Evaluation of two PCR methodologies for the detection of *Aspergillus* DNA

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Summary We investigated the application of two different nested PCR methodologies for the diagnosis of invasive aspergillosis, by studying the possible cross-reactions among different fungal species and genera. The sensitivity obtained using both techniques can be considered adequate, although we obtained amplified products from fungal genera other than *Aspergillus*, showing the presence of cross-reactions with fungal DNA.

Key words *Aspergillus*, DNA, PCR, Sera, Cross-reactions

Evaluación de dos metodologías PCR para la determinación de ADN de *Aspergillus*

Resumen En el presente trabajo hemos estudiado la aplicación de dos diferentes metodologías de PCR anidada para el diagnóstico de la aspergilosis invasiva, analizando las posibles reacciones cruzadas entre diferentes especies y géneros fúngicos. La sensibilidad obtenida utilizando ambas técnicas puede considerarse aceptable, aun cuando se obtuvieron productos de amplificación de otros géneros distintos de *Aspergillus*, apareciendo por tanto reacciones cruzadas.

Palabras clave *Aspergillus*, ADN, PCR, Suero, Reacciones cruzadas

Aspergillosis is an increasingly prevalent disease both in human and in veterinary medicine [1]. The incidence of systemic aspergillosis in neutropenic patients is only exceeded by *Candida* yeast infections [1,12]. It mainly affects immunocompromised individuals (neutropenic patients, solid organ and bone marrow transplant recipients receiving high doses of corticosteroids) [2,14]. Currently, one of the main problems is that the disease is diagnosed too late for the patient, with an unfavorable prognosis.

Therefore, clinical diagnosis is very unspecific. Microbiological diagnosis is laborious because culture of the fungus can lead to false positives, since *Aspergillus* is ubiquitous in the environment. False negatives may also occur because the fungus, even if it is responsible for the disease, is not always recovered in culture during primary isolation. Histopathology is the most reliable technique, but it is unspecific, time consuming and, above all, invasive. Immunologic diagnosis has provided promising results in veterinary medicine [9] and in certain human processes such as ABPA or aspergilloma, although this is not the case in invasive aspergillosis.

Faced with these diagnostic shortcomings, we decided to investigate the specificity of the PCR technique, that is, the possible cross-reactions among different fungal species and genera.

To evaluate this procedure, genomic DNA was obtained according to the method previously described [8] from 27 strains of the following fungal species: *Acremonium chrysogenum*, *Alternaria alternata*, *Aspergillus flavus, Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus niger, Aspergillus oryzae, Aspergillus restrictus, Aspergillus terreus*, *Aspergillus terreus var. terreus*, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Cladosporium cladosporioides*, *Cryptococcus laurentii, Cunninghamella elegans, Fusarium graminearum, Geotrichum lactis, Microsporum canis, Mucor racemosus, Penicillium chrysogenum, Penicillium griseofulvum, Rhizopus oryzae, Rhizopus stolonifer*, *Syncephalastrum racemosus*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*.

The amount of DNA obtained was calculated spectrophotometrically (Biophotometer, Eppendorf, Germany) by measuring the optical density (OD) at 260 nm and the quality was estimated using the relation between the OD at 260 nm and at 280 nm (OD_{260} / OD_{280}). The concentrations were then adjusted to determine the sensitivity and specificity of each technique.

