Analytical comparison of *in vitro* spiked human serum and plasma for the PCR-based detection of *Aspergillus fumigatus* DNA – a study by the European Aspergillus PCR Initiative

Juergen Loeffler[a]*, Carlo Mengoli[b], Jan Springer[a], Stephane Bretagne[c], Manuel Cuenca Estrella[d], Lena Klingspor[e], Katrien Lagrou[f], Willem JG Melchers[g], C. Oliver Morton[h], Rosemary A. Barnes[i], J. Peter Donnelly[g], and P. Lewis White[j], on behalf of the European Aspergillus PCR Initiative

Affiliations: [a]Wuerzburg University, Wuerzburg, Germany, [b]University of Padua, Padua, Italy, [c]Paris Diderot University, Saint Louis Hospital-APHP, Paris, France; [d]Spanish National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain; [e]Karolinska University Hospital, Stockholm, Sweden; [f]National Reference Center for Mycosis, University Hospitals Leuven, Leuven, Belgium and Department of Microbiology and Immunology, KU Leuven, Leuven, Belgium; [g]Radboud University Medical Centre, Nijmegen, The Netherlands, [h]University of Western Sydney, Sydney, NSW, Australia; [i]Cardiff University, UHW, Cardiff; [j]Public Health Wales Microbiology Cardiff, UK.

*Correspondence
Prof. Dr. Jürgen Löffler
University Hospital Wuerzburg
Medical Hospital II, Building C11
97080 Wuerzburg, Germany
Telephone +49 931 20136412, Fax: +49 931 20136409
Mailto: Loeffler_J@ukw.de

Abstract

The use of serum or plasma for *Aspergillus* PCR testing facilitates automated and standardised technology. Recommendations for serum testing are available, and while serum and plasma are regularly considered inter-changeable for fungal diagnostics, differences in GM-ELISA performance have been reported and attributed to clot formation. Therefore it is important to assess plasma PCR testing to determine if previous recommendations for serum are applicable, and also compare analytical performance with serum PCR.

Molecular methods testing serum and plasma were compared through multi-centre 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 regression analysis performed to identify variables that enhanced plasma PCR performance.

When testing plasma, sample volume, pre/post-extraction volume ratio, PCR reaction volume, duplicate testing and the use of an internal control PCR were positively associated with performance. When whole blood samples were spiked 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 ($p = 0.028$).

Summary: Technically there are no major differences for 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 could improve performance while maintaining the methodological simplicity of serum testing.

Introduction

Invasive aspergillosis (IA) represents a serious health problem for the immunocompromised patient, especially those undergoing cancer chemotherapy, receiving corticosteroid therapy or immunosuppression to avoid allograft rejection or solid organ rejection. *Aspergillus fumigatus* is a ubiquitous mould, and the most frequent cause of Aspergillosis in humans causing a wide spectrum of diseases ranging from allergic 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, that can be as high as 90% with cerebral disease (5). Hence, there is an urgent need for an accurate and standardized, diagnostic approach for IA.

Performance of mycological diagnostics when testing serum or plasma samples is assumed to be similar. The revised EORTC-MSG definitions include galactomannan (GM) EIA for testing of both serum and plasma (3), although, the testing of plasma 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 vs 0.279, *P*: 0.0398 (13)]. Moreover, four possible IA cases would have been classified as probable IA had plasma been tested. The authors hypothesised 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, affecting PCR performance.

Journal of Clinical Microbiology

This manuscript 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 (12, 15). We report data on differences in analytical performances associated with the 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.

As a companion to this analytical *in vitro* study, a further multi-centre clinical study, which compares performances of plasma and serum was performed in parallel (11).

Material and Methods

DNA source material

DNA was obtained from a sporulating culture of *Aspergillus fumigatus* (ATCC strain 1022). Conidia were harvested and DNA was extracted as described before (15).

Participants

The participants comprised of 8 laboratories representing the EAPCRI core facilities and an extended group of a further 15 laboratories. To maintain impartiality throughout the analytical process, all centres were given a numerical code to allow 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, Wuerzburg University Hospital, (Wuerzburg, Germany) and approved by the local ethical review board. Serum and plasma were fractionated by centrifugation and pooled respectively, before being distributed to Public Health Wales, Microbiology Cardiff, 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 (14). Panels were distributed by courier on dry ice. Participating centres were asked to confirm receipt, comment on the state of the panel (frozen or thawed) and keep specimens frozen at -80°C until testing.

Analytical testing was performed by using a four part process, as follows:

i) Assessing the analytical performance of *Aspergillus* PCR methods when testing serum

Initially, serum was divided into 3 x 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 ml of serum, respectively. The 35 ml serum batches were further divided into 1ml aliquots and frozen at -80°C until distribution on dry ice to the 23 centres. 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 comparison with the plasma results, but also determining centres with optimal results for the analyses of further panels, including the analysis of plasma samples (15).

ii) Assessing the analytical performance of *Aspergillus* PCR methods when testing plasma

Plasma was divided in 8 x 12 ml aliquots; two aliquots were retained to provide negative control samples, while six aliquots were spiked with varying concentrations of *A. fumigatus* genomic DNA (1000, 100, 50, 10, 5, 1 and 0 ge/ml) and divided into 1ml aliquots. Panels were distributed on dry ice to the 8 EAPCRI core centres for testing.

