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Comparison of the utility of five commercial kits for extraction of DNA from *Aspergillus fumigatus* spores★

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The aim of this study was to compare the efficiency of DNA extraction from water as well as from blood samples spiked with *A. fumigatus* spores, using selected commercial kits. Extraction of DNA according to manufacturer's protocols was preceded by blood cells lysis and disruption of fungal cells by enzymatic digestion or bead beating. The efficiency of DNA extraction was measured by PCR using *Aspergillus*-specific primers and SYBR Green I dye or TaqMan probes targeting 28S rRNA gene. All methods allowed the detection of *Aspergillus* at the lowest tested density of water suspensions of spores (10^1 cells/ml). The highest DNA yield was obtained using the ZR Fungal/Bacterial DNA kit, YeastStar Genomic DNA kit, and QIAamp DNA Mini kit with mechanical cell disruption. The ZR Fungal/Bacterial DNA and YeastStar kits showed the highest sensitivity in examination of blood samples spiked with *Aspergillus* (100% for the detection of 10^2 spores and 75% for 10^1 spores). Recently, the enzymatic method ceased to be recommended for examination of blood samples for *Aspergillus*, thus ZR Fungal/Bacterial DNA kit and QIAamp DNA Mini kit with mechanical cell disruption could be used for extraction of *Aspergillus* DNA from clinical samples.

Keywords: *Aspergillus*, aspergillosis, DNA extraction

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INTRODUCTION

Invasive aspergillosis (IA) is a serious, life-threatening mycosis with a number of cases increasing notably over the last two decades. The infection usually occurs in deeply immuno-suppressed patients, e.g., patients with leukaemia, after bone marrow transplantation, and treated with high doses of corticosteroids (Karkowska-Kuleta *et al*., 2009). Despite the improved efficiency of antifungal therapy and introduction of new drugs (e.g., echinocandins, new triazoles, posaconazole, voriconazole, new forms of amphothericine B), the mortality in IA remains high, up to 60–90% (Hardak *et al*., 2009). Early recognition of the infection and rapid initiation of antifungal therapy play a crucial role in patients' recovery and survival. Unfortunately, early diagnosis is an extremely difficult task in the case of IA in immunosuppressed patients. Weak and uncharacteristic clinical signs of the beginning of the infection together with negative results of mycological examinations of routinely tested clinical specimens (sputum, blood) make the diagnosis very difficult. The most relevant specimens are the infected tissues taken by biopsy, however, the patients' poor condition very often stymies the possibility of performing such invasive procedures. Current diagnostic strategies include examination by computer tomography, which detects fungal lesions earlier than traditional X-ray examination, or screening of blood samples for the presence of galactomannan — a cell-wall component of *Aspergillus* (Ascioglu *et al*., 2002; Bhatti *et al*., 2006).

Over the last two decades many molecular methods have been extensively tested for detection of fungal DNA in blood and in other clinical samples (Williamson *et al*., 2000; Loeffler *et al*., 2000b; Kami *et al*., 2001; White *et al*., 2006). Clinical materials usually contain few fungal cells, which stresses the necessity for applying the most sensitive and specific molecular methods, e.g., real-time PCR. In addition to using the most specific primers and probes, suitable for detection of low level of *Aspergillus* DNA, it is extremely important to use the most efficient method of DNA extraction. Generally, obtaining DNA from fungi is regarded to be more difficult than from bacteria or from mammalian cells. Additional procedures leading to disruption of the fungal cell wall (e.g., by mechanical, enzymatic and/or chemical methods) are required (Löffler *et al*., 1997; Loeffler *et al*., 2000a). Poor efficiency of DNA extraction as well as the presence of PCR inhibitors may be the reasons for obtaining false negative results from clinical specimens (Loeffler *et al*., 1999). Apart from the difficulties with the effectiveness of DNA extraction, another problem is the avoidance of contamination with environmental fungi. Because of the ubiquitous distribution of fungal spores, many reagents (sometimes even those certified for molecular biology as DNA-free ones) may be contaminated by airborne fungi and their DNA (Loeffler *et al*., 1999). Such a contamination, usually of no concern when human or bacterial DNA is tested, in the case of detection of pathogenic fungi may cause false positivity, especially in the highly sensitive PCR procedures, such as real-time PCR.

