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

4
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26 **Abstract**

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

33 Molecular methods testing serum and plasma were compared through multi-centre
34 distribution of quality control panels, with additional studies to investigate the effect of clot
35 formation and blood fractionation on DNA availability. Analytical sensitivity and time to
36 positivity (TTP) were compared and regression analysis performed to identify variables that
37 enhanced plasma PCR performance.

38 When testing plasma, sample volume, pre/post-extraction volume ratio, PCR reaction volume,
39 duplicate testing and the use of an internal control PCR were positively associated with
40 performance. When whole blood samples were spiked then fractionated, the analytical
41 sensitivity and TTP were superior when testing plasma. Centrifugation had no effect on DNA
42 availability, whereas the presence of clot material significantly lowered the concentration ($p =$
43 0.028).

44 **Summary:** Technically there are no major differences for molecular processing of serum and
45 plasma, but the formation of clot material potentially reduces available DNA in serum.
46 During disease *Aspergillus* DNA burdens in blood are often at the limits of PCR performance.
47 Using plasma could improve performance while maintaining the methodological simplicity of
48 serum testing.

49

50 **Introduction**

51 Invasive aspergillosis (IA) represents a serious health problem for the immunocompromised
52 patient, especially those undergoing cancer chemotherapy, receiving corticosteroid therapy or
53 immunosuppression to avoid allograft rejection or solid organ rejection. *Aspergillus fumigatus*
54 is a ubiquitous mould, and the most frequent cause of Aspergillosis in humans causing a wide
55 spectrum of diseases ranging from allergic to severe life-threatening invasive manifestations.
56 Spores of *A. fumigatus* enter the body through inhalation and infection primarily occurs in the
57 lungs. Accurate diagnosis of IA remains difficult. Given the limitations of clinical signs and
58 the difficulty in obtaining appropriate specimens for diagnosis a significant proportion of
59 cases remain undetected, resulting in late or inappropriate therapy, and increased mortality
60 rates, that can be as high as 90% with cerebral disease (5). Hence, there is an urgent need for
61 an accurate and standardized, diagnostic approach for IA.

62 Performance of mycological diagnostics when testing serum or plasma samples is assumed to
63 be similar. The revised EORTC-MSG definitions include galactomannan (GM) EIA for
64 testing of both serum and plasma (3), although, the testing of plasma has not been validated
65 by the manufacturer. A study comparing GM EIA performance in serum and plasma
66 confirmed that the testing of plasma using the same thresholds was appropriate, but
67 interestingly the mean index generated by testing plasma was significantly higher than that for
68 serum [0.315 vs 0.279, *P*: 0.0398 (13)]. Moreover, four possible IA cases would have been
69 classified as probable IA had plasma been tested. The authors hypothesised that differences in
70 indices could be attributed to the formation of the blood clot potentially ensnaring some of the
71 GM within the sample taken for serum testing. It is conceivable that the same might be true
72 for extracellular DNA, the *Aspergillus* target in the cell free fraction of host blood, affecting
73 PCR performance.

74 This manuscript describes further efforts of the European *Aspergillus* PCR Initiative
75 (EAPCRI) to evaluate the analytical performance of plasma and serum samples through the
76 blinded distribution of simulated panels, as described previously for whole blood and plasma
77 (12, 15). We report data on differences in analytical performances associated with the
78 different sample types (plasma versus serum) and on how differences in the initial formation
79 of the sample types (clot versus no clot) and sample processing (whole blood centrifugation)
80 affected the availability of DNA within the cell free sample itself.

