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Note

Variation in copy number of the 28S rDNA of *Aspergillus fumigatus* measured by droplet digital PCR and analog quantitative real-time PCR

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ABSTRACT

Droplet digital PCR (ddPCR) after DNA digestion yielded a 28S rDNA copy number of 61 to 86 copies/genome when testing 10 unrelated *Aspergillus fumigatus* isolates, higher than with quantitative PCR. Unfortunately, ddPCR after DNA digestion did not improve the sensitivity of our PCR assay when testing serum patients with invasive aspergillosis.

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The DNA detection for the diagnosis of invasive aspergillosis (IA) are dominated by laboratory specific assays based mostly on ribosomal DNA (rDNA) targets (White et al., 2015b) with varying performance (Cruciani et al., 2015; White et al., 2015a). Among the possible causes of this variability is the known copy number variation (CNV) of the rDNA of *Aspergillus fumigatus* (Herrera et al., 2009), the main species involved in IA (Lortholary et al., 2011). The results generated by quantitative PCR (qPCR) are then dependent on CNV of the rDNA of each isolate and could participate to some discrepancies observed between different quantitative PCR (qPCR) assays. A small variation in sensitivity of the PCR assays can lead to false negative results because the fungal DNA load in blood or serum during IA is most often very low (Costa et al., 2002; Loeffler et al., 2000).

Relative quantification of CNV using qPCR is often used because of its easiness (D'haene et al., 2010; Herrera et al., 2009). However, droplet digital PCR (ddPCR) intrinsically allows better accuracy of DNA quantification (Huggett et al., 2013, 2015). The first step in ddPCR is to isolate single molecules in droplets with some of them containing the target molecule and some not. It allows independent amplification of each target that will lead at the end to an absolute count of the number of target molecules in the sample. The sample preparation of genomic DNA for ddPCR requires restriction enzyme digestion (RED) to separate tandem gene copies and to then ensure proper random partitioning into droplets (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf). This method particularly fits with measurement of small percentage differences and quantification of rare variants (Huggett et al., 2015). Therefore, we investigated the potential of ddPCR in comparison with qPCR for *A. fumigatus* rDNA CNV. We secondarily tested serum samples from patients to know whether ddPCR can improve the performance of PCR for IA diagnosis or can give clues on the nature of the DNA amplified.

DNA from the *A. fumigatus* reference strain AF293 (CBS 101355) and nine isolates from nine patients with IA was extracted as already described (Alanio et al., 2011). All had different genotypes (Bart-Delabesse et al., 1998) and belonged to different clonal complexes (Alanio et al., 2012).

A duplex PCR assay was designed to amplify either the 28S rDNA (Challier et al., 2004) or the single copy gene FKS1 of *A. fumigatus* (Herrera et al., 2009) with primers and probe sequences as reported by the authors. The 28S rDNA probe was labeled with 6-carboxyfluorescein (FAM) and the FKS1 probe with hexachloro-fluorescein (HEX). Primer and probe concentrations were set at 0.3 and 0.1 μM, respectively, in the ddPCR Supermix for Probes (Biorad, Marnes-la-Coquette, France) for the ddPCR and the 480 probe Master (Roche, Meylan, France) for the qPCR.

For the ddPCR assay, after mixing 2.2 μL of DNA with 19.8 μL of premix, 20 μL of solution were used to generate droplets using the QX100® droplet generator (Biorad), amplified in a C1000 Touch® thermocycler (Biorad) and then analyzed in a QX100® droplet reader (Biorad). The

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absolute quantification of each target was analyzed using Quantasoft® software and expressed as number of copies/μL. Indeed, the number of positive and negative droplets was determined using specific thresholds based on the dotplot and histogram analysis of droplets' fluorescence (Fig. 1A). A preliminary experiment using temperature gradient and *A. fumigatus* Af293 DNA without and with RED (Fig. 1B) allowed to fix the optimal annealing temperature at 59 °C, which was used thereafter for qPCR and ddPCR (activation at 95 °C 10 min, 45 cycles of 95 °C for 15 s and 59 °C for 60 s). This optimized annealing temperature is the one resulting in the largest fluorescence amplitude difference of the droplets between the positive and negative wells (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf).

The qPCR assay was performed in a LightCycler 480 instrument (Roche). Calibration curves and efficacies were obtained upon serial 10-fold dilutions. 28S and FKS1 PCR efficiencies were 1.919 and 1.929, respectively. Determination of the 28S rDNA copy number using qPCR was performed with modification of Pfaffl method (Pfaffl, 2001) as: $(28S\ efficiency^{-(28S\ qPCR\ Cq)})$ / $(FKS1\ efficiency^{-(FKS1\ qPCR\ Cq)})$.