Numerous protocols for extraction of fungal DNA from clinical specimens employing different commercial kits have been described in the medical literature (van

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Abbreviations: BCCM/IHEM, Belgian Co-ordinated Collections of Micro-organisms/Biomedical Fungi and Yeast Collection; fumigatus, *Aspergillus fumigatus*; EAPCRI, European Aspergillus PCR Initiative; IA, invasive aspergillosis; rRNA, ribosomal RNA; qPCR, real-time quantitative PCR

Burik *et al.*, 1998; Loeffler *et al.*, 2002; Maaroufi *et al.*, 2004; Metwally *et al.*, 2008). Currently, the market offers kits usually intended for isolation of human DNA from clinical samples. Their application to mycological investigations requires additional pre-treatment for fungal cell disruption. Another option is to use kits destined for extraction of DNA from fungal cultures and to modify the procedure with the step of blood cell lysis. In this study we used quantitative PCR (q-PCR) to compare the efficiency of DNA extraction from water suspensions of *Aspergillus fumigatus* spores and from blood samples spiked with *Aspergillus* using different commercial kits and rupturing fungal cells by bead beating or enzymatic treatment with lyticase.

MATERIALS AND METHODS

Strains and blood spiking. The study was performed on *Aspergillus fumigatus* BCCM/IHEM 13934. For each experiment the fungi were cultivated on Sabouraud dextrose agar slants at 37 °C for 72 h. One millilitre of sterilised MilliQ water supplemented with Tween 20 (2 µl per 5 ml) was added to each slant. Then the tubes were vortexed gently to obtain a cell suspension which was next collected in a new tube. After washing with water, suspensions of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 spores per millilitre were prepared. The cell number was measured by counting in a Burker's camera and by quantitative evaluation of the culture on Sabouroud plates. The suspensions of 10^7–10^3 cells/ml were used to spike human blood with EDTA to obtain final concentrations of 10^5–10^1 cells/ml (10 µl of appropriate suspension were added to 1 ml of blood).

DNA Extraction. Extraction of DNA from spiked blood samples was performed using a blood cell lysis procedure according to Löffler *et al.* (1997). Namely, 1 ml of blood was incubated with 40 ml of red blood cells lysis buffer (10 mM Tris pH 7.6, 5 mM $MgCl_2$, 10 mM NaCl) for 10 min and then centrifuged for 10 min at 5 000 × *g*. The supernatant was discarded and the pellet was suspended in 1 ml of white blood cells lysis buffer (10 mM Tris pH 7.6, 10 mM EDTA pH 8.0, 50 mM NaCl, 0.2% SDS, proteinase K 200 µg), incubated for 45 min at 65 °C, and subsequently centrifuged for 10 min at 5 000 × *g*. The pellet obtained by this procedure was used for DNA extraction with different commercial kits. All DNA extraction procedures were performed four times in triplicate. A negative control (control of reagent contamination) was performed in each experiment.

Method A. QIAmpDNA Mini kit (Qiagen) and treatment with lyticase (Löffler *et al.*, 1997). The pellet was treated with 300 µl of buffer for spheroplasts (10 mM Tris pH 7.5, 1 mM EDTA, 0.2% β-mercaptoetanol, 0.1% lyticase), incubated for 50 min at 37 °C, and centrifuged for 10 min at 5 000 × *g*. Supernatant was discarded and the pellet suspended in 180 µl of ATL buffer supplemented with 20 µl (12 mAU) of proteinase K (ATL buffer and proteinase K provided in the kit). Starting from this step, the extraction was carried out following exactly the manufacturer's procedure. Briefly, after incubation in ATL buffer (20 min, 55 °C) the AL buffer (200 µl, 10 min incubation at 70 °C) and subsequently ethanol (200 µl) were added. The mixture obtained was loaded on a spin column and washed with AW1 followed by AW2 buffers. DNA was eluted with 60 µl of AE buffer and preserved until use at –28 °C.

Method B. QIAmpDNA Mini kit and bead beating (Löffler *et al.*, 1997; Griffiths *et al.*, 2006). Instead of the treatment with lyticase the pellet was suspended in 180 µl of ATL buffer supplemented with 20 µl (12 mAU) of proteinase K. After incubation at 55 °C for 20 min, the mixture was transferred to a new tube containing 250 mg of sterile acid-washed glass beads of 710–1 180 µm diameter (Sigma G1152-10G) and vortexed 2 × 30 s on a Mini-BeadBeater-8 (Biospec, USA) at the maximal speed. The sample was centrifuged (10 min, 5 000 × *g*) and the supernatant transferred to a new tube. From this point the extraction was carried out following the manufacturer's procedure, according to the description given for method A.