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

83

84 **Material and Methods**

85

86 **DNA source material**

87 DNA was obtained from a sporulating culture of *Aspergillus fumigatus* (ATCC strain 1022).

88 Conidia were harvested and DNA was extracted as described before (15).

89

90 **Participants**

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

95

96 **Panels**

97 All EDTA whole blood and clotted blood samples were obtained from consenting healthy
98 volunteers, and screened for the presence of infectious agents as per protocol of the Institute
99 of Transfusion medicine, Wuerzburg University Hospital, (Wuerzburg, Germany) and
100 approved by the local ethical review board. Serum and plasma were fractionated by
101 centrifugation and pooled respectively, before being distributed to Public Health Wales,
102 Microbiology Cardiff, to develop the panel. To avoid airborne contamination all processing of
103 material took place in a category 2 laminar flow cabinet. The panel was validated through
104 testing with an “in-house” *Aspergillus* specific real-time PCR (14). Panels were distributed by
105 courier on dry ice. Participating centres were asked to confirm receipt, comment on the state
106 of the panel (frozen or thawed) and keep specimens frozen at -80°C until testing.

107

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

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

111 Initially, serum was divided into 3 x 35 ml aliquots; one aliquot was retained to provide a
112 negative control sample, while two aliquots were spiked with 100 and 10 genome equivalents
113 (ge) of *A. fumigatus* DNA per ml of serum, respectively. The 35 ml serum batches were
114 further divided into 1ml aliquots and frozen at -80°C until distribution on dry ice to the 23
115 centres. This panel was a quality control (QC) exercise designed to confirm the findings of the
116 previous research investigating serum, in doing so providing a reference for comparison with
117 the plasma results, but also determining centres with optimal results for the analyses of further
118 panels, including the analysis of plasma samples (15).

119

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

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

126 In keeping with previous studies, the threshold of detection, based on 95% variance was set at
127 10 ge/ml (15).

128

129 **iii) Assessing the DNA availability in serum/plasma – Whole blood spiking**

130 DNA was spiked into blood collected into an EDTA vacutainer and into a vacutainer without
131 any anticoagulant, immediately after sampling, but before centrifugation. Blood was spiked
132 with varying concentrations of genomic *A. fumigatus* DNA (100, 50, 10 and 0 ge/ml, Figure
133 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

159 protocols comply with the EAPCRI recommendations for testing serum (15). PCR was
160 performed in duplicate and included an internal control to measure PCR inhibition.

161

162 **Impact of clot formation**

163 Immediately after blood was drawn, 4 ml of EDTA whole blood and 4 ml of clotted blood
164 were spiked with 100 ge of *A. fumigatus* DNA. Clotted blood was left at ambient temperature
165 for 30 mins to coagulate, while EDTA blood remained untouched at room temperature for the
166 same period. Samples were then centrifuged at 3500 g for 5 mins. After centrifugation, a
167 minimum of three 0.5 ml aliquots of plasma and serum, as well as the cell pellet from the
168 EDTA whole blood samples were extracted using local protocols and DNA was eluted in
169 <100 µl. The clot was not processed as no EAPCRI validated procedures are currently
170 available. PCR was performed in both centres using in-house amplification protocols and an
171 inhibition control, all in accordance with EAPCRI guidelines (15).

172

173 **Statistical analysis**

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

180 The correlation between Cq and genomic load was estimated by linear regression. The Cq
181 was the dependent variable, whereas the explanatory variables were i) the genomic load (as
182 log₁₀ genomic DNA), ii) the spiked sample type (as a binary variable for serum or plasma),
183 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

190 **Results**

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

193 The serum analysis in this QC panel compared favourably with the previous EAPCRI serum
194 evaluation (15). All the 23 centres were able to reproducibly detect the sample that was spiked
195 with 100ge and generate negative results when testing the sample not containing *Aspergillus*-
196 DNA. Samples spiked with 10 ge / ml generated an overall positivity rate of 74% (17 / 23
197 centres) and a positivity rate of 82% (14 / 17) in centres that strictly followed EAPCRI
198 recommendations for serum. Five out of the 6 centres who did not follow EAPCRI
199 recommendations were unable to detect 10 ge / ml. Centres that were unable to detect the 10
200 ge / ml did not routinely process serum samples (6/6) or used serum volumes below 0.5 ml
201 (5/6, range 0.1 – 0.2 ml serum). In addition, these centres used only a small portion of their
202 original serum sample volume in the subsequent PCR assay (mean 7.9%, range 1% - 25%),
203 compared to a mean volume of 24.4% (range 16% - 82%) for centres that were able to detect
204 10 ge / ml ($p = 0.028$).

