnostic ambiguity. The baseline patient demographics are shown in Table 1.

Galactomannan EIA. GM-EIA testing was performed using the Platelia *Aspergillus* EIA (Bio-Rad), according to the manufacturer's instructions, and all runs were required to meet the designated validity criteria. The GM-EIA was considered positive when the optical density was ≥0.5.

DNA extraction. All manual DNA extraction steps were performed in a class 2 laminar flow cabinet and were compliant with EAPCRI recom-

mendations (2). At PHW, DNA was extracted from 0.5 ml of undiluted serum and plasma using the commercially available DSP virus kit on the Qiagen EZ1 XL Advance automated extractor, according to the manufacturer's instructions, and DNA was eluted in 60 μl. At UKW, DNA was extracted from 1.0 ml of undiluted serum and plasma using the commercially available Qiagen UltraSens virus kit, according to the manufacturer's instructions, with the following modifications: (i) no carrier RNA was used, (ii) lysate centrifugation was adjusted to 3,000 × g, and (iii) the elution buffer volume was increased to 70 μl and was incubated on the column at room temperature for 2 min, followed by centrifugation of the tubes for 2 min (3). At PHW, extractions included a positive control (serum/plasma spiked with 20 genome equivalents [ge] of *A. fumigatus* DNA) and a negative control (serum/plasma from a healthy donor), while at UKW, only a negative control was utilized.

PCR amplification. The PCR methods showed comparable performance when testing EAPRCI panels and were conducted in accordance with the EAPCRI recommendations, including the use of an internal control PCR to monitor inhibition (1, 2).

At UKW, an *Aspergillus*-specific real-time PCR assay targeting the internal transcribed spacer 1 (ITS1)/5.8S rRNA gene region was used as previously described (3). The final reaction volume was 21 μl, containing 0.3 μM forward primer, 0.3 μM reverse primer, 0.15 μM hydrolysis probe, 10 μl of TaqMan gene expression master mix (Applied Biosystems), and 10 μl of template DNA. Amplification was performed using a StepOnePlus machine (Applied Biosystems), with the following conditions: 95°C for 10 min, and 60 cycles of 95°C for 5 s, 54°C for 15 s, and 72°C for 1 s. Negative and positive PCR controls were included for every run.

At PHW, the *Aspergillus* real-time PCR test was performed as a single-round assay using a Rotor-Gene Q high-resolution melting (HRM) instrument (Qiagen, United Kingdom) targeting the 28S rRNA gene, as previously described (11). The final reaction volume was 50 μl, containing 0.75 μM primers, 0.4 μM hydrolysis probe, 4 mM MgCl$_2$, 5 μl of Roche LightCycler hybridization master mix (Roche, United Kingdom), 9 μl of molecular-grade water, and 15 μl of template DNA. PCR controls in the form of cloned PCR products (300, 30, and 3 input copies) and no-template molecular-grade water were included to monitor PCR performance.

Both centers tested each sample in duplicate. The analytical threshold was defined by at least one replicate generating a positive signal at any cycle during the PCR.

Statistical analysis. Patients with proven and probable IA were classified as cases, and patients with no evidence of fungal disease were categorized as controls. The clinical performance was defined using both single-positive PCR and multiple-positive (≥2) PCR thresholds. The validity of positive PCR was determined by comparing the rate of positivity in samples originating from cases compared to the rate of false positivity in control samples. Sensitivity, specificity, positive and negative likelihood ratios, and the diagnostic odds ratios of the PCR assays were calculated using 2 by 2 tables. Predictive values were not calculated because of the artificially high prevalence of IA (proven/probable IA, 19/62 [30.6%]), which is typical of case-control studies. Ninety-five percent confidence intervals were generated for each proportionate value and, where required, P values (Fisher's exact test; significance threshold, P = 0.05) were calculated to determine the significance of the difference between rates (12). A Kappa statistic was calculated to test the agreement between PCR results and was applied individually to the total population and for cases and controls. The observed agreement between PCR results and between PCR and GM-EIA results was also calculated. For cases of IA, the first assay result from serum PCR, plasma PCR, and GM-EIA to become positive was identified, and the timing of PCR positivity was compared to that of a diagnosis of IA, as determined by the date at which histology or both radiology and GM-EIA were positive.

C_q values for PCR-positive results and for cases and controls were also compared, with both C_q values being included for the confirmed positive results. The Kolmogorov-Smirnov test, D'Agostino-Pearson test, and Shapiro-Wilk test were applied to all populations to determine normality.