Method C. QIAmpDNA Micro kit (Qiagen) and treatment with lyticase. The extraction was performed similarly to method A, but using reagents from QIAmpDNA Micro kit. The procedure differed from the former in the addition of Carrier RNA to buffer AL.

Method D. QIAmpDNA Micro kit and bead beating. Extraction was performed following method B, using reagents from QIAmpDNA Micro kit and Carrier RNA.

Method E. Dynabeads DNA DIRECT Blood (Dynal Biotech) and bead beating. Portions of 200 µl of lysis buffer containing magnetic beads (Dynabeads) were dispensed to 1.5-ml tubes. The tubes were placed in a Dynal MPC-S magnetic rack to allow the Dynabeads to move to one side of the tube. The supernatant (lysis buffer without the Dynabeats) was transferred to a tube containing pellet formed after lysis of blood cells. After 10 min of incubation at room temp. the mixture was added to a tube with glass beads and vortexed similarly as in method B (2 × 30 s at maximal speed). The samples were centrifuged (10 min, 5000 × *g*) and the supernatant transferred to the previously prepared tubes with Dynabeads. The tubes were again incubated for 10 min at room temperature and placed in Dynal MPC-S to separate the Dynabeats coated with DNA. The supernatant was discarded and DNA eluted in 60 µl of resuspension buffer provided in the kit. Because of a significant loss in DNA yield observed the washing step was omitted.

Method F. ZR Fungal/Bacterial DNA Kit (Zymo Research). The procedure was carried out according to the manufacturer's instruction. Briefly, the pellet was resuspended in 200 µl of PBS (phosphate-buffered saline) and transferred to a ZR Bashing Bead Lysis Tube (tubes with beads provided in the kit). After adding 750 µl of lysis solution the tubes were vortexed on a Mini-BeadBeater-8 at the maximal speed for 3 × 45 s and subsequently centrifuged (10 000 × *g*, 1 min). The supernatant was transferred first to a Zymo-Spin IV Spin Filter and centrifuged at 7 000 × *g* for 1 min and after adding Fungal/Bacterial DNA binding buffer to a Zymo-Spin IIC Column. After washing with appropriate buffers DNA was eluted with 60 µl of DNA elution buffer.

Method G. YeastStar Genomic DNA Kit (Zymo Research). The kit was used in accordance with the manufacturer's instruction. Briefly, the pellet was suspended in 120 µl of YD digestion buffer supplemented with 5 µl of R-Zymolyase and incubated for 30 min at 37 °C. In the next step, 120 µl of YD lysis buffer was added and the sample was vortexed vigorously before addition of 250 µl of chloroform. The content of the tube was mixed and the tube was centrifuged for 2 min at 14 000 × *g*. Supernatant was loaded onto a Zymo-spin column and washed two times with DNA wash buffer. Finally, DNA was eluted with 60 µl of TE.