In keeping with previous studies, the threshold of detection, based on 95% variance was set at 10 ge/ml (15).

iii) Assessing the DNA availability in serum/plasma – 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 varying concentrations of genomic *A. fumigatus* DNA (100, 50, 10 and 0 ge/ml, Figure 1). Both, EDTA and clotted blood vacutainers were left for a minimum of 30 mins at room

134 temperature to allow the blood without anticoagulants to clot. Vacutainers were then
135 centrifuged (3500 *g* for 5 mins) to separate the cell free fractions, which were pooled
136 according to sample type and initial concentration of fungal burden. Each sample was then
137 divided into 0.5 ml aliquots (potential target burden range 50, 25 5 and 0 ge), frozen at -80°C
138 prior to distribution on dry ice. Serum and plasma samples were shipped on dry ice to the 8
139 EAPCRI core centres.
140
141 **iv) Investigating the effects of clot formation and blood fractionation on the**
142 **availability of *A. fumigatus* genomic DNA**
143 The following experiments were performed in a two-centre study in Cardiff and Wuerzburg
144 using the following identical spiking protocols, respectively.
145
146 **Impact of centrifugation**
147 Two 4 ml aliquots of serum and plasma were spiked with 100 genome equivalents of *A.*
148 *fumigatus* DNA, respectively. An aliquot of plasma and an aliquot of serum were centrifuged
149 at 3500 *g* for 5 mins, mimicking the centrifugation process necessary to fractionate blood
150 samples and potentially identifying any losses associated with this process. The other aliquot
151 of plasma and serum, equivalent to a direct spike control remained untouched at ambient
152 temperature until further processing by extraction. DNA was extracted from a minimum of
153 three replicates of 0.5 ml of serum and 0.5 ml plasma spiked but not centrifuged, and
154 minimum of three replicates of 0.5 ml of serum and 0.5 ml plasma spiked and centrifuged.
155 DNA was extracted using the in-house protocols of both centres (10, 16). Briefly, in Cardiff,
156 *Aspergillus* DNA was extracted using the Qiagen EZ1 DSP virus kit as per manufacturer's
157 instructions, with DNA eluted in 60 µl, in Wuerzburg, the QIAamp UltraSens Virus kit
158 (Qiagen) was used as described by the manufacturer and DNA was eluted in 70 µl. Both

protocols comply with the EAPCRI recommendations for testing serum (15). 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. Clotted blood was left at ambient temperature for 30 mins to coagulate, while EDTA blood remained untouched at room temperature for the same period. Samples were then centrifuged at 3500 *g* for 5 mins. After centrifugation, a minimum of three 0.5 ml aliquots of plasma and serum, as well as the cell pellet from the EDTA whole blood samples were extracted using local protocols and DNA was eluted in <100 µl. The clot was not processed as no EAPCRI validated procedures are currently available. PCR was performed in both centres using in-house amplification protocols and an inhibition control, all in accordance with EAPCRI guidelines (15).

Statistical analysis

Centres were requested to return both qualitative (positive/negative) and quantitative (quantification cycle [Cq value]) results within a designated time frame and provide detailed protocols for their DNA extraction and PCR amplification systems. Information required included sample volume used, extraction method, DNA elution volume, PCR method, PCR target, PCR template input volume, PCR total reaction volume, PCR amplification platform and internal control PCR results.

The correlation between Cq and genomic load was estimated by linear regression. The Cq was the dependent variable, whereas the explanatory variables were i) the genomic load (as log10 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

184 performed, using the centres as a grouping variable. This model was comparable to a
185 simplified calibration curve, also comparing the calibration lines concerning serum and
186 plasma. The model was graphically reported.
187 In addition, bivariate linear regression was performed to analyze possible associations
188 between PCR sensitivity and selected covariates (Table 1).
189

Results

i) Assessing the analytical performance of *Aspergillus* PCR methods when testing serum

The serum analysis in this QC panel compared favourably with the previous EAPCRI serum evaluation (15). All the 23 centres were able to reproducibly detect the sample that was spiked with 100ge 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 centres) and a positivity rate of 82% (14 / 17) in centres that strictly followed EAPCRI recommendations for serum. Five out of the 6 centres who did not follow EAPCRI recommendations were unable to detect 10 ge / ml. Centres that were unable to detect the 10 ge / ml did not routinely process serum samples (6/6) or used serum volumes below 0.5 ml (5/6, range 0.1 – 0.2 ml serum). In addition, these centres used only a small portion of their original serum sample volume in the subsequent PCR assay (mean 7.9%, range 1% - 25%), compared to a mean volume of 24.4% (range 16% - 82%) for centres that were able to detect 10 ge / ml ($p = 0.028$).

ii) Assessing the analytical performance of *Aspergillus* PCR methods when testing plasma

All centres that had received this plasma panel were requested to follow the EAPCRI guidelines for serum testing and their positivity rates in the related QC serum panel were 100%, 100% and 0% (100, 10, 0 genome equivalents / ml, respectively).