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We applied two nested PCR techniques to the DNA extracted. Technique 1 used as external primers ASP 5 (5'GATAACGAACGAGACCTCGG3') and ASP 8 (5' TGCCAACTCCCCTGAGCCAG 3'), which amplify a 384 bp sequence, and as internal primers we used ASP 1 (5'CGGCCCTTAAATAGCCCGGTC3') and ASP 7 (5'CCTGAGCCAGTCCGAAGGCC3'), which amplify a 357 bp sequence and are based on primers used elsewhere [18]. The primers come from the sequence of the variable region V7-V9 of the genes of the rRNA subunit 18S. The mix of the first amplification consisted of 50 mM KCl, 15 mM Tris HCl (pH=8), 1.5 mM $MgCl_2$, 250 µM of each dNTP (dATP, dCTP, dTTP, dGTP) and 0.02 U/µl of Taq DNA polymerase (ampliTaq Gold, Applied Biosystems, USA). Two pmol/µl of each external primer was combined with 10 µl of DNA solution (0.3 ng/µl), giving a final volume of 50 µl. The PCR was carried out on an automatic thermocycler (GeneAmp PCR System 9700, PE Biosystem, USA). The amplification reaction consisted of an initial cycle at 95 °C for 10 min, followed by 30 denaturation cycles at 94 °C for 1 min, hybridization at 50 °C for 1 min and extension at 72 °C for 3 min, finishing with an extension cycle at 72 °C for 10 min. One µl of the product obtained in the first reaction was taken and added to a new mix identical to the previous one, but with internal primers. The times and temperatures of the second amplification reaction were equal to those of the first one, except that the hybridization of the primers was carried out at 65 °C for 1 min.

Technique 2 used as external primers AF4 (5'GCGCACAAGTAGAGTGATC3') and AR1 (5'GCGTTCCTCGGTCCA3'), and as internal primers ASF1 (5'GCACGTGAAATTGTTGAAAGG3') and ADR1 (5'CAGGCTGGCCGCATTG3'), which amplify a 180 bp sequence and are based on primers used elsewhere [17]. The primer sequence comes from region D1-D2 of the genes of the large ribosomal subunit. The reaction mixes were the same as in the previous case, except that the amount of external and internal primers was reduced by half (1 pmol/µl). The reactions were carried out in the same thermocycler. The conditions for both amplifications were an initial cycle at 95 °C for 10 min, followed by 30 denaturation cycles at 94 °C for 30 s, hybridization of the primers at 58 °C for 1 min and extension at 72 °C for 1 min. Final extension was at 72 °C for 7 min.

The amplified products were detected using electrophoresis at 85 V for 1 h in 1.6% agarose gel in TAE buffer (0.04 M Tris Acetate, 0.01 M EDTA) containing 10 mg/ml of ethidium bromide.

With technique 1, detected up to 1 pg of fungal DNA. All strains of the genus *Aspergillus* from which DNA was extracted and on which this nested PCR was performed (*A. flavus, A. fumigatus*, *A. nidulans*, *A. niger, A. oryzae, A. restrictus, A. terreus* and *A. terreus var. terreus*) showed the presence of the amplified product, that is, a 357 bp band in the second amplification. Of those strains that did not belong to the genus *Aspergillus*, no bands were obtained from the following fungal species: *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. laurentii*, *F. graminearum*, *M. canis*, *R. oryzae*, *R. stolonifer* and *T. rubrum*, whereas amplified product was obtained from the following species: *A. chrysogenum*, *A. alternata*, *C. cladosporioides*, *C. elegans, G. lactis*, *M. racemosus*, *P. chrysogenum*, *P. griseofulvum*, *S. racemosus* and *T. mentagrophytes* (Figure 1).

Technique 2 detected up to 65 fg of fungal DNA. The strains belonging to the genus *Aspergillus* from which DNA was obtained and on which this nested PCR was performed presented the 180 bp band corresponding to the

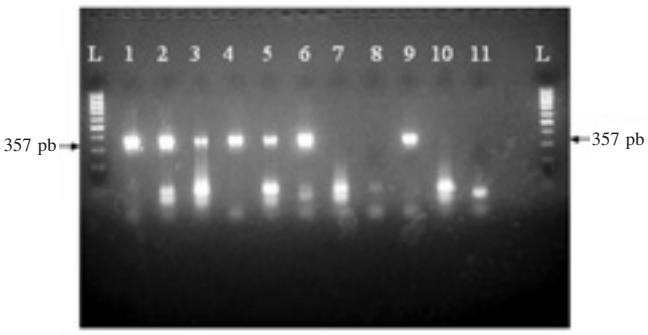

Figure 1. PCR by technique 1 with DNA from different fungal species. L: ladder; 1: positive control; 2: *A. fumigatus;* 3: *A. nidulans*; 4: *A. flavus;* 5: *A. terreus*; 6: *A. terreus* var. *terreus;* 7: *C. albicans;* 8: *C. tropicalis*; 9: *A. fumigatus*; 10: *R. oryzae*; 11: negative control.