TABLE 2 Sample concordance when testing serum and plasma specimens by *Aspergillus* PCR

Plasma PCR results by sample group (no.)	No. of samples with serum PCR result of:	
	Positive	Negative
All samples ($n = 393$)		
Positive	22	31
Negative	26	314
IA cases ($n = 190$)		
Positive	22	24
Negative	14	130
NEF patients ($n = 203$)		
Positive	0	7
Negative	12	184

TABLE 3 Sample concordance between serum or plasma PCR and GM-EIA

No. of PCR results of ($n = 393$):	No. with GM-EIA result of:	
	Positive	Negative
Serum positive	15	33
Serum negative	30	315
Plasma positive	19	33
Plasma negative	26	315

To overcome differences in the population distribution, the significance of differences in serum-positive PCR results in cases and controls were compared using an unpaired Student *t* test, whereas the significance of differences between plasma-positive PCR results in cases and controls were compared using a Mann-Whitney test. For both investigations, significance was defined by a *P* value of 0.05 using a two-tailed test. A receiver operating characteristic (ROC) curve was generated using the earliest C_q value generated per patient to determine whether a particular C_q threshold provided optimal PCR performance when testing both serum and plasma. For this analysis, in patients negative by PCR, a C_q value of 50 cycles was applied, representing the lowest number of PCR cycles performed.

RESULTS

Sample population. One case of proven IA, 18 episodes (17 cases, as one patient had two episodes 1 year apart and was considered to have clinically improved between episodes) of probable IA, and 42 patients with no evidence of invasive fungal disease formed the controls. A total of 393 paired serum and plasma samples were tested. One hundred ninety samples (median per patient, 10 samples [range, 3 to 25 samples]; median sampling period, 58 days [range, 6 to 119 days]) originated from proven/probable cases of IA, and samples prior to and after diagnosis were included to determine any differences in sample type performance. Two hundred three samples were from control patients (median per patient, 4 [range, 3 to 9]; median sampling period, 31 days [range, 7 to 297]) and were temporally interspersed between the episodes of proven/probable IA. Although the median sampling period for the controls was less than that for the cases, it represented the typical period of time in which multiple-PCR-positive results were encountered with respect to a clinical diagnosis (median, 24 days; range, 6 to 80 days).

Sample positivity rates. The overall positivity rates for PCR testing of plasma and serum were 53/393 (13.5%; 95% confidence interval [CI], 10.5 to 17.2%) and 48/393 (12.2%; 95% CI, 9.3 to 15.8%), respectively, and were not significantly different ($P = 0.7483$). The true-positive rates in cases were significantly higher than the false-positive rates in the control population for both sample types ($P = <0.0001$), indicating that PCR positivity was associated with IA. For plasma PCR, the positivity rate in cases was 46/190 (24.2%; 95% CI, 18.7 to 30.8%) compared to 7/203 (3.4%; 95% CI, 1.7 to 7.0%) in the control population. Serum PCR positivity in cases was 36/190 (18.9%; 95% CI, 14.0 to 25.1%) com-

pared to a false-positive rate of 12/203 (5.9%; 95% CI, 3.4 to 10.1%). There was no significant difference between plasma and serum PCR positivity rates when testing samples originating from cases ($P = 0.2617$) or controls ($P = 0.3476$).

Sample concordance. The observed sample agreement between PCR testing of serum and plasma for the total population was 85.5% (95% CI, 80.4 to 89.5%), generating a Kappa statistic of 0.353, which represents fair agreement (Table 2). For the samples originating from cases, 152/190 (80.0%; 95% CI, 71.6 to 86.5%; 22 PCR positive and 130 PCR negative) were concordant in plasma and serum, and 38/190 (24 plasma PCR positive/serum PCR negative and 14 plasma PCR negative/serum PCR positive) were conflicting, yielding a Kappa of 0.411, which represents moderate agreement. For the samples originating from the controls, 184/203 (90.6%; 95% CI, 85.9 to 93.9%) that were negative by both plasma and serum PCR and 19 (7 plasma PCR positive/serum PCR negative and 12 plasma PCR negative/serum PCR positive) gave conflicting results. It was not possible to calculate a Kappa statistic for these.

The overall observed agreement between plasma PCR and GM-EIA testing and serum PCR between and GM-EIA was 85.0% (95% CI, 79.8 to 89.1%) and 84.0% (95% CI, 78.7 to 88.2%), respectively, yielding Kappa statistics of 0.232 and 0.307, which indicate poor and fair agreement, respectively (Table 3).