205

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

208 All centres that had received this plasma panel were requested to follow the EAPCRI
209 guidelines for serum testing and their positivity rates in the related QC serum panel were
210 100%, 100% and 0% (100, 10, 0 genome equivalents / ml, respectively).

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

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

217 Centres used between 0.1 ml and 1 ml (mean 0.5 ml) of plasma for subsequent DNA
218 extraction, 50 – 100 μ l (mean 60 μ l) of elution volume and 2 – 15 μ l (mean 8.7 μ l) of
219 template volume for their PCR assays. In bivariate analyses, we observed significant positive
220 correlations between PCR sensitivity and the volume of plasma used for DNA extraction, the
221 ratio between the volume of plasma used for extraction and the subsequent elution volume,
222 the reaction volume used for PCR, the analysis of ≥ 2 replicates and the use of an internal
223 control (Table 1).

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

229

230 **iii) Assessing the DNA availability in serum/plasma – Whole blood spiking**

231 In order to measure the availability of DNA in the cell free fraction post whole blood
232 processing, an additional panel, consisting of 4 serum samples and 4 plasma samples was
233 shipped to the 8 core EAPCRI centres. Our linear model showed that while the slope for PCR
234 from plasma and serum did not differ significantly ($p = 0.381$), the plasma intercept was 4.3
235 cycles lower (standard error = 1.38, 95% confidence interval -6.996 / -1.585) than the serum
236 intercept ($z = -3.11$, $p = 0.002$). Interestingly, this shift in Cq values was achieved at all DNA
237 concentrations tested (50, 25, 5 ge / ml, Figure 3). No inhibition was observed. Centre-
238 specific methodological details are shown in Table 4. Individual performance of centres in
239 this panel was significantly associated with the volume of sample used for DNA extraction.

240 While both centres using 0.2 ml of serum or plasma achieved suboptimal sensitivity not
241 detecting the serum sample spiked with 5 ge / ml, centres using ≥ 0.5 ml of plasma and serum
242 were able to detect this sample as positive ($p < 0.001$ [Table 4]). In addition, we observed a
243 trend that laboratories, which routinely test serum or plasma by *Aspergillus* PCR achieved
244 higher sensitivity ($p = 0.07$).

245
246 **iv) Investigating the effects of clot formation and blood fractionation on the**
247 **availability of *A. fumigatus* genomic DNA**

248 In order to identify any potential losses of DNA during centrifugation necessary to fractionate
249 blood samples, the recovery of DNA from spiked serum and plasma specimens that were
250 centrifuged at 3500 g were compared to spiked samples that were not centrifuged. In both
251 centres, there were no significant differences in Cq values between centrifuged or non-
252 centrifuged samples, for either plasma or serum (Table 5). These results demonstrate that
253 centrifugation at 3500 g is not sufficient to sediment free DNA from serum and plasma.

254 Interestingly, in both centres, plasma, obtained after centrifugation of spiked EDTA blood
255 samples, showed earlier Cq values, compared to serum obtained from the corresponding
256 clotted blood samples (difference of the mean Cq values between plasma and serum $\Delta = 2.26$,
257 standard error = 0.45, $p = 0.0002$). The concentration of genomic DNA added to the PCR
258 reaction was significantly lower ($p = 0.028$) in serum samples post clot formation (mean 1.9
259 copies, SD: 0.8) compared to DNA extracted from serum not influenced by clot formation
260 (4.2 copies, SD: 1.3). Although it is likely that a substantial amount of *A. fumigatus* DNA is
261 bound, during blood clot formation, and consequently is unavailable for DNA extractions
262 using serum, it was not possible to test the clot material as optimized protocols are not
263 available, and technical limitations prevent the processing of the entire clot material, which in
264 this scenario was > 2 ml in volume. However, whole blood cell pellets from centrifuged