Table 2 summarizes the results of the 8 centres. No samples were found to be inhibitory to PCR amplification. All 8 centres achieved 100% positivity for fungal burdens between 5 ge/ml and 1000 ge/ml, while the sample spiked with 1 ge/ml was positive in a single sample of one centre only (Figure 2). Six out of 8 centres stated that they use either serum or plasma

or both in their routine diagnostic procedures (2 centres mainly tested bronchoalveolar lavages).

Centres used between 0.1 ml and 1 ml (mean 0.5 ml) of plasma for subsequent DNA extraction, 50 – 100 µl (mean 60 µl) of elution volume and 2 – 15 µl (mean 8.7 µl) of template volume for 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 volume used for PCR, the analysis of ≥2 replicates and the use of an internal control (Table 1).

To provide complete Cq values for a range of different plasma volumes, a linear mixed model was used, which predicts Cq values for hypothetical plasma volumes (0.1 ml – 0.5 ml) and various *A. fumigatus* DNA concentrations (5 – 1000 ge / ml). The model shows that both, plasma volume and DNA load influence Cq values (e. g. predicted Cq values for 5 ge / ml ranged depending on the plasma volume between 37.6 and 40.7 [Table 3]).

iii) Assessing the DNA availability in serum/plasma – 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 centres. 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 / -1.585) than the serum intercept ($z = -3.11$, $p = 0.002$). Interestingly, this shift in Cq values was achieved at all DNA concentrations tested (50, 25, 5 ge / ml, Figure 3). No inhibition was observed. Centre-specific methodological details are shown in Table 4. Individual performance of centres in this panel was significantly associated with the volume of sample used for DNA extraction.

While both centres using 0.2 ml of serum or plasma achieved suboptimal sensitivity not detecting the serum sample spiked with 5 ge / ml, centres using ≥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, which 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 losses of DNA during centrifugation necessary to fractionate blood samples, the recovery of DNA from spiked serum and plasma specimens that were centrifuged at 3500 *g* were compared to spiked samples that were not centrifuged. In both centres, there were no significant differences in Cq values between centrifuged or non-centrifuged samples, for either plasma or serum (Table 5). These results demonstrate that centrifugation at 3500 *g* is not sufficient to sediment free DNA from serum and plasma.

Interestingly, in both centres, plasma, obtained after centrifugation of spiked EDTA blood samples, showed earlier Cq values, compared to serum obtained from the corresponding clotted blood samples (difference of the mean Cq values between plasma and serum $\Delta = 2.26$, standard error = 0.45, $p = 0.0002$). The concentration of genomic DNA added to the PCR reaction was significantly lower ($p = 0.028$) in serum samples post clot formation (mean 1.9 copies, SD: 0.8) compared to DNA extracted from serum not influenced by clot formation (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 > 2ml 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 centres (centre 1: 1/4 samples positive [Ct value= 51.9], centre 2: 2/5 samples positive [Ct values=40.2, 42]), 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 (12) and serum (15). 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 multi-centre clinical study was performed to compare detection of *A. fumigatus* DNA isolated from plasma and serum obtained from haematological patients (11).

Plasma specimens were spiked with genomic *A. fumigatus* DNA. The group of 8 EAPCRI core laboratories were able to demonstrate that Cq values obtained from plasma were significantly lower ($p = 0.002$, Figure 3) than Cq values from sera (50, 25, 5 genome equivalents).

Almost 15 years ago, Loeffler *et al.* compared sensitivities of *A. fumigatus* DNA detection from plasma and whole blood (7). 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 has 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 (2) 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 towards superior sensitivity when testing whole blood, but this did not reach statistical significance, and any benefit was outweighed by the simplicity of testing serum (10).

The current study demonstrated that after spiking whole blood the PCR testing of plasma samples showed superior sensitivity to that of serum ($p = 0.002$, Figure 3). To our knowledge, no data comparing the detection of *A. fumigatus* DNA detection in both serum and plasma exists. Lau *et al.* 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 (6). 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; n=24), 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 the formation of clot (13).

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 formation. Fibrinogen is a soluble protein, which is exclusively found in plasma (normal range 150-400 mg/dL), but not in serum and has been shown to interact with magnesium, which is also an essential component of the PCR reaction (9). If this interaction results in lowering the magnesium concentration, it could result in PCR failure. All DNA extraction protocols used by the 8 EAPCRI study centres (Table 4) provided plasma DNA eluates that yielded superior sensitivity and lower Cq values compared to serum indicating that the additional components in plasma had no effect on PCR efficiency. In addition, the source of anticoagulant is also important; Garcia *et al*. (4) showed in rat blood that when comparing sodium citrate, heparin

and tripotassium-EDTA as anticoagulants, only the latter did not interfere with PCR used to diagnose IA.