Comparison of crossing point (C_q) values. For plasma, C_q values for positive samples occurred significantly earlier for cases (mean, 40.8 cycles; 95% CI, 40.1 to 41.5 cycles) than for controls (mean, 44.5 cycles; 95% CI, 43.0 to 45.9 cycles) (Mann-Whitney test, $P = 0.0005$). There was no difference in a comparison of C_q values for PCR-positive serum samples originating from cases (mean, 40.9 cycles; 95% CI, 40.3 to 41.5 cycles) or controls (mean, 41.1 cycles; 95% CI, 40.2 to 42.1 cycles) (unpaired *t* test $P = 0.7478$) (Fig. 1). C_q values for false-positive plasma PCR results originating from the control population occurred significantly later than those for false-positive serum PCR results (Mann-Whitney test, $P = 0.0009$). There was no difference in C_q values in the plasma or serum PCR-positive results of cases of IA (Mann-Whitney test, $P = 0.2925$).

For plasma, a threshold of 42.3 cycles appeared to be optimal, generating sensitivity and specificity values of 79.0% (95% CI, 54.4 to 94.0%) and 100% (95% CI, 91.6 to 100%), respectively (Fig. 2). A positivity threshold of 45 cycles provides sufficient sensitivity to exclude IA when testing plasma, resulting in a sensitivity of 94.7% and a negative likelihood ratio of 0.06. As the C_q values generated by serum PCR were similar for cases and controls, it was not possible to determine an optimal C_q threshold using ROC analysis (Fig. 2), and a determination of the performance was better achieved by qualitative interpretation of results (positive/negative) (Table 4). The area under the ROC curve was signifi-
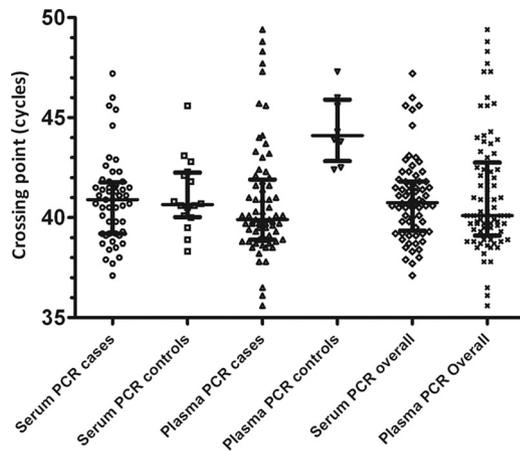
FIG 1 Comparison of crossing point values for positive PCR results when testing serum and plasma. The horizontal bar represents the median value and the error bars the interquartile range.


FIG 2 Receiver operating characteristic (ROC) curve for *Aspergillus* PCR testing of serum and plasma. The ROC curve was calculated using the earliest C_q value per patient, with patients negative by PCR given a C_q value of 50 cycles. Each point represents a C_q value.

cantly greater for plasma (area under the concentration-time curve [AUC], 0.9555; 95% CI, 0.8889 to 1.0000) than that for serum PCR (AUC, 0.7318; 95% CI, 0.5886 to 0.8751).

Clinical performance. The clinical performance characteristics for PCR when testing plasma and serum are shown in Table 4. There was a trend toward increasing sensitivity when testing plasma (94.7%) compared to serum (68.4%). Using a clinical threshold of a single-PCR-positive result provided optimal sensitivity but at the expense of specificity, and a single positive result could not be used to accurately rule in IA in either plasma (likelihood positive, 5.7) or serum (likelihood positive, 2.9). The use of a clinical threshold of ≥2 positive PCR results within the same episode improved PCR specificity when testing plasma (100%) and serum (95.2%) and provided the best diagnostic odds ratio for *Aspergillus* PCR. For cases with multiple PCR-positive results (plasma PCR testing, 13/19; serum PCR testing, 8/19), all had ≥2 positive results within a 3-week period of testing, but when testing plasma, 8/13 patients had consecutive PCR-positive results compared to 6/8 when testing serum. A diagnostic approach requiring a PCR-positive result in both plasma and serum results in a sensitivity and specificity of 68.4% (95% CI, 46.0 to 84.6%) and 95.2% (95% CI, 79.0 to 99.4%), respectively, but provided no benefit over testing plasma alone using a clinical threshold of ≥2 positive PCR results within the same episode.