PCR. qPCR was performed in a Corbett Rotor-Gene (Corbett Research Ltd, Cambridge, UK) system using a real-time PCR protocol with *Aspergillus*-specific primers ASF1 5'-GCA CGT GAA ATT GTT GAA AGG-3' and ADR1 5'-CAG GCT GGC CGC ATT G-3' targeting the 28S rRNA gene (Williamson *et al.*, 2000) and SYBR Green dye. The SYBR Green I PCR amplification mixture of 20 µl contained 10 µl of Maxima SYBRs Green Master Mix (Fermentas), 1 µl of each primer (5 mM), 2 µl of DNA template and 6 µl of water. Selected samples were tested additionally with the *Aspergillus*-specific hydrolysis probe ASP 28P 5'-FAM-CAT TCG TGC CGG TGT ACT TCC CCG-TAMRA-3' (White *et al.*, 2006b). The TaqMan PCR amplification mixture of 20 µl contained 10 µl of Platinum Quantitative PCR Super Mix-UDG (Invitrogen), 1 µl of each primer (5 mM), 0.5 µl of probe (20 mM), 2 µl of DNA template and 5.5 µl of deionized water. The PCR amplification protocol used in both cases was as follows: one cycle of 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. In each run of the SYBR Green and TaqMan methods negative (sterile water instead of template DNA) as well as positive (*A. fumigatus* DNA) controls were included. The presence of PCR inhibitors in DNA samples obtained from blood (without spores) was tested by adding of exogenous DNA (2 pg of *A. fumigatus* DNA) to the PCR mixture. A difference of more than two PCR cycles was considered a significant inhibition. Genomic DNA of *A. fumigatus* BCCM/IHEM 13934 of a concentration estimated basing on absorbance at 260 nm (NanoDrop ND-1000 Spectrophotometer; TK Biotech) was used as a standard. The Ct values obtained for eight ten-fold dilutions of fungal DNA (100 ng–10 fg) were used to prepare standard curves with Rotor-Gene Software Version 1.7.87 and assess the amount of DNA in tested samples. Sequencing of PCR products was performed by standard methods in the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Warsaw, Poland). The sequences obtained were analysed with the use of the Blast software and Genbank-EMBL database.

The statistical analysis of obtained data was performed with the use of Student's *t*-test and software system STATISTICA, version 9.0. (StatSoft, Inc. 2009).

RESULTS

The efficiency of the extraction methods under study is presented in Fig. 1. The results of qPCR are expressed as C_T values read out with the threshold selected manually at 0.1, which each time was above the negative controls. All the methods succeeded in extracting of DNA from water suspensions of *A. fumigatus* spores of the lowest density (10^1 cells/ml). Method B (mechanical lysis followed by extraction with QIAamp DNA mini kit) showed the highest sensitivity (100% of positive results), whereas the other methods were successful in 75% (method F), 50% (methods A and G) or 25% (methods C, D, E) of experiments with such a low number of *Aspergillus* spores. The efficiency of extraction with the QIAamp DNA Mini kit was better when the cell disruption was performed mechanically (method B) than using enzymatic lysis (method A). Replacing QIAamp DNA Mini with QIAamp DNA Micro (methods C and D), a kit for small volume samples, did not improve the yield of DNA extraction at any concentration of fungal spores. Similarly, extraction performed with the help of magnetic beads (method E; Dynabeads DNA DIRECT Blood) showed the same level of efficiency as method A. Methods F and G employed Zymo Research kits designed for fungi and were performed according to manufacturer's instruction, which included either mechanical disruption (method F) or enzymatic treatment (method G). Both tests resulted in significantly higher DNA yield than method A from inocula of 10^7–10^3 cells/ml ($P<0.05$; calculated on the basis of 12 readings obtained in four experiments).

The methods listed above (except for QIAamp DNA Micro) were used to extract DNA from blood samples spiked with 10^5–10^1 *A. fumigatus* spores. qPCR performed with non-spiked blood revealed the reactivity of the ADR/ASF primers with human DNA resulting in the presence of an amplicon with a melting temperature of 85 °C. This additional PCR product could be easily distinguished from the amplicon obtained with *Aspergillus* DNA, which had a melting temperature of 89 °C (Fig. 2). The identity of both amplicons was examined by sequencing and subsequent analysis with the use of the Blast software and the Genbank-EMBL database. It was found that the non-specific PCR product corresponds to a fragment of reference sequence NT 030059.13 representing part of human chromosome 10, and the product obtained with *Aspergillus* DNA was confirmed to be 100% identical with reference sequences of *A. fumigatus* (as an example *A. fumigatus* ATCC16907 — Gen Bank number AY216670.1). The presence of dual amplification was observed for DNA samples obtained from blood spiked with a low (100–10) number of fungal spores. Retesting of those samples with the *Aspergillus*-specific TaqMan probe confirmed the presence of approximately 100 fg (mean Ct value 40.6) of *Aspergillus* DNA in 50% of samples. The analysis of DNA yield obtained from blood samples spiked with 10^5–10^3 spores indicated the highest efficiency for methods F and G. Both methods showed also the highest sensitivity, which was 100% for the detection of 10^2 spores and 75% for 10^1 spores. The methods based on a Qiagen kit (A and B) succeed in 75% of samples with 10^2 and 50% of sam-