The ten *A. fumigatus* DNAs underwent RED using *Eco*RI and *Rsa*I (New England Biolabs, Ipswich, USA), after verification of the absence of specific recognition sites in the 28S and FKS1 amplified regions. RED was designed to calculate the number of independent copies of 28S rDNA on the same DNA strand. The ten DNAs were then tested undigested and digested.

In parallel, ten frozen sera from ten patients with probable IA already known to be qPCR-positive were selected. After thawing, DNA from one milliliter of serum was extracted using the Qiasymphony DSP virus/ Pathogen Mini kit (Qiagen, Hilden, Germany) on a Qiasymphony apparatus (Qiagen), eluted in 85 μL of water and tested using the 28S rDNA PCR (Challier et al., 2004). The ten patients DNAs were then tested undigested and digested as above.

When testing the DNAs of the ten stain/isolates, the number of 28S rDNA copies/genome varied from 20 to 49 and from 46 to 68 using qPCR (Fig. 2A) and from 34 to 76 and from 61 to 86 using ddPCR (Fig. 2B), without and with RED, respectively, The copy numbers were significantly higher using ddPCR compared to qPCR (Wilcoxon paired *t*-test,

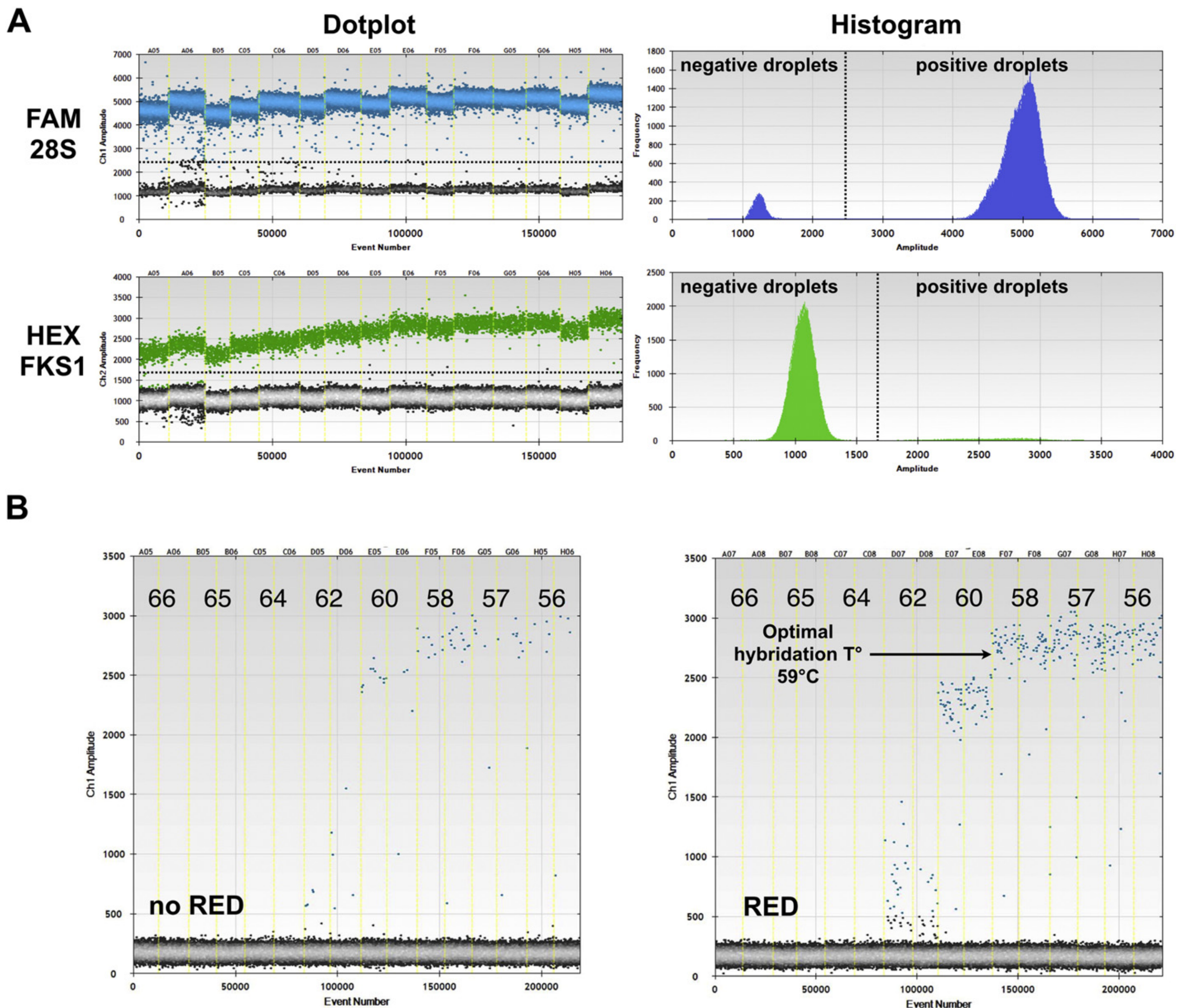

