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Critical Stages of Extracting DNA from *Aspergillus fumigatus* in Whole-Blood Specimens▽†

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A standardized protocol for extracting DNA from *Aspergillus fumigatus* has been proposed by the European *Aspergillus* PCR Initiative (EAPCRI). Using meta-regression analysis, the EAPCRI showed certain stages of the process to be critical to providing a satisfactory analytical sensitivity. The study investigated each step of the EAPCRI protocol by elimination and monitored the influence on *Aspergillus* PCR performance.

The European *Aspergillus* PCR Initiative (EAPCRI) recently published their research evaluating fungal DNA extraction and *Aspergillus* PCR amplification techniques (5). The paper focused on DNA extraction from whole blood with the aim of providing a standard that could be used to assess clinical validity and utility to allow its inclusion in future disease-defining criteria (1). The results showed that the DNA extraction process was critical to the success of most PCR amplification systems that performed adequately with similar analytical sensitivities.

The standardized whole-blood fungal DNA extraction protocol involved lysis of human blood cells before lysis of the fungal cell by mechanical disruption and DNA purification, precipitation, and elution using commercial kits or instruments. Individual steps and parameters in the DNA extraction process, namely, white cell lysis, fungal lysis by bead beating, and determination of the elution volume, were shown statistically to be crucial to its success, whereas other steps, such as using the entire specimen and red cell lysis, were not critical but were associated with better assay performance. The focus of this report is the evaluation of the performance of the recommended EAPCRI whole-blood DNA extraction protocol with stepwise exclusion/modification of individual steps with the aim of corroborating the critical stages of fungal DNA extraction and the rationale behind the EAPCRI recommendations.

EDTA-whole blood was spiked with *A. fumigatus* (ATCC strain 1022) conidia at two clinically representative fungal burdens (33 conidia/ml and 10 conidia/ml) and then frozen at −80°C. Five replicates of 3 ml EDTA-blood with each fungal concentration were tested for each extraction variant, and controls were included to monitor interexperimental variation and possible contamination. The full DNA extraction protocol was that recommended by the EAPCRI, and on this occasion, the Roche High Pure template DNA kit was used for final DNA purification (5). For most methods, DNA was eluted in a final volume of 60 μl, providing DNA eluate concentrations of 8.8 to 88.3 rRNA copies per μl, dependent on the fungal burden and methodology (Table 1). DNA extraction was then performed, with individual steps of the protocol omitted or modified as described in Table 1. To monitor PCR inhibition, an internal control (IC), a plasmid containing the capsular transport (CTRA) gene of *Neisseria meningitidis*, was introduced at the start of the process that used the High Pure kit. As the IC was not included from the start, it could not be used to determine extraction efficiency. The EAPCRI protocol was also modified to include the additional step of a hot NaOH incubation to help permeabilize the fungal cell wall as previously described (3). *Aspergillus* PCR was performed in duplicate by using the method of White et al., albeit using volumes that allowed PCR to be performed using 15 μl of DNA template in a final volume of 50 μl amplified using the Corbett Rotorgene 3000 system (6). PCR standards were included to monitor amplification efficiency and maintain interexperimental threshold consistency. The IC PCR used 10 μl of DNA template in a final volume of 20 μl, and PCR inhibition was determined by comparison of the individual threshold cycle (C_T) for each specimen to that of a positive plasmid control.

At the higher fungal burden (33 conidia/ml), only the omission of bead beating significantly affected *Aspergillus* PCR detection rates compared to those attained using the EAPCRI protocol (Table 2). Four modifications affected PCR amplification, indicating suboptimal quality and/or quantity of DNA

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TABLE 1. Extraction variables assessed for their influence on fungal DNA extraction efficiency[a]