The EAPCRI specific recommendations for *Aspergillus* PCR in serum included the use of a minimum volume of 0.5 ml serum as starting material, DNA elution in a volume <100 µl ($p = 0.003$) and the use of an internal control (15). For plasma, bivariate linear regression was used to evaluate similar covariates, finding a statistically significant positive association between PCR sensitivity and larger sample volumes (≥0.5 ml plasma), the use of ≥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 ($z = -2.32$, $p = 0.02$, Table 1). All 8 core centres complied with EAPCRI serum recommendations on elution volume (<100 µl (15)) so correlation with PCR sensitivity is only 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 minutes was detectable in small amounts in some leukocyte pellets as well as 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*. showed that there is a common binding site for DNA on white blood cells (1). However, when comparing this to the detection of *A. fumigatus* DNA in serum clots, only 3/9 leukocyte pellets showed weak PCR-positive results whereas McCulloch *et al* reported significantly more *Aspergillus* DNA detectable in serum clots compared to the cell-free fraction ($p<0.001$, 95% CI 2.24 to 6.48 [8]). These authors found that the average Cq value for the clot sample was 2.38 cycles lower than for EDTA blood and 3.69 cycles lower than for serum. While this

equates to a 7.9- and 12-fold increase in DNA yield from the clot, respectively, difficulties in processing these samples limits their use in routine practice. This observation might partially explain our observation that Cq values from native serum (used before clotting) developed consistently later compared to Cq values obtained from native plasma samples, both spiked with identical number of genome equivalents.

In conclusion, the analytical sensitivity of plasma, as determined by multi-centre evaluation of PCR-based detection of *A. fumigatus* DNA is superior compared to serum. Recommendations published by EAPCRI for serum (15), 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 multi-centre clinical study was performed. The retrospective case-control study supports the findings of this analytical manuscript and comes to the conclusion that there is a trend towards increased sensitivity of plasma, although this did not reach significance (P = 0.0897), and positivity was most frequently earliest when testing plasma by PCR (11).

Acknowledgements

We acknowledge the European Aspergillus Initiative (www.eapcri.eu) of the ISHAM.

We thank the following members of the participating laboratories: Martina Lengerova, Central Molecular Biology, Gene Therapy and Haematology Clinic, Brno, Czech Republic; Melinda Paholcsek, University of Debrecen Medical and Health Science Center; Cornelia Lass Floerl and Wolfgang Mutschlechner, Medical University Innsbruck; Laurence Millon, Laboratoire de Parasitologie-Mycologie Centre Hospitalier Universitaire, Besancon, France; Birgit Willinger, Division of Clinical Microbiology, Medical University Vienna, Vienna, Austria; Rebecca Gorton, Royal Free Hospital, NHS Trust, London, UK; Elaine McCulloch, Department of Microbiology, Royal Hospital for Sick Children, Glasgow, UK; Yvette

Debets-Ossenkopp, Department of Medical Microbiology and Infection Control, University Centre, Amsterdam, Netherlands; Chris Linton and Elizabeth Johnson, UK Mycology Reference Lab, HPA Southwest, Bristol, UK; Kathleen Harvey-Wood, Southern General Hospital, Glasgow, UK; Phillippe Hauser, CHUV, Institute de Microbiologie, Lausanne, Switzerland; Angie Caliendo and Deborah Abdul-Ali, Emory University, Atlanta, GA, USA; Jose Palomares, Sección de Microbiología Molecular, Universidad de Seville, Seville, Spain; Petr Hamal, Department of Microbiology, Palacky University, Olomouc, Czech Republic; Ferry Hagen, Department of Medical Microbiology, Canisius-Wilhelmina Hospital, Nijmegen, Netherlands; Gemma Johnson, Barts and the London Hospital, London, UK; Massimo Cogliati, Università degli Studi di Milano, Milano, Italy.

Conflicts of Interest

JL is a founding member of the EAPCRI, received an educational grant and scientific fellowship award from Pfizer, and was sponsored by Astellas to attend international meetings.

RAB is a founding member of the EAPCRI, received an educational grant and scientific fellowship award from Gilead Sciences and Pfizer, is a member of the advisory board and speaker bureau for Gilead Sciences, MSD, Astellas, and Pfizer, and was sponsored by Gilead Sciences and Pfizer to attend international meetings.

PLW is a founding member of the EAPCRI, received project funding from Myconostica, Luminex, and Renishaw diagnostics, was sponsored by Myconostica, MSD and Gilead Sciences to attend international meetings, and provided consultancy for Renishaw Diagnostics Limited.

JPD is a founding member of the EAPCRI, is a member of the advisory board for Gilead Sciences, and Pfizer, and has been on a speaker's bureau for Gilead Sciences, MSD and Pfizer.

LK has been a consultant to Astellas Pharma, Gilead Sciences, Merck & Co., and Schering-Plough. She has received research grants from Gilead and Schering-Plough/Merck & Co.

MCE has received grant support from Astellas Pharma, Basilea, bioMerieux, Gilead Sciences, Merck Sharp and Dohme, Pfizer, Schering Plough, Soria Melguizo SA, Ferrer International, the European Union, the ALBAN program, the Spanish Agency for International Cooperation, the Spanish Ministry of Culture and Education, The Spanish Health Research Fund, The Instituto de Salud Carlos III, The Ramon Areces Foundation, The Mutua Madrileña Foundation. He has been an advisor/consultant to the Panamerican Health Organization, Astellas Pharma, Gilead Sciences, Merck Sharp and Dohme, Pfizer, and Schering Plough. He has been paid for talks on behalf of Gilead Sciences, Merck Sharp and Dohme, Pfizer, Astellas Pharma, and Schering Plough.

SB is a founding member of the EAPCRI, received project funding from Renishaw diagnostics, was sponsored by Pfizer and MSD to attend international meetings, and provided consultancy for Gilead.