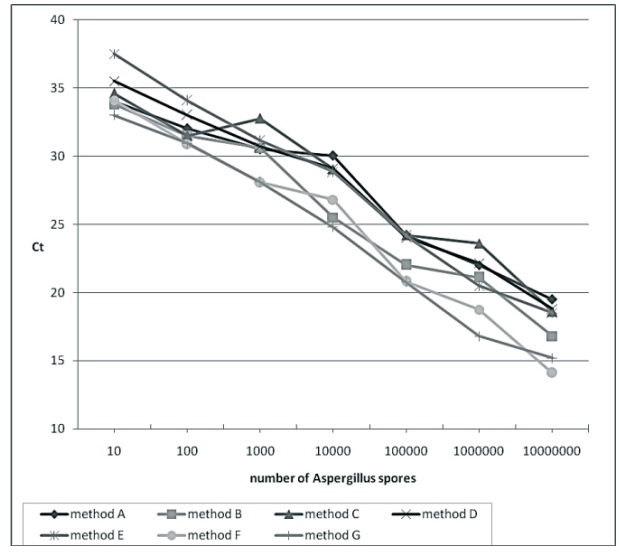

Figure 1. Results of qPCR examination of DNA samples obtained from water suspension of *Aspergillus fumigatus* spores
Mean Ct values obtained from four experiments performed in triplicate are shown.

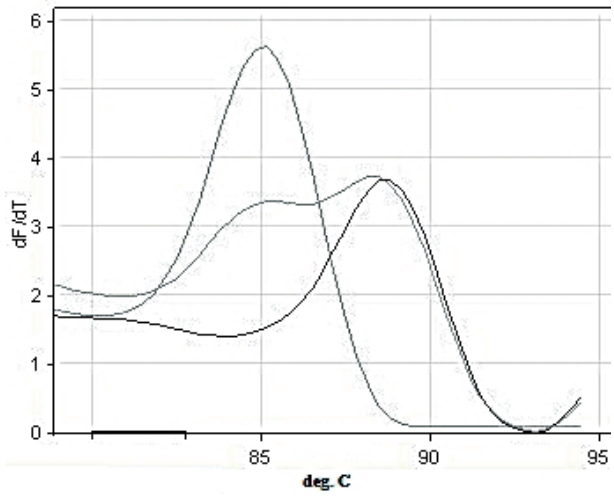

Figure 2. Melt data of PCR products obtained with primers ASF1 /ADR1 and DNA from *Aspergillus* (89 °C) and from human blood (85 °C).

ples with 10^1 spores. Lower sensitivity was observed for method E, which was 50% for 10^2 and failed to detect 10^1 spores (Table 1). The PCR inhibition test performed for DNA isolated from non-spiked blood was negative for all methods tested (differences between the Ct values obtained in the presence of tested human DNA and fungal DNA alone were 0–1 cycle; not shown).