Extraction protocol[g]	Description	Theoretical maximum eluate DNA concn (rRNA copies/μl)[b]	
		10 conidia/ml blood	33 conidia/ml blood
EAPCRI method	DNA extraction as described by the EAPCRI[c]	26.5	88.3
Modified EAPCRI method			
Plus NaOH	DNA extraction as described by the EAPCRI[c] but with the additional step of 95°C incubation in 50 mM NaOH[d]	26.5	88.3
Using 1 ml of 3-ml sample	DNA extraction as described by the EAPCRI[c] but using 1 ml of a 3-ml sample	8.8 (8.2–9.5[e])	29.4
Using 2 ml of 3-ml sample	DNA extraction as described by the EAPCRI[c] but using 2 ml of a 3-ml sample	17.7(17.0–18.2[f])	58.8
Minus one RCL	DNA extraction as described by the EAPCRI[c] but using only one rather than two red cell lysis steps	26.5	88.3
No RCL	DNA extraction as described by the EAPCRI[c] but without red cell lysis	26.5	88.3
No WCL	DNA extraction as described by the EAPCRI[c] but without white cell lysis	26.5	88.3
No bead beating	DNA extraction as described by the EAPCRI[c] but without bead beating	26.5	88.3
WCL replaced with NaOH	DNA extraction as described by the EAPCRI[c] but with white cell lysis buffer replaced with a 50 mM NaOH incubation	26.5	88.3
Using 100-μl elution vol	DNA extraction as described by the EAPCRI[c] but with DNA eluted in 100 μl	15.9	53.0
Using 200-μl elution vol	DNA extraction as described by the EAPCRI[c] but with DNA eluted in 200 μl	8.0	26.5

[a] DNA eluted in 60 μl unless otherwise stated. The theoretical maximum eluate DNA concentrations are shown for both fungal burdens and show the influence of using smaller sample volumes and eluting in larger volumes.
[b] Assuming 100% extraction efficiency, 1 genome/conidium, and 53 rRNA copies/genome (per reference 2).
[c] See reference 5.
[d] See reference 3.
[e] 95% CI for using one-third of the specimen.
[f] 95% CI for using two-thirds of the specimen.
[g] RCL, red cell lysis; WCL, white cell lysis.

TABLE 2. Influence of the DNA extraction protocol on *Aspergillus* PCR performance[a]

Extraction protocol[e]	10 conidia/ml[d]					33 conidia/ml[d]				
	Aspergillus PCR			Internal control PCR		*Aspergillus* PCR			Internal control PCR	
	Detection rate (%)	Mean C_T (range)	Difference from EAPCRI value	Referenced to PCR standard (cycles)[b]	Difference from EAPCRI value	Detection rate (%)	Mean C_T (range)	Difference from EAPCRI value	Referenced to PCR standard (cycles)[b]	Difference from EAPCRI value
EAPCRI method	100	37.2 (35.3–39.8)	NA	−0.18	NA	100	35.0 (33.3–36.8)	NA	0.66	NA
Modified EAPCRI method										
Plus NaOH	100	36.3 (35.6–37.1)	−0.9	0.14	0.32	100	34.4 (32.3–36.9)	−0.6	0.68	0.02
Minus one RCL	100	37.2 (36.4–38.2)	0.0	0.08	0.26	100	34.8 (34.0–35.5)	−0.2	0.5	0.16
No RCL	90	39.1 (37.7–43.0)	1.9*	0.76[c]	0.94[c]	100	35.8 (33.9–36.9)	0.8	−0.02	0.68*
WCL replaced with NaOH	60*	40.1 (36.3–44.5)	2.9**	0.74	0.92**	100	38.9 (36.9–41.8)	3.9***	2.74	2.08***
Using 100-μl elution vol	50*	40.0 (38.1–45.1)	2.8*	−0.12	0.06	—	—	—	—	—
No WCL	40*	40.6 (39.9–41.3)	3.4**	1.9	2.08**	100	34.8 (33.7–35.6)	−0.2	2.9	2.24***
Using 200-μl elution vol	40*	41.5 (39.0–43.4)	4.3***	1.14	1.32**	90	38.0 (36.1–39.6)	3.0***	2.94	2.28**
Using 2 ml of sample	30**	38.8 (37.5–41.3)	1.6	−0.56	0.38	—	—	—	—	—
Using 1 ml of sample	0***	NA	NA	−0.38	−0.20	100	37.4 (35.9–38.7)	2.4***	1.08	0.42
No bead beating	—	—	—	—	—	30**	38.8 (37.1–40.2)	3.8**	1.08	0.42