JS, CM, WM, COM and KL have no conflicts of interest.

References

1. **Bennett, R. M., G. T. Gabor, and M. M. Merritt.** 1985. DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. J Clin Invest **76:**2182-90.
2. **Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer.** 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem **55:**611-22.
3. **De Pauw, B., T. J. Walsh, J. P. Donnelly, D. A. Stevens, J. E. Edwards, T. Calandra, P. G. Pappas, J. Maertens, O. Lortholary, C. A. Kauffman, D. W. Denning, T. F. Patterson, G. Maschmeyer, J. Bille, W. E. Dismukes, R. Herbrecht, W. W. Hope, C. C. Kibbler, B. J. Kullberg, K. A. Marr, P. Munoz, F. C. Odds, J. R. Perfect, A. Restrepo, M. Ruhnke, B. H. Segal, J. D. Sobel, T. C. Sorrell, C. Viscoli, J. R. Wingard, T. Zaoutis, and J. E. Bennett.** 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis **46:**1813-21.
4. **Garcia, M. E., J. L. Blanco, J. Caballero, and D. Gargallo-Viola.** 2002. Anticoagulants interfere with PCR used to diagnose invasive aspergillosis. J Clin Microbiol **40:**1567-8.
5. **Kosmidis, C., and D. W. Denning.** 2014. The clinical spectrum of pulmonary aspergillosis. Thorax.
6. **Lau, A., C. Halliday, S. C. Chen, E. G. Playford, K. Stanley, and T. C. Sorrell.** 2010. Comparison of whole blood, serum, and plasma for early detection of candidemia by multiplex-tandem PCR. J Clin Microbiol **48:**811-6.
7. **Loeffler, J., H. Hebart, U. Brauchle, U. Schumacher, and H. Einsele.** 2000. Comparison between plasma and whole blood specimens for detection of Aspergillus DNA by PCR. J Clin Microbiol **38:**3830-3.
8. **McCulloch, E., G. Ramage, B. Jones, P. Warn, W. R. Kirkpatrick, T. F. Patterson, and C. Williams.** 2009. Don't throw your blood clots away: use of blood clot may improve sensitivity of PCR diagnosis in invasive aspergillosis. J Clin Pathol **62:**539-41.
9. **Okada, M., and B. Blomback.** 1983. Factors influencing fibrin gel structure studied by flow measurement. Ann N Y Acad Sci **408:**233-53.
10. **Springer, J., C. O. Morton, M. Perry, W. J. Heinz, M. Paholcsek, M. Alzheimer, T. R. Rogers, R. A. Barnes, H. Einsele, J. Loeffler, and P. L. White.** 2013. Multicenter comparison of serum and whole-blood specimens for detection of Aspergillus DNA in high-risk hematological patients. J Clin Microbiol **51:**1445-50.
11. **White, P. L., R. A. Barnes, J. Springer, L. Klingspor, M. Cuenca-Estrella, C. O. Morton, K. Lagrou, S. Bretagne, W. J. G. Melchers, C. Mengoli, J. P. Donnelly, W. J. Heinz, and J. Loeffler.** 2015. An evaluation of the clinical performance of Aspergillus PCR when testing serum and plasma. J Clin Microbiol.
12. **White, P. L., S. Bretagne, L. Klingspor, W. J. Melchers, E. McCulloch, B. Schulz, N. Finnstrom, C. Mengoli, R. A. Barnes, J. P. Donnelly, and J. Loeffler.** 2010. Aspergillus PCR: one step closer to standardization. J Clin Microbiol **48:**1231-40.
13. **White, P. L., T. Jones, K. Whittle, J. Watkins, and R. A. Barnes.** 2013. Comparison of galactomannan enzyme immunoassay performance levels when testing serum and plasma samples. Clin Vaccine Immunol **20:**636-8.
14. **White, P. L., C. J. Linton, M. D. Perry, E. M. Johnson, and R. A. Barnes.** 2006. The evolution and evaluation of a whole blood polymerase chain reaction assay for the detection of

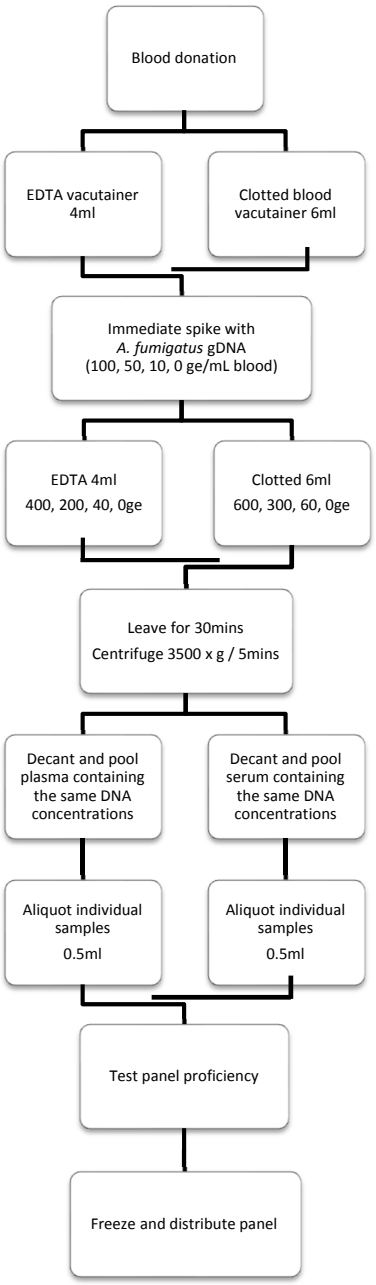
invasive aspergillosis in hematology patients in a routine clinical setting. Clin Infect Dis **42:**479-86.