265 EDTA vacutainers, containing peripheral blood mononuclear cells were tested. Relatively
266 small amounts of *A. fumigatus* DNA (Mean: 0.4 input copies) were detectable in samples at
267 both centres (centre 1: 1/4 samples positive [Ct value= 51.9], centre 2: 2/5 samples positive
268 [Ct values=40.2, 42]), although it appears that the majority of the DNA remains in the plasma
269 fraction (Mean: 3.3 input copies).
270

271 **Discussion**

272 The European *Aspergillus* PCR Initiative (EAPCRI) has published standards for *Aspergillus*
273 PCR and protocols for detecting *Aspergillus* DNA in whole blood (12) and serum (15). The
274 identification of the critical stages of *Aspergillus* DNA extraction from both specimen types
275 allowed the EAPCRI to propose a protocol that helps ensure optimal performance of
276 *Aspergillus* PCR across laboratories. However, no such data exist for plasma and no direct
277 comparison between plasma and serum specimens has been performed. Thus, EAPCRI
278 continued its efforts to evaluate the analytical performance of plasma, compared with serum
279 samples through the blinded distribution of simulated panels. In parallel, a multi-centre
280 clinical study was performed to compare detection of *A. fumigatus* DNA isolated from plasma
281 and serum obtained from haematological patients (11).

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

286 Almost 15 years ago, Loeffler *et al.* compared sensitivities of *A. fumigatus* DNA detection
287 from plasma and whole blood (7). Although plasma and whole blood samples spiked with
288 *Aspergillus* conidia showed an identical lower detection limit (10 CFU), the sensitivity of
289 plasma PCR was inferior to that of PCR performed on whole blood samples obtained from
290 patients with proven IA. However, both DNA extraction and PCR amplification technology
291 has advanced over the past decade. In addition, the quality of molecular diagnostics has
292 advanced through numerous External Quality Assessment initiatives, including the EAPCRI,
293 the Minimum Information for Publication of Quantitative Real-Time PCR Experiments
294 (MIQE) guidelines (2) and the Quality Control for Molecular Diagnostics (QCMD;
295 <http://www.qcmd.org>). In a recent study comparing EAPCRI methods for the testing of serum

296 and whole blood there was a trend towards superior sensitivity when testing whole blood, but
297 this did not reach statistical significance, and any benefit was outweighed by the simplicity of
298 testing serum (10).

299 The current study demonstrated that after spiking whole blood the PCR testing of plasma
300 samples showed superior sensitivity to that of serum ($p = 0.002$, Figure 3). To our knowledge,
301 no data comparing the detection of *A. fumigatus* DNA detection in both serum and plasma
302 exists. Lau *et al.* showed that *Candida* DNA was detected more often in serum (71%) and
303 plasma (75%) than in whole blood (54%) in a study of 109 patients with candidemia (6).
304 However, there was no significant difference between plasma and serum, possibly because of
305 the relatively small numbers of serum and plasma specimens that were tested ($n=29$; $n=24$), or
306 the differences in fungal disease manifestation. With respect to IA, the concentration of
307 galactomannan as determined by EIA was shown in another study to be significantly higher in
308 plasma than in serum ($P: 0.0398$), and may have been associated with the formation of clot
309 (13).

310 The presence of compounds that may interfere with molecular assays is always a concern
311 when processing clinical samples. Compared to serum, plasma samples contain various
312 coagulation factors that lead to the conversion of fibrinogen to fibrin and clot formation.
313 Fibrinogen is a soluble protein, which is exclusively found in plasma (normal range 150-400
314 mg/dL), but not in serum and has been shown to interact with magnesium, which is also an
315 essential component of the PCR reaction (9). If this interaction results in lowering the
316 magnesium concentration, it could result in PCR failure. All DNA extraction protocols used
317 by the 8 EAPCRI study centres (Table 4) provided plasma DNA eluates that yielded superior
318 sensitivity and lower Cq values compared to serum indicating that the additional components
319 in plasma had no effect on PCR efficiency. In addition, the source of anticoagulant is also
320 important; Garcia *et al.* (4) showed in rat blood that when comparing sodium citrate, heparin