On 13 occasions, plasma PCR was the earliest assay with a positive result; it was the sole first positive assay result on eight occasions, and it was concurrently positive in combination with other assays on five occasions (Fig. 3). GM-EIA was the earliest positive assay result on seven occasions, it was the sole first positive result on three occasions, and it was concurrently positive with other assay results on four occasions (Fig. 3). Serum PCR was the earliest positive result on six occasions; it was the sole first positive result on three occasions, and it was concurrently positive with other assays on three occasions. The first PCR-positive result was, on average, 16.8 (95% CI, 3.6 to 30.0) and 10.8 (95% CI, −8.5 to 30.1) days, respectively, earlier than the diagnosis of IA, when testing plasma and serum. However, at a prevalence of 10%, a single positive plasma or serum PCR result provides probabilities of IA of 38.8% and 24.4%, respectively. Multiple-positive plasma and serum PCR results improve the probability of IA to >88.4%
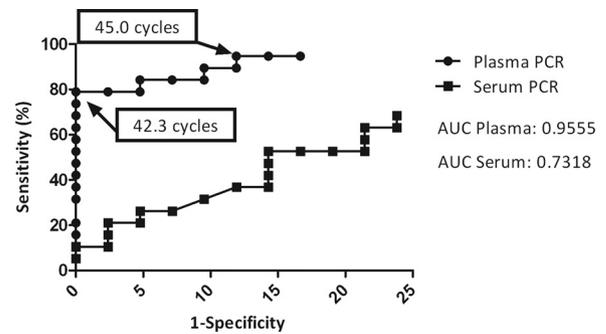
and 49.4%, respectively. In waiting for these confirmatory results, the mean time to positivity prior to a combined radiological/serological diagnosis is reduced to 7.3 (95% CI, −5.7 to 20.3) and 4.0 (95% CI, −3.0 to 11.0) days for plasma and serum PCR, respectively.

DISCUSSION

This clinical evaluation supports the analytical evidence confirming free DNA to be more abundant in plasma than in serum. Plasma PCR sensitivity (94.7%) was higher than that of serum PCR (68.4%), generating a negative likelihood ratio of 0.06, which is sufficient to exclude disease in patients not receiving mold-active prophylaxis/treatment (13). Unfortunately, it was not possible to identify whether individual patients were receiving mold-active prophylaxis without breaking anonymity. However, the effects of prophylaxis on decreasing the available fungal burden and assay sensitivity would apply equally to serum and plasma. The sensitivity of serum PCR in this current study was comparable to that for serum in a previous study comparing whole-blood (WB) and serum PCR performance, whereas the current plasma PCR sensitivity was comparable to that of previous WB PCR (3). Increasing assay stringency by using a clinical threshold of ≥2 PCR-positive results improved plasma PCR specificity to 100%, with a positive likelihood ratio of >68.4; this would allow IA to be reliably diagnosed (Table 4), although it would delay the diagnosis by a median of 14 days (range, 2 to 24 days) compared to that with a single PCR-positive result, limiting clinical utility. Plasma was the first indicator of IA in 68% of cases, albeit in combination with other biomarker tests in just under half.

In the analytical study, the C_q values associated with true-positive PCR results with plasma occurred significantly earlier than those with serum. Conversely, in the clinical study, there was no significant difference in C_q values for true-positive results when testing plasma or serum, although false-positive results occurred significantly later when testing plasma, which allowed a positivity threshold to be determined. In the analytical study, the majority of samples (23/27) gave concordant positive results. In the clinical study, most positive samples (53/74) were either positive in serum or plasma, with only 21 being positive in both. A comparison of only concordantly positive serum and plasma samples showed a median value for plasma of 39.8 cycles, which occurred approximately 1 cycle earlier than that found for serum (median C_q, 40.5), ($P = 0.4373$).

TABLE 4 Performance of *Aspergillus* PCR when testing serum and plasma samples

Parameter[a]	Serum		Plasma	
	Single PCR positive threshold	Multiple (≥2) PCR positive threshold	Single PCR positive threshold	Multiple (≥2) PCR positive threshold
Sensitivity (no./total no., % [95% CI])	13/19, 68.4 (46.0–84.6)	8/19, 42.1 (23.1–63.7)	18/19, 94.7 (75.4–99.1)	13/19, 68.4 (46.0–84.6)
Specificity (no./total no., % [95% CI])	32/42, 76.2 (61.5–86.5)	40/42, 95.2 (84.2–98.7)	35/42, 83.3 (69.4–91.7)	42/42, 100 (85.1–100)
Positive LR	2.9	8.8	5.7	>68.4[a]
Negative LR	0.4	0.6	0.06	0.32
DOR	7.5	14.7	95.0	>213.8[a]

[a] 95% CI, 95% confidence interval; LR, likelihood ratio; DOR, diagnostic odds ratio. Positive LR and DOR values were calculated with a specificity of 99% to overcome ∞ that would be generated with 100% specificity.