15. **White, P. L., C. Mengoli, S. Bretagne, M. Cuenca-Estrella, N. Finnstrom, L. Klingspor, W. J. Melchers, E. McCulloch, R. A. Barnes, J. P. Donnelly, and J. Loeffler.** 2011. Evaluation of Aspergillus PCR protocols for testing serum specimens. J Clin Microbiol **49:**3842-8.

16. **White, P. L., M. D. Perry, A. Moody, S. A. Follett, G. Morgan, and R. A. Barnes.** 2011. Evaluation of analytical and preliminary clinical performance of Myconostica MycAssay Aspergillus when testing serum specimens for diagnosis of invasive Aspergillosis. J Clin Microbiol **49:**2169-74.

473 **Figure 1:** Flow diagram highlighting the process for determining the availability of

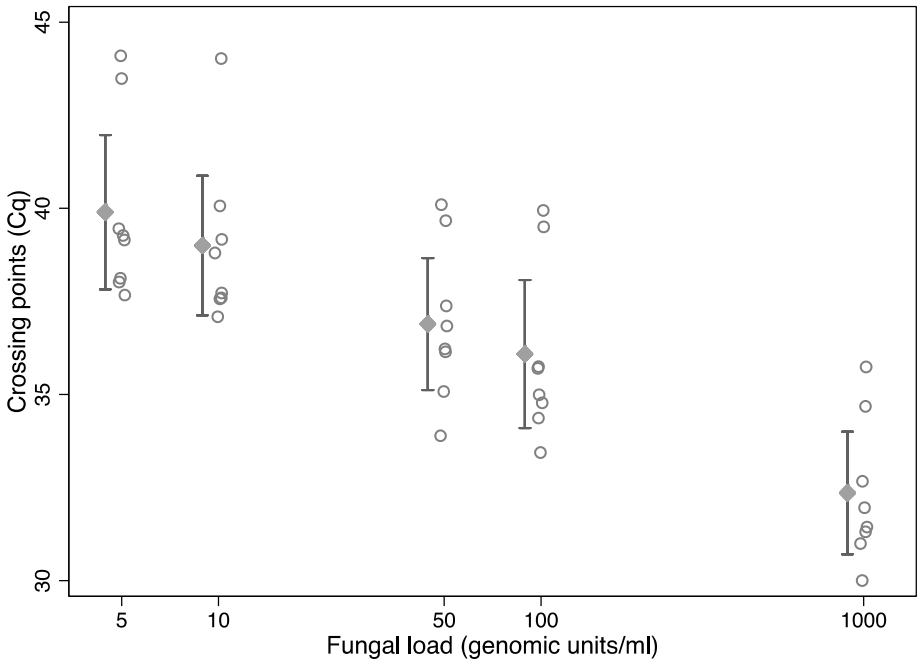
474 *Aspergillus* DNA in serum and plasma samples post blood fractionation



475

476 **Figure 2:** Descriptive statistics for *Aspergillus* real-time PCR crossing points when testing
477 plasma samples containing various concentrations of *Aspergillus fumigatus* genomic DNA.
478 Means (solid dots), 95% confidence intervals of the means (vertical bars), and single
479 observations (empty circles) are shown for each fungal load.