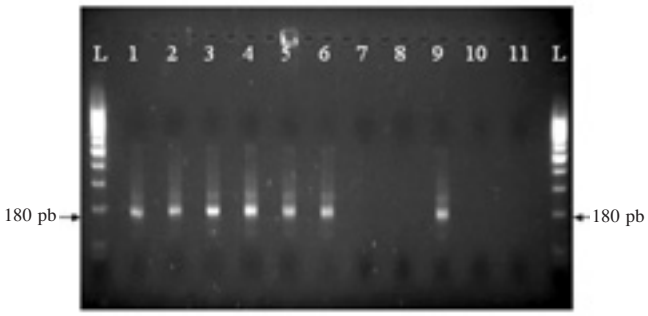

Figure 2. PCR by technique 2 with DNA from different fungal species. L: ladder; 1: positive control; 2: *A. fumigatus;* 3: *A. nidulans*; 4: *A. flavus;* 5: *A. terreus*; 6: *A. terreus* var. *terreus;* 7: *C. albicans;* 8: *C. tropicalis*; 9: *A. fumigatus*; 10: *R. oryzae*; 11: negative control.

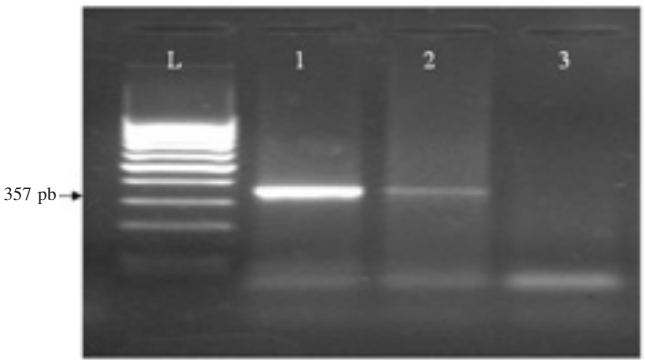

Figure 3. PCR of sera from experimentally inoculated mouse by technique 1. L: ladder; 1: positive control; 2: infected mouse serum; 3: non-infected mouse serum.

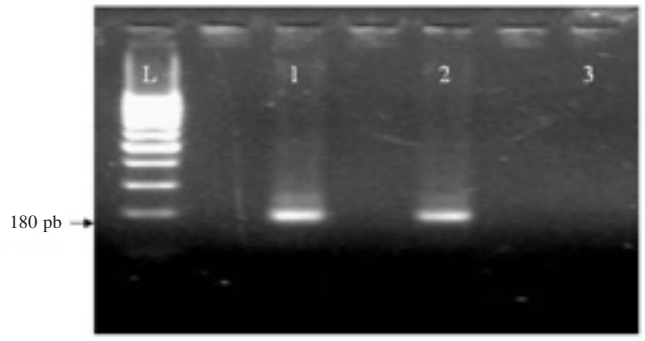

Figure 4. PCR of sera from experimentally inoculated mouse by technique 2. L: ladder; 1: positive control; 2: infected mouse serum; 3: non-infected mouse serum.

amplified product, except for *A. restrictus*, which did not. Of the strains not belonging to the genus *Aspergillus* no bands were obtained for the following fungal species: *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. laurentii*, *C. elegans*, *F. graminearum*, *R. oryzae*, *R. stolonifer, T. rubrum* and *T. mentagrophytes,* whereas amplified product was obtained from the following species: *A. chrysogenum, A. alternata*, *C. cladosporioides*, *G. lactis*, *M. canis*, *M. racemosus, P. chrysogenum*, *P. griseofulvum* and *S. racemosus* (Figure 2).