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

323 The EAPCRI specific recommendations for *Aspergillus* PCR in serum included the use of a
324 minimum volume of 0.5 ml serum as starting material, DNA elution in a volume <100 μ l ($p =$
325 0.003) and the use of an internal control (15). For plasma, bivariate linear regression was used
326 to evaluate similar covariates, finding a statistically significant positive association between
327 PCR sensitivity and larger sample volumes (≥ 0.5 ml plasma), the use of ≥ 2 replicates (from a
328 single eluate) and an internal control. While the DNA elution volume itself is not significantly
329 associated with PCR sensitivity, the ratio of the initial plasma sample volume to the
330 subsequent elution volume was significantly associated ($z = -2.32$, $p = 0.02$, Table 1). All 8
331 core centres complied with EAPCRI serum recommendations on elution volume (<100 μ l
332 (15)) so correlation with PCR sensitivity is only to be expected. However, the use of an
333 internal control was correlated and found to be statistically significant. Although this
334 parameter is only indirectly associated with the detection of *A. fumigatus* DNA, it reflects the
335 degree of diligence and accuracy of an individual laboratory.

336 Interestingly, *A. fumigatus* genomic DNA, spiked into EDTA whole blood and left untouched
337 for 30 minutes was detectable in small amounts in some leukocyte pellets as well as the cell
338 free fraction. This suggests that circulating extracellular DNA reaches the cell pellet, either by
339 gravitational force or due to binding to leukocyte surface receptors. Indeed, Bennett *et al.*
340 showed that there is a common binding site for DNA on white blood cells (1). However, when
341 comparing this to the detection of *A. fumigatus* DNA in serum clots, only 3/9 leukocyte
342 pellets showed weak PCR-positive results whereas McCulloch *et al.* reported significantly
343 more *Aspergillus* DNA detectable in serum clots compared to the cell-free fraction ($p < 0.001$,
344 95% CI 2.24 to 6.48 [8]). These authors found that the average Cq value for the clot sample
345 was 2.38 cycles lower than for EDTA blood and 3.69 cycles lower than for serum. While this

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

351 In conclusion, the analytical sensitivity of plasma, as determined by multi-centre evaluation of
352 PCR-based detection of *A. fumigatus* DNA is superior compared to serum. Recommendations
353 published by EAPCRI for serum (15), including sample volume, a minimum of duplicate
354 PCR testing of each DNA extract and an internal control can be applied to plasma.

355 To confirm the analytical findings described here, a parallel multi-centre clinical study was
356 performed. The retrospective case-control study supports the findings of this analytical
357 manuscript and comes to the conclusion that there is a trend towards increased sensitivity of
358 plasma, although this did not reach significance ($P = 0.0897$), and positivity was most
359 frequently earliest when testing plasma by PCR (11).

360

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381

382 **Conflicts of Interest**

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

385 RAB is a founding member of the EAPCRI, received an educational grant and
386 scientific fellowship award from Gilead Sciences and Pfizer, is a member of the advisory
387 board and speaker bureau for Gilead Sciences, MSD, Astellas, and Pfizer, and was sponsored
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394 Gilead Sciences, and Pfizer, and has been on a speaker's bureau for Gilead Sciences, MSD
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411 JS, CM, WM, COM and KL have no conflicts of interest.