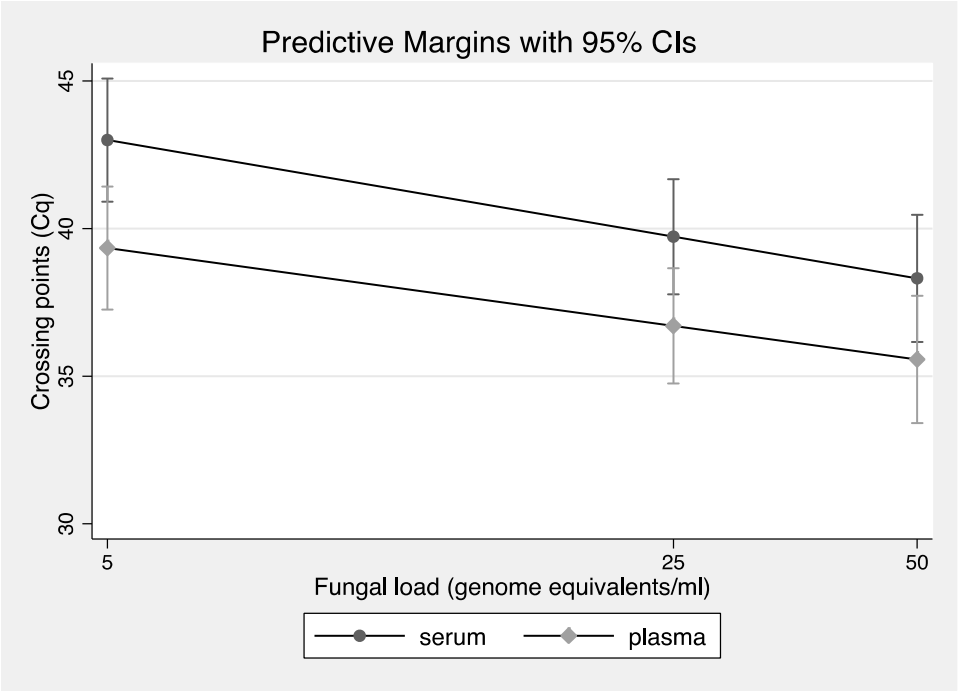
480



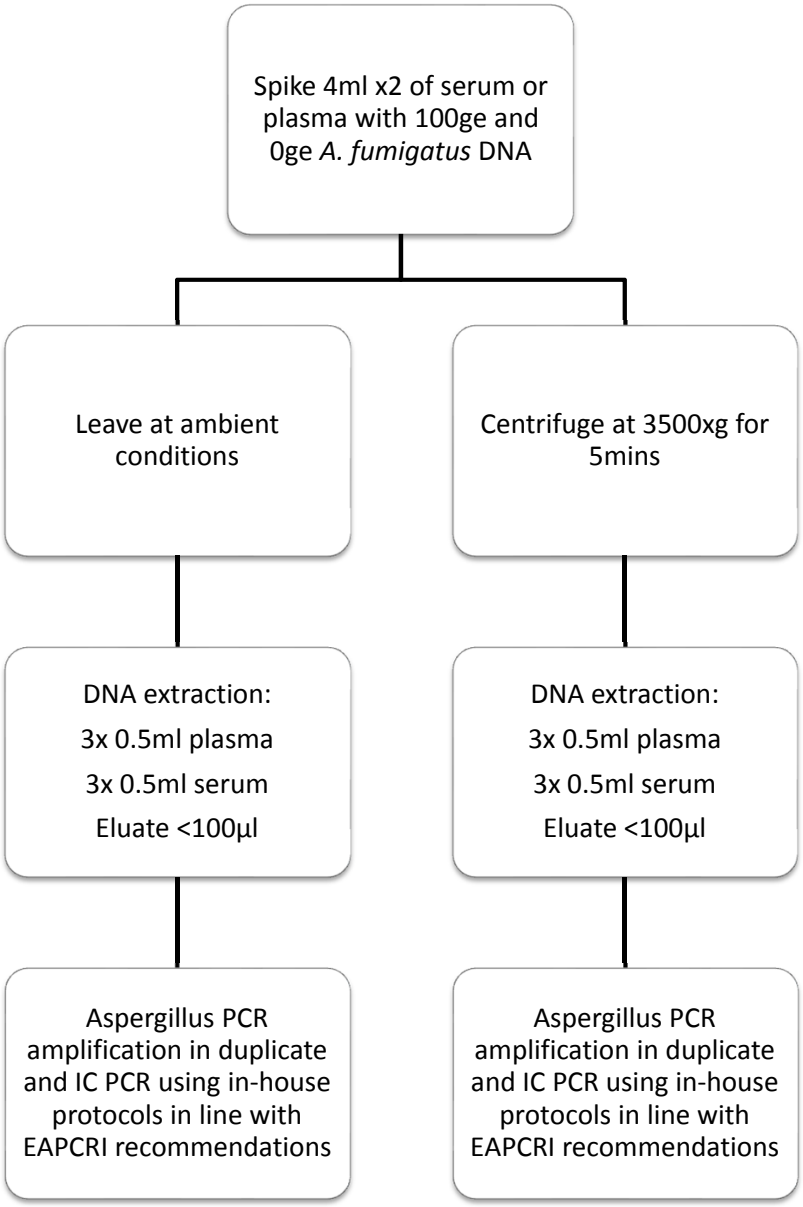
481

482

Figure 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 Cq value versus the fungal genomic burden, with serum or plasma as a binary covariate.