Fig. 1. Specific analysis of ddPCR data on the Quantasoft® software (Biorad) including gradient analysis dotplot (A) and an example of dotplot and histogram analysis for quantification of the number of positive and negative droplets (B). The analysis of the gradient of hybridization temperature showed that 59 °C was the optimal temperature for the 28S assay since the fluorescence was higher in the droplets at 59 °C with no gain at higher temperatures. The number of droplets was also higher in RED compared to no RED tests. Positive and negative droplets were determined using dotplot and histogram analysis of each experiment by applying a florescence threshold (2400 for 28S and 1650 for FKS1).

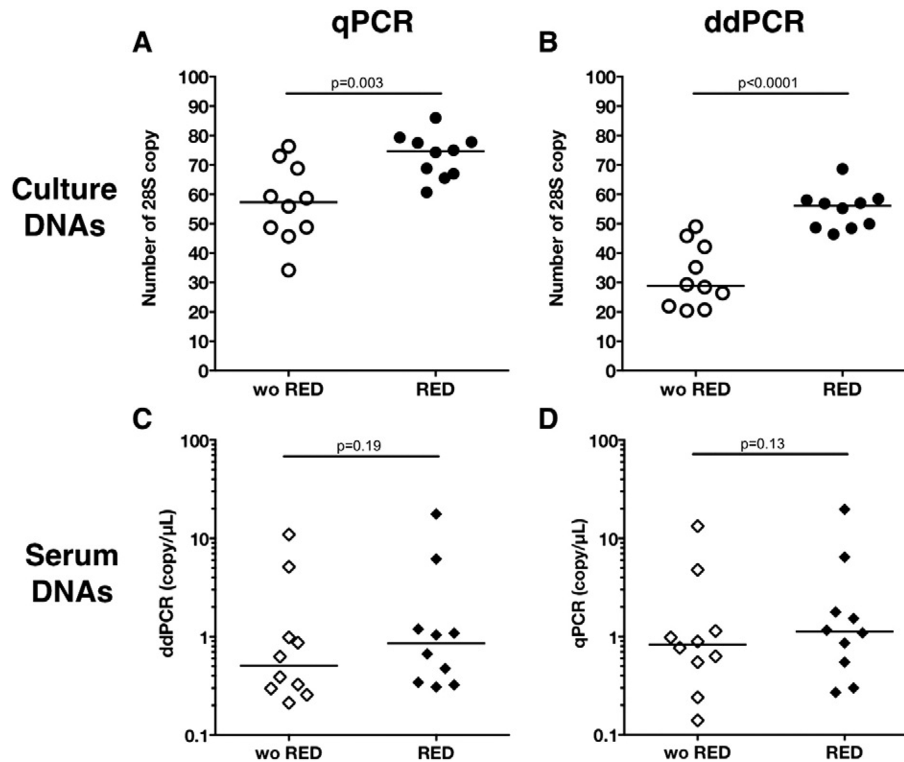

Fig. 2. Distribution of the number of number of 28S copies/genome of nine *A. fumigatus* clinical isolates and Af293 using qPCR (A) or ddPCR (B) and the number of 28S copies/μL of the DNA of 10 sera of patients with invasive aspergillosis using qPCR (C) or ddPCR (D) regarding DNA treated with or without restriction enzyme digestion (RED). For strains analysis in qPCR, one dot corresponds to the mean of four independent experiments tested at different concentrations (pure, 1:20 and 1:200) in duplicates (eight values per strains). For strains analysis in ddPCR, one dot corresponds to the mean of three independent experiments tested at different concentrations (1:10, 1:40 and 1:100) and duplicates (six values per strains). For patient analysis, one dot corresponds to the mean of one duplicate.