412

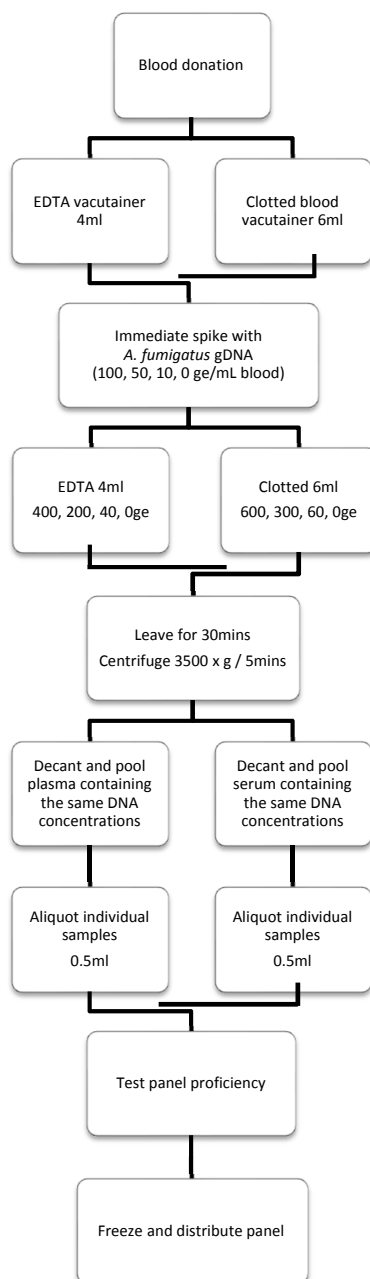
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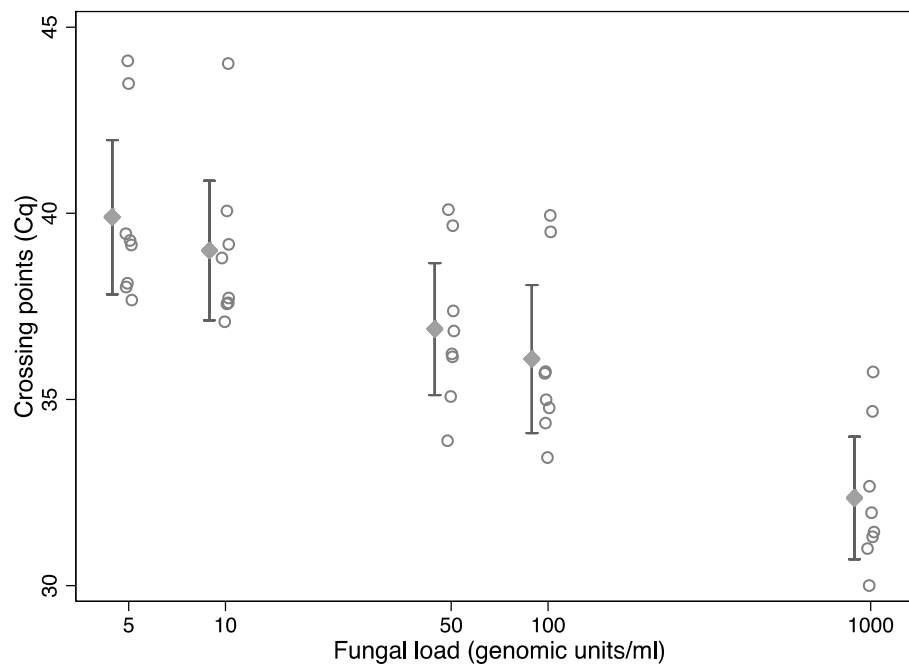
473 **Figure 1:** Flow diagram highlighting the process for determining the availability of
474 *Aspergillus* DNA in serum and plasma samples post blood fractionation