Both techniques were also applied to sera from mice immunosuppressed and infected with *A. fumigatus*, and also to sera from control mice. These sera were stored at –80 °C before use, and come from previous studies performed in our laboratory. With both techniques, the respective amplified band was observed in the sera from inoculated mice, whereas the sera from non-infected mice did not present these bands (Figures 3 and 4).

The sensitivity obtained with each of the techniques differs from that reported by other authors who used the same primers. Thus, Yamakami et al. [18] point out that they detected up to 50 fg using their nested PCR, whereas we only detected 1 pg of genomic DNA. The same occurred with technique 2, in which Williamson et al. [17] point out that they detected from 1 to 10 fg of *Aspergillus* DNA whereas we managed to detect up to 65 fg. We do not believe that these differences are significant, especially if we take into account the fact that detection limits similar to ours have been achieved by other authors [10,15] and considered adequate.

The specificity we obtained in both cases does not coincide with that obtained by the previously cited authors [17,18]. We obtained amplified product in almost all the species belonging to the genus *Aspergillus* (*A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. terreus* and *A. terreus var. terreus*), with the exception of *A. restrictus*, which did not show the amplified product by either of the techniques.

Yamakami et al. [18] used the sequence of the variable region V7-V9 of the 18 S subunit to design their primers and point out that they only obtained a positive result from those fungal species belonging to the genus *Aspergillus* (*A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger* and *A. terreus*). They did not obtain amplified product from species belonging to other fungal genera (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. neoformans*, *Trichosporon beigelii*, *Penicillium citreoviridae*, *P. expansum* and *P. crustosum*). Skladny et al. [13] carried out a nested PCR using the same variable region to synthesize their primers and only obtained amplified product from the genus *Aspergillus*. However, despite the fact that region V7-V9 is considered variable within the 18S subunit of ribosomal DNA, we found cross-reactions with other fungal species which do not belong to the genus *Aspergillus*, such as those mentioned above. Other authors such as Melchers et al. [11] also used primers synthesized from this sequence and obtained cross-reactions with species such as *Paecilomyces variotii*, *Penicillium marneffei* or *P. chrysogenum*.

Williamson et al. [17] used the sequence from the variable region D1-D2 of the large ribosomal subunit to design their primers, with positive results for the genus *Aspergillus* (*A. flavus*, *A. niger* and *A. terreus*) and negative results for the remaining fungal species tested (*Absidia corymbifera*, *Acremonium strictum*, *Bipolaris hawaiiensis*, *C. albicans*, *C. parapsilosis*, *C. glabrata*, *Cryptococcus neoformans*, *Exophiala jeanselmei*, *E. dermatidis*, *Fusarium solanii*, *F. oxysporum*, *Penicillium chrysogenum*, *Scedosporium apiospermum*, *Tricophyton interdigitale*, *T. rubrum* and *T. soudanense*) except for *P. variotii*, which presented a cross-reaction. As in the previous case, and in spite of the fact that this region is variable within the 26S subunit, we obtained cross-reactions with other fungal species not belonging to the genus *Aspergillus*.

This study and those published by other authors [4,18], consider serum to be the sample of choice to carry out nested PCR techniques. In the case of aspergillosis, it is essential that the sample can be obtained easily and non-invasively, thus allowing repeated sampling and optimal clinical follow-up. Although different authors have carried out their studies in whole blood samples [5,6,13,16], Bougnoux et al. [3] achieved better results with serum than with blood in the diagnosis of systemic candidiasis by nested PCR. We have already reported that we do not recommend using whole blood or plasma for PCR in the diagnosis of invasive aspergillosis in order to ensure the absence of any inhibitory effect by anticoagulants [7].

In the light of our results, detection of *Aspergillus* DNA using nested PCR is a valid alternative in the laboratory diagnosis of this disease. The favorable results obtained in experimentally inoculated mice encourage its use in humans. Nevertheless, we must also bear in mind the possibility of cross-reactions between fungal species other than *Aspergillus*.

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