$p < 0.0001$, with and without RED) and with RED compared to without RED (Wilcoxon paired *t*-test, $p = 0.003$ for qPCR and $p < 0.0001$ for ddPCR). When considering the Af293 reference strain, we found a higher copy number using ddPCR (69 copies) for the 28S copy number than the 35 copies obtained after whole genome sequencing (Nierman et al., 2005) and the 38 copies of 18S rDNA obtained using qPCR without RED (Herrera et al., 2009). Of note, using qPCR without RED, we obtained closer results (46 28S rDNA copies versus 38 18S rDNA copies) than previously found (Herrera et al., 2009), knowing that the same copy number is expected for 18S and 28S rDNA since the large and small ribosomal subunits are collinear and transcribed as a single transcript (Iwen et al., 2002; Warner, 1989).

In ddPCR, the proportion of positive droplets allows precise quantification independently of the PCR efficiency, in contrast to qPCR for which relative quantification is susceptible to the PCR efficiency. A small variation in the efficiency of one of the two assays can lead to huge differences in the quantification (Pfaffl, 2001). Therefore, calculation of the copy number using relative quantification based on a exponential formula introduced bias, that are intrinsically not observed with ddPCR, Interestingly, the gain in the number of 28S copies using RED was also observed using qPCR, although lower than with ddPCR. Thus, RED could act to make more targeted molecules accessible and then amplifiable with a possible improvement of sensitivity of PCR assays.

Therefore, we tested ten serum samples from patients with IA with and without RED. The number of 28S copies ranged from 0.1 to 13 and from 0.3 to 20 copies/μL using qPCR with and without RED, respectively (Fig. 2C), whereas the number of 28S copies in the serum of patients ranged from 0.2 to 11 and from 0.3 to 18 copies/μL using ddPCR with and without RED, respectively (Fig. 2D). The number of circulating 28S copies was not significantly different using ddPCR compared to qPCR and with and without RED (Wilcoxon paired *t*-test, $p > 0.05$). The lower gain with DNA extracted from patient serum samples than from fungal cultures suggests that circulating *A. fumigatus* DNA in human serum is already fragmented. Indeed, the mean length of circulating DNA fragment is usually short, mainly below 500 bp, when investigated in human genetics and prenatal diagnosis (Breitbach et al., 2014). As a consequence, digestion of DNA present in serum does not increase the number of copies, which could have increased the sensitivity of the qPCR assays for the diagnosis of IA.

In conclusion, CNV may be an important factor that affects the sensitivity of PCR assays since different clinical strains can harbor different number of 28S qPCR copies. This could be one of the explanations of the different performances observed in the clinical evaluation of these PCR assays (Cruciani et al., 2015; White et al., 2015a). CNV mainly depends on the *A. fumigatus* strain since the other parameters such as culture duration, temperature culture, antifungal exposure, fungal morphology (pure conidia and hyphae) have already been shown not to impact on the copy number of 18S rRNA in *A. fumigatus* (Herrera et al., 2009). For intrinsic technical reasons, quantification using ddPCR is more reliable than when using qPCR, although the results are very parallel.

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References

Alanio, A., Sitterlé, E., Liance, M., Farrugia, C., Foulet, F., Botterel, F., Hicheri, Y., Cordonnier, C., Costa, J.-M., Bretagne, S., 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. J. Antimicrob. Chemother. 66, 371–374.

Alanio, A., Cabaret, O., Sitterlé, E., Costa, J.-M., Brisse, S., Cordonnier, C., Bretagne, S., 2012. Azole preexposure affects the *Aspergillus fumigatus* population in patients. Antimicrob. Agents Chemother. 56, 4948–4950.

Bart-Delabesse, E., Humbert, J.F., Delabesse, E., Bretagne, S., 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J. Clin. Microbiol. 36, 2413–2418.

Breitbach, S., Tug, S., Helmig, S., Zahn, D., Kubiak, T., Michal, M., Gori, T., Ehlert, T., Beiter, T., Simon, P., 2014. Direct quantification of cell-free, circulating DNA from unpurified plasma. PLoS One 9, e87838.

Challier, S., Boyer, S., Abachin, E., Berche, P., 2004. Development of a serum-based Taqman real-time PCR assay for diagnosis of invasive aspergillosis. J. Clin. Microbiol. 42, 844–846.

Costa, C., Costa, J.-M., Desterke, C., Botterel, F., Cordonnier, C., Bretagne, S., 2002. Real-time PCR coupled with automated DNA extraction and detection of galactomannan antigen in serum by enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis. J. Clin. Microbiol. 40, 2224–2227.