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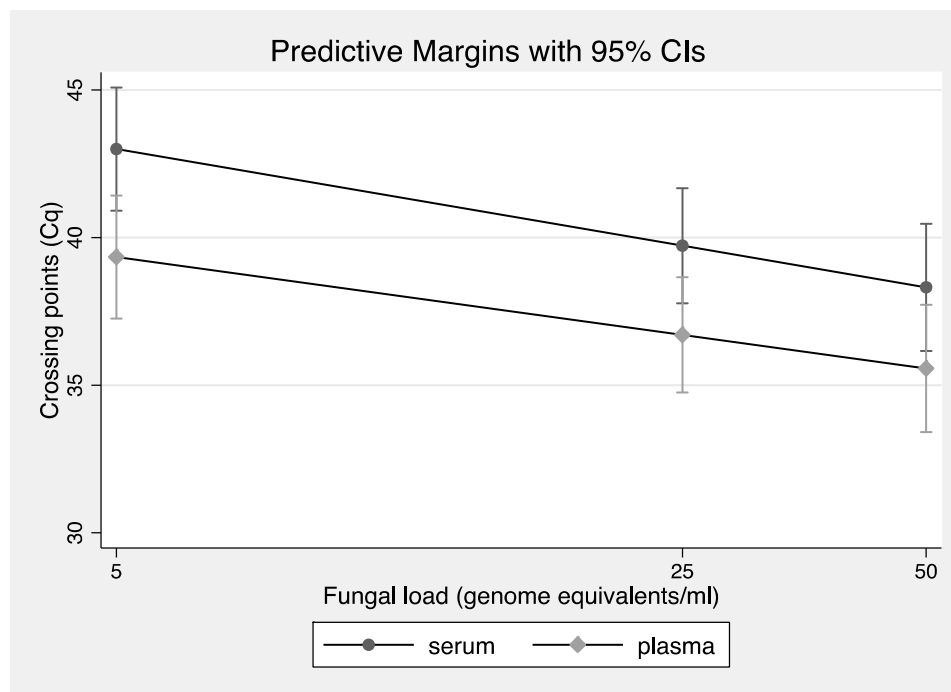
476 **Figure 2:** Descriptive statistics for *Aspergillus* real-time PCR crossing points when testing
477 plasma samples containing various concentrations of *Aspergillus fumigatus* genomic DNA.
478 Means (solid dots), 95% confidence intervals of the means (vertical bars), and single
479 observations (empty circles) are shown for each fungal load.
480