The limited concordance between serum and plasma PCR and between PCR and GM-EIA likely reflects that levels of *Aspergillus* DNA in the circulation are low and at the limit of detection of real-time PCR. As a result, PCR positivity is often not reproducible, although it is still considered significant ([14]). Furthermore, the samples had been stored at −80°C for an average of 9 months. Consequently, degradation of the DNA target was a possibility ([15]). For serum, no prior testing had been performed, although for plasma PCR, positives before and after C_q values were not significantly different ($P = 0.097$). Given that the overall sample positivity rates were similar and the mean C_q values for cases of IA when testing serum (40.9 cycles) and plasma (40.8 cycles) were almost identical, the effect of storage appears to be minimal. One caveat is that the variability of real-time PCR C_q values at this late stage of amplification is wide, and prospective clinical comparison is required to overcome any associated limitations.

In the clinical study, the concentration of circulatory DNA was lower than that encountered in the analytical evaluation. The lack of reproducibility and the variability of C_q values associated with testing DNA at these concentrations might explain why the earlier C_q values associated with plasma PCR analytical testing were not as evident in the clinical study. From an analytical perspective, it is essential that all DNA concentrations be within the reproducible detection range, as testing outside these boundaries might generate random erroneous results leading to false conclusions. Nevertheless, clinical performance appeared to be superior in plasma, as the sensitivity was comparable to that found for WB PCR testing (86%; 95% CI, 73 to 93%) ([16]). However, automated sample processing is simpler, being the same as that used for serum.

Targeting free circulating *Aspergillus* DNA (DNAemia) is tech-nically less demanding than targeting DNA associated within the circulating fungal cells ([2]). The analytical evaluation showed that the amount of cell-free DNA is greater in plasma than that in serum, which is likely the result of DNA becoming trapped during clot formation ([9]). DNA concentrations in clot material have been shown to be higher than those found in serum, supporting this hypothesis ([8]). Moreover, the use of clots in daily practice is technically challenging. Usually, blood is drawn into 4- to 6-ml Vacutainers, and subsequent clot formation will occupy approximately half of this volume, so it cannot be processed unless it is divided into smaller amounts. Testing a single aliquot of this divided material would be preferable, but it assumes an equal distribution of *Aspergillus* DNA within the clot. Whether this is the case has not been determined and might result in false negativity. Testing multiple aliquots increases costs and the complexity of interpretation, as positivity may vary between aliquots. Since the use of plasma precludes clot formation, there may be a reduction in the loss of DNA.
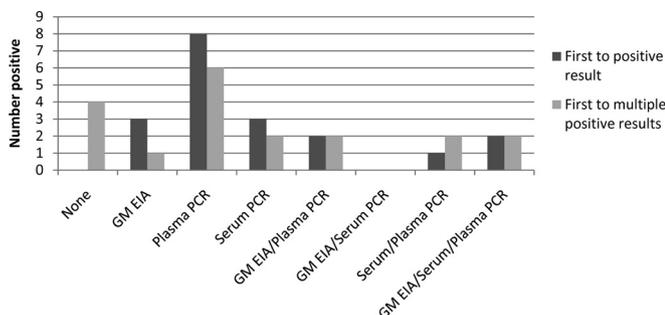
In summary, testing of serum and plasma by PCR is technically less demanding than that of WB and allows *Aspergillus* PCR to be performed in routine molecular diagnostic laboratories alongside viral and bacterial assays. This multicenter clinical evaluation supports the analytical findings in the accompanying manuscript ([9]), in which analytical sensitivity in plasma appeared superior to that of serum. In the clinical evaluation, there was a trend toward increased sensitivity, and positivity occurred earlier when testing plasma. Despite the limitations (population size and retrospective testing) of this case-control study, the results justify further prospective cohort or randomized control studies to confirm clinical validity and accurately define the clinical utility of testing different types of specimens.

FIG 3 Breakdown of the first biomarker assay to be positive among the 19 cases of invasive aspergillosis.

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