Cruciani, M., Mengoli, C., Loeffler, J., Donnelly, P., Barnes, R., Jones, B.L., Klingspor, L., Morton, O., Maertens, J., 2015. Polymerase chain reaction blood tests for the diagnosis of invasive aspergillosis in immunocompromised people. Cochrane Database Syst. Rev. 9, CD009551.

D'haene, B., Vandesompele, J., Hellemans, J., 2010. Accurate and objective copy number profiling using real-time quantitative PCR. Methods 50, 262–270.

Herrera, M.L., Vallor, A.C., Gelfond, J.A., Patterson, T.F., Wickes, B.L., 2009. Strain-dependent variation in 18S ribosomal DNA copy numbers in *Aspergillus fumigatus*. J. Clin. Microbiol. 47, 1325–1332.

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., Bustin, S.A., 2013. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin. Chem. 59, 892–902.

Huggett, J.F., Cowen, S., Foy, C.A., 2015. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin. Chem. 61, 79–88.

Iwen, P.C., Hinrichs, S.H., Rupp, M.E., 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med. Mycol. 40, 87–109.

Loeffler, J., Henke, N., Hebart, H., Schmidt, D., Hagmeyer, L., Schumacher, U., Einsele, H., 2000. Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. J. Clin. Microbiol. 38, 586–590.

Lortholary, O., Gangneux, J.-P., Sitbon, K., Lebeau, B., de Monbrison, F., Le Strat, Y., Coignard, B., Dromer, F., Bretagne, S., French Mycosis Study Group, 2011. Epidemiological trends in invasive aspergillosis in France: the SAIF network (2005–2007). Clin. Microbiol. Infect. 17, 1882–1889.

Nierman, W.C., Pain, A., Anderson, M.J., Wortman, J.R., Kim, H.S., Arroyo, J., Berriman, M., Abe, K., Archer, D.B., Bermejo, C., Bennett, J., Bowyer, P., Chen, D., Collins, M., Coulsen, R., Davies, R., Dyer, P.S., Farman, M., Fedorova, N., Fedorova, N., Feldblyum, T.V., Fischer, R., Fosker, N., Fraser, A., García, J.L., García, M.J., Goble, A., Goldman, G.H., Gomi, K., Griffith-Jones, S., Gwilliam, R., Haas, B., Haas, H., Harris, D., Horiuchi, H., Huang, J., Humphray, S., Jiménez, J., Keller, N., Khouri, H., Kitamoto, K., Kobayashi, T., Konzack, S., Kulkarni, R., Kumagai, T., Lafon, A., Lafton, A., Latgé, J.-P., Li, W., Lord, A., Lu, C., Majoros, W.H., May, G.S., Miller, B.L., Mohamoud, Y., Molina, M., Monod, M., Mouyna, I., Mulligan, S., Murphy, L., O'Neil, S., Paulsen, I., Peñalva, M.A., Pertea, M., Price, C., Pritchard, B.L., Quail, M.A., Rabbinowitsch, E., Rawlins, N., Rajandream, M.-A., Reichard, U., Renauld, H., Robson, G.D., Rodriguez de Córdoba, S., Rodríguez-Peña, J.M., Ronning, C.M., Rutter, S., Salzberg, S.L., Sanchez, M., Sánchez-Ferrero, J.C., Saunders, D., Seeger, K., Squares, R., Squares, S., Takeuchi, M., Tekaia, F., Turner, G., Vazquez de Aldana, C.R., Weidman, J., White, O., Woodward, J., Yu, J.-H., Fraser, C., Galagan, J.E., Asai, K., Machida, M., Hall, N., Barrell, B., Denning, D.W., 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438, 1151–1156.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Warner, J.R., 1989. Synthesis of ribosomes in *Saccharomyces cerevisiae*. Microbiol. Rev. 53, 256–271.

White, P.L., Barnes, R.A., Springer, J., Klingspor, L., Cuenca-Estrella, M., Morton, C.O., Lagrou, K., Bretagne, S., Melchers, W.J.G., Mengoli, C., Donnelly, J.P., Heinz, W.J., Loeffler, J., 2015a. Clinical performance of *Aspergillus* PCR for testing serum and plasma: a study by the European *Aspergillus* PCR initiative. J. Clin. Microbiol. 53, 2832–2837.

White, P.L., Wingard, J.R., Bretagne, S., Löffler, J., Patterson, T.F., Slavin, M.A., Barnes, R.A., Pappas, P.G., Donnelly, J.P., 2015b. *Aspergillus* polymerase chain reaction: systematic review of evidence for clinical use in comparison with antigen testing. Clin. Infect. Dis. 61, 1293–1303.