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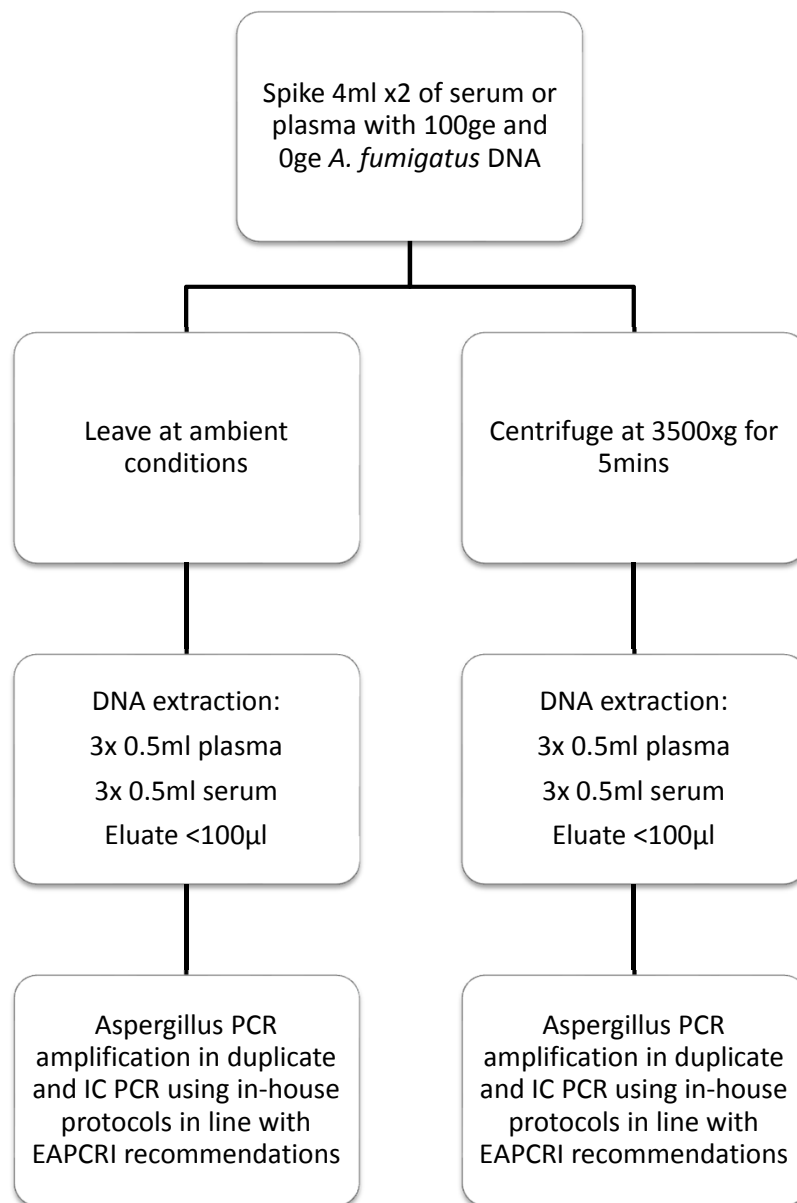
483 **Figure 3:** Comparison of *Aspergillus* real-time PCR when testing serum and plasma samples
484 containing various fungal loads using a linear mixed model. Data are shown after the
485 regression of the Cq value versus the fungal genomic burden, with serum or plasma as a
486 binary covariate.
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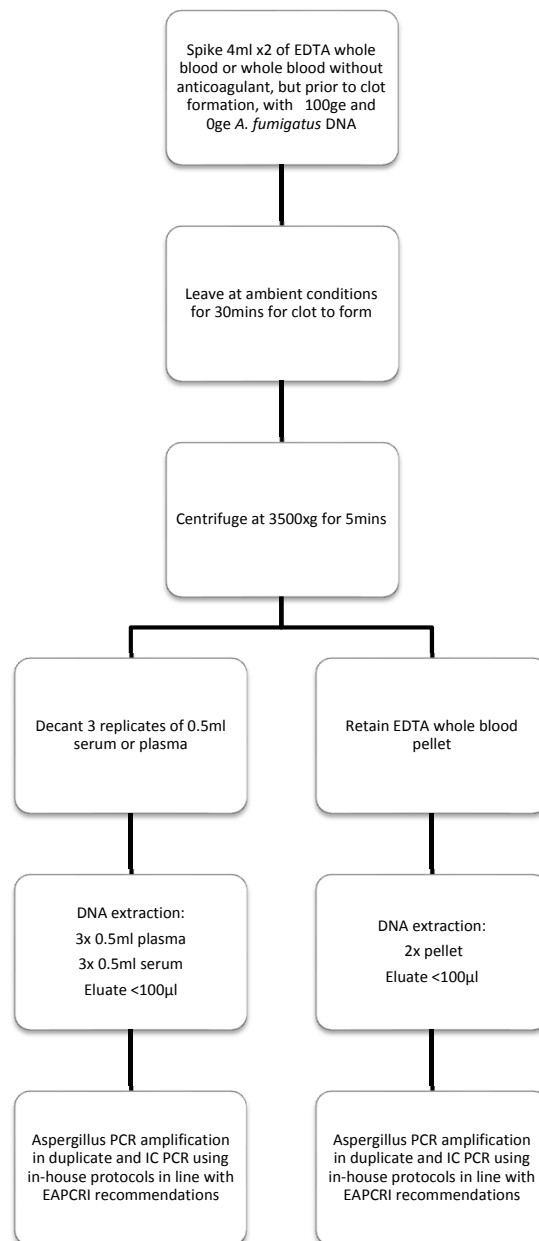
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490 **Figure 4a:** Flow diagram highlighting the process for determining the effect of centrifugation
491 on the availability of *Aspergillus* DNA in serum and plasma samples.
492



493

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

26

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 volume [ml]	Fungal load [ge / ml plasma]				
	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	50	10	20	18S	100	100
			NA Large Volume						
6	240	0.5	EZ1 DSP Virus	60	15	50	28S	100	100
7	50	0.5	MagNAPure Total	100	5	20	28S	100	100
			NA Large Volume						
8	10	0.5	MagNAPure Viral	50	10	50	28S	100	100
			NA Large Volume						

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