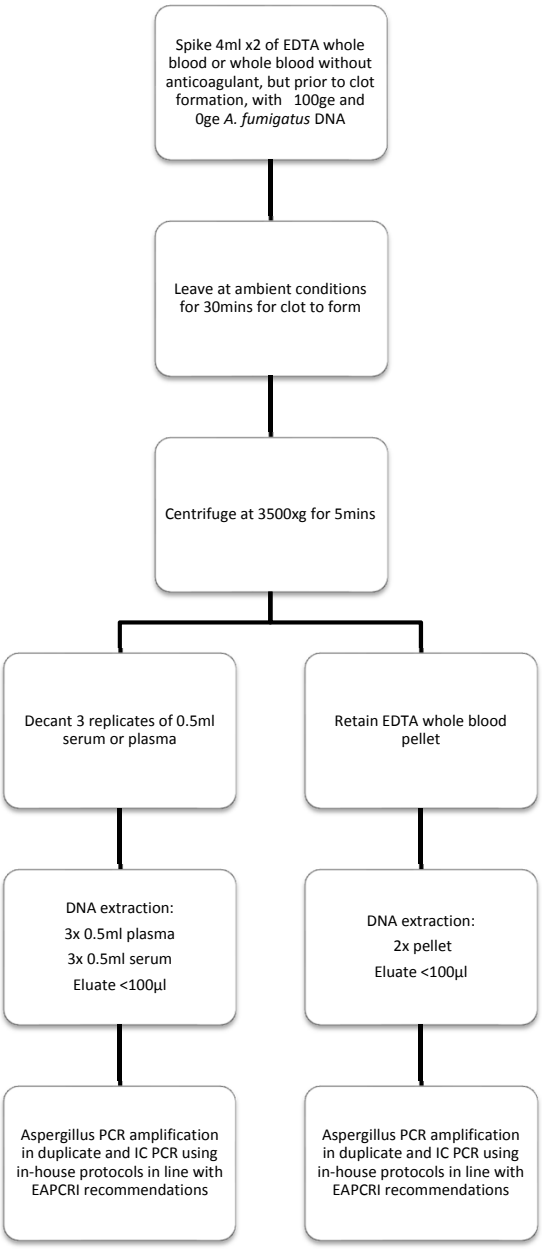
490 **Figure 4a:** Flow diagram highlighting the process for determining the effect of centrifugation
491 on the availability of *Aspergillus* DNA in serum and plasma samples.
492



493

Journal of Clinical Microbiology

494 **Figure 4b:** Flow diagram highlighting the process for determining the effect of clot formation
495 on the availability of *Aspergillus* DNA in serum compared to the processing of plasma
496 samples.



497

498 **Table 1:** Bivariate analysis for the sensitivity of *Aspergillus* PCR testing spiked plasma
499 specimens distributed to 8 EAPCRI core centres. All continuous and binary center-specific
500 covariates were included into the basic model. With a negative sign of the z-score, the
501 variable tended to exert a favorable effect on the PCR assay. The covariates exerting a
502 significant effect are indicated by a $P < 0.05$ (*) and an absolute z-score > 1.96
503

Variable	Z-Score	P-Value
No of plasma samples analyzed / month	-1.61	0.107
Plasma starting volume	-2.50	0.01*
DNA elution volume	-0.65	0.513
Ratio starting plasma volume : elution volume	-2.32	0.02*
Total PCR reaction volume	-2.64	0.01*
Template volume	-1.47	0.141
Use of internal control	-4.90	0.00*
Use of ≥2 replicates	-2.31	0.02*
Percentage of eluate volume used in PCR	-0.95	0.343

504

505 **Table 2:** *Aspergillus* PCR performance using plasma samples spiked with different fungal
506 loads
507

Sample No	Burden genomes/ml	95% sample variance	Positivity rate (% /95% CI)	Mean Cq (95% CI)	Std. Err.
1	1000	939.6-1060.4	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*	n. a.
7, 8	0 x2	n. a.	0	No signal	n. a.

508
509 *Plasma 6 was positive in one single centre in a single sample only
510 Plasma samples 1 – 8 were spiked with different fungal burdens (genome equivalents [ge] /
511 ml plasma); results shown as mean Cq values, standard errors (Std. Err.) and 95% Confidence
512 intervals (Conf. Interval); n. a. = not available
513

514 **Table 3:** The influence of sample volume (0.1-0.5 ml) on *Aspergillus* real-time PCR crossing
515 points when testing plasma samples containing a range of *Aspergillus fumigatus* genomic
516 DNA concentrations (5-1000 ge/ml). Prediction of Cq values calculated using a linear mixed
517 model.
518

Plasma	**Fungal load [ge / ml plasma]**				
volume [ml]	5	10	50	100	1000
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

519

520 **Table 4:** Details of the molecular procedures used by the EAPCRI centres investigating the performance of *Aspergillus* PCR when testing serum
521 and plasma
522

EAPCRI Centre #	Monthly serum sample numbers	DNA extraction volume [ml]	DNA extraction system	Elution volume [µl]	Template volume [µl]	PCR reaction volume [µl]	PCR target gene	Sensitivity Serum [%]	Sensitivity Plasma [%]
1	0	0.2	MagNAPure	50	10	20	18S	66.7	100
2	80	1	QIAamp UltraSense Virus	70	10	21	ITS	100	100
3	40	1	QIAamp UltraSense Virus	38	10	21	ITS	100	100
4	15	0.2	QIAamp DNA Mini	50	2	20	ITS	0	100

5	70	1	MagNAPure 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	MagNAPure Total NA Large Volume	100	5	20	28S	100	100
8	10	0.5	MagNAPure Viral NA Large Volume	50	10	50	28S	100	100

523

524

525 **Table 5:** The effect of centrifugation at 3500 x g on DNA recovery (as indicated by mean Cq values) from spiked serum and plasma specimens.
526 Both centres revealed no significant differences in Cq values between centrifuged and non-centrifuged plasma and serum samples, respectively, P >
527 0.05 (*)
528

	Centre 1	Centre 2
Material	**[Mean Cq values]**	**[Mean Cq Values]**
Plasma centrifuged	47.1*	37.8*
Plasma non-centrifuged	46.9*	37.6*
Serum centrifuged	46.1*	37.2*
Serum non-centrifuged	47.2*	37.0*

529
530