DISCUSSION

Over the past 20 years several protocols for detection and identification of *Aspergillus* by PCR have been developed, but a standard procedure for examination of clinical samples remains to be established. Experts from the United Kingdom Fungal PCR Consensus Study Group (White *et al*., 2006a) and the European Aspergillus PCR Initiative (EAPCRI) working group of the International Society of Human and Animal Mycoses (White *et al*., 2010) — organisations purposed to offer recommendations for the use of PCR in the diagnosis of mycoses agree that extraction procedure is the main factor limiting the efficiency of *Aspergillus* PCR. Recently the EAPCRI published recommendations for extraction of fungal DNA from blood samples (White *et al*., 2010). The protocol recommends the use of 3–4 ml of EDTA-whole blood specimens and the following extraction procedure: lysis of red and white blood cells, disruption of fungal cells by bead beating, and DNA purification with commercial kits for manual or automated processing. The present study had been performed before those recommendations were published. We aimed to evaluate the impact of the method of fungal cell disruption and the use of different commercial kits on the efficiency of DNA extraction. The initial step of blood cell lysis used in this study is in agreement with the described standard, except for sample volume, which was 1 ml. The comparison between the enzymatic and mechanical cell disruption (extraction using Qiagen kit; methods A and B) indicated that bead beating is more efficient than enzymatic lysis, especially at the highest concentrations of spores tested (Fig. 1). Method B was the most sensitive and allowed fungal DNA to be detected in 100% of water samples inoculated with 10^1 spores, but its sensitivity with blood samples was only 50% (Table 1). In this method the bead beating was performed in the AL buffer as described by Griffiths *et al*. (2006) and the extraction was carried out using the QIAamp DNA mini kit. Griffiths and co-workes found this method the most successful with the detection limit of 10 conidia *versus* 10^3 obtained for enzymatic lysis. Other authors (Lugert *et al*., 2006) achieved detection limit of 10^4 spores using an enzymatic method. In the present study a combination of a enzymatic lysis and Qiagen test detected 10 cells with the sensitivity of 50%. It should be emphasized that the enzymatic method gives a higher DNA yield from *Candida* than from moulds and probably it can be more suitable for examinations of yeasts (Fredricks *et al*., 2005). The other two commercial tests used in this study are designed for extraction of DNA from cultures of yeast (method G) and from fungi (method F) with the use of enzymatic lysis or bead beating, respectively. Both tests showed similar efficiency and detection limit of 10 conidia, which indicates that the same sensitivity of *Aspergillus* detection is achievable using mechanical cell disruption and enzymatic lysis. The yield of DNA obtained with methods F and G was higher than with method B. The better result of method F comparing to B may be related to some differences in the pre-treatment procedure (type of beads, longer duration of bead beating). Currently, the EAPCRI recommends that enzymatic digestion be replaced by bead beating. The justification for this is the higher expense and the longer working-time required for enzymatic methods compared to mechanical ones. Enzymatic methods are also connected with a potentially higher risk of contamination, because of the more complicated procedure and the use of recombinant lyticase (often contaminated with fungal DNA). Contamination with fungal DNA was detected in many commercial kits including those from Qiagen (Loeffler *et al*., 1999). Working with an *Aspergillus*-specific probe we did not notice any contamination problems with the Qiagen or Zymo Research kits, while the panfungal PCR did detect the presence of fungal DNA in reagents and

Table 1. Results of real-time PCR examination of DNA samples obtained from blood spiked with *A. fumigatus* spores.
Inocula 10^5–10^3 were tested by the SYBR Green method and inocula 10^2 and 10^1 by the TaqMan method.

Method of DNA isolation	*Ct values (± standard deviation) for DNA samples obtained from blood spiked with following numbers of *A. fumigatus* spores:				
	10^5	10^4	10^3	10^2 (% of positive results)	10^1 (% of positive results)
A	25.75 ± 2.05	29.4 ± 0.5	31.2 ± 0.95	40 (75%)	42 (50%)
B	24.45 ± 2.19	29 ± 0.8	29.7 ± 0.66	41 (75%)	42 (50%)
E	25.7 ± 1.3	29.25 ± 2.47	34.4 ± 2.46	42 (50%)	no amplification
F	21.08 ± 1.43	23.09 ± 0.80	28.35 ± 1.93	39 ± 0.7 (100%)	40.5 (75%)
G	23.35 ± 1.05	27.6 ± 0.28	30.45 ± 0.35	39.3 ± 0.84 (100%)	42.4 (75%)

*mean Ct values obtained from four experiments performed in triplicate

spin columns of the ZR Fungal/Bacterial DNA Kit (not shown). In most commercial kits the DNA purification step is performed with the help of spin columns. Method E is based on magnetic separation of DNA adsorbed on magnetic beads. The magnetic method of DNA purification is used in the automated system MagNA Pure LC, which has been reported as highly sensitive in mycological examination of blood samples (Loeffler *et al*., 2002) and is also listed among kits recommended by the EAPCRI. The usefulness of manual magnetic method in extraction of DNA from yeasts and dermatophytes has also been reported (Faggi *et al*., 2005). In this study we found the magnetic method less sensitive and thus not applicable for examination of clinical samples. According to the EAPCRI the detection limit achieved in examination of blood samples should be no more than 50 fungal cells. Among the methods tested in this study only methods B and F satisfied all requirements of new recommendation (detection limit, bead beating). Considering that method F (ZR Fungal/Bacterial DNA Kit) is more sensitive and less expensive that method B (QIAmpDNA Mini kit) we conclude that this is the more appropriate method for testing *Aspergillus* in blood. However, all of the methods studied, including the less efficient method E (Dynabeads), may be used for DNA extraction from high concentration of fungal cells.

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