[a] Five replicate 3-ml EDTA-blood specimens were spiked with each fungal burden, and *Aspergillus* PCR was performed in duplicate.
[b] The results shown represent the mean differences between the internal control PCR C_T value for the five replicates and that for the positive PCR standard used to calibrate the individual PCR experiments.
[c] The mean difference is influenced by a single outlying value of 3.1 cycles. The other 4 replicates had a difference in C_T of 0.1 to 0.2 cycles. Consequently, the difference between the mean IC PCR C_T values for this method and the EAPCRI method is not statistically significant.
[d] NA, not applicable; —, not performed; *, $0.01 < P$ value < 0.05; **, $0.001 < P$ value < 0.01; ***, P value of <0.001 (measuring the difference between the values with the asterisks and the value for the unmodified EAPCRI method).
[e] RCL, red cell lysis; WCL, white cell lysis.

as represented by later *Aspergillus* PCR C_T values (Table 2). In using 1 ml of the 3-ml specimen, the fungal burden potentially available was reduced from 33 conidia/ml to 11 conidia/ml, a load almost identical to that of the 3-ml specimen spiked with 10 conidia/ml extracted using the EAPCRI method (Table 1). C_T values for these specimens were also the same (Table 2). Removal of a bead-beating step reduced the quantity of fungal DNA extracted, not only affecting reproducibility but also the *Aspergillus* PCR C_T values. Omission of white cell lysis affected the IC, indicating the presence of an inhibitor, and without prior lysis, human DNA survived the extraction, possibly in DNA concentrations potentially detrimental to PCR amplification, although the higher burden was still reproducibly detected. It is postulated that the high fungal DNA concentration combined with a large *Aspergillus* PCR volume compared to those of the IC PCR limited the inhibitory effect. Elution in a larger volume, 200 μl, resulted in a DNA concentration that was lower than those attained using the EAPCRI method and is reflected by later C_T values in both the *Aspergillus* PCR and IC and a slight reduction in *Aspergillus* PCR reproducibility (Tables 1 and 2).

At the lower fungal burden (10 conidia/ml), seven modifications affected *Aspergillus* PCR performance (Table 2). As bead beating was so detrimental for the detection of the higher fungal burden, it was not evaluated at this lower load. Using less than the entire 3-ml specimen, excluding white cell lysis, and eluting in volumes of ≥100 μl greatly reduced detection rates compared to those obtained using the EAPCRI protocol. Assuming 100% extraction efficiency and elution in 60 μl, using a 15-μl template input for PCR incorporates 7.5 (95% confidence interval [CI], 2.7 to 12.3) *A. fumigatus* genomes per reaction, certifying the presence of DNA within each reaction, whereas elutions in 100-μl and 200-μl volumes incorporate 4.5 (95% CI, 0.5 to 8.5) and 2.25 (95% CI, 0 to 5.0) genomes per reaction, respectively, reducing replicate reproducibility.

A reduced detection rate was coupled with later C_T values compared to those obtained with the EAPCRI method, and four modifications resulted in mean C_T values greater than 40 cycles, a threshold that reduces real-time PCR reproducibility below 100% (4). Later C_T values were also seen if red cell lysis was not done, and this was coupled with a slight reduction in the detection rate. Again, the IC was affected by the absence of the white cell lysis step and by the elution of DNA in 200-μl volumes (Table 2).

The removal of one red cell lysis step had no affect on PCR performance, but in this investigation, the blood was frozen prior to testing, resulting in the lysis of the red cells. It is likely that when using fresh blood, two red cell lysis steps are required. Incorporating incubation with hot NaOH after white cell lysis did not improve PCR reproducibility or C_T values for either fungal burden. The primary function of a NaOH incubation is to permeabilize the fungal cell wall and, in so doing, aid downstream lyticase digestion. The use of bead beating obviates this additional step, though hot alkali treatment may still be beneficial when testing blood from nonneutropenic patients, as a greater abundance of white cells may persist despite attempts at conventional lysis (personal practical experience).

In conclusion, to improve analytical sensitivity, the EAPCRI recommends the use of ≥3 ml EDTA blood specimens, a red and white cell lysis step, bead beating to lyse the fungal element, and elution volumes of less than 100 μl. This research investigating the influence of the individual steps of the EAPCRI protocol supports the original findings and emphasizes their importance at low fungal burdens.

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