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Anticoagulants Interfere with PCR Used To Diagnose Invasive Aspergillosis

Invasive aspergillosis is a disease that affects immunodepressed individuals and has become one of the leading causes of death in many transplant centers (1). Owing to the difficulties encountered in diagnosis of the disease and to the high sensitivity of the PCR technique, PCR-based methods have been developed since 1993 to detect the presence of *Aspergillus* DNA in patients.

In our laboratory, we began to use the PCR technique to detect the DNA of *Aspergillus fumigatus* in the blood of experimentally infected rats. In our first studies, the negative results obtained for almost all the plasma samples from the infected rats led us to suspect that the anticoagulant used could be interfering in the PCR. Therefore, we decided to ascertain the effect of the most widely used anticoagulants on this method for diagnosing invasive aspergillosis.

DNA was obtained using the strain *A. fumigatus* 48238, isolated from a human case of invasive aspergillosis and obtained from a culture collection from Glaxo Smith Kline (Greenford, United Kingdom). The DNA of the samples was extracted according to the method used by Tokimatsu et al. (16).

In order to prepare the samples used in the reaction, blood was obtained from two male Spragüe-Dawley rats by cardiac puncture. This blood was quickly separated into four aliquots, of which the first three contained sodium citrate (Sarstedt, Granollers, Spain), tripotassium-EDTA (Sarstedt), and heparin (Rovi, Barcelona, Spain), respectively, in order to obtain plasma. The last aliquot remained free of anticoagulant so that serum could be obtained. The tubes were centrifuged (3,000 × *g* for 10 min), aliquots (100 μl) of each type of plasma or serum were taken, and 1 μl of a solution of genomic DNA (15 mg/ml) was added to each one, in such a way that each plasma and serum sample contained a 0.15-mg/ml concentration of DNA.

The oligonucleotides used were obtained from genes of sequence 18S of the rRNA belonging to *Aspergillus* (20). PCR was performed using a nested PCR technique in which the internal primers were Asp.5 (5′ GATAACGAACGAGAC CTCGG 3′) and Asp.8 (5′ TGCCAACTCCCCTGAGCCAG 3′), which amplify a sequence of 384 bp, and the external primers were Asp.1 (5′ CGGCCCTTAAATAGCCCGGTC 3′) and Asp.7 (5′ CCTGAGCCAGTCCGAAGGCC 3′), which amplify a sequence of 357 bp. The reaction mixture and the conditions of both cycles were the same as those described previously (20).

In all cases, the initial concentration of DNA in the PCR mixture was 30 μg/ml (1.5 μg in 50 μl of the reaction mixture). The first PCR was performed starting with 10-μl samples of treated plasma, to reach a final volume of 50 μl. The second reaction was performed with 1 μl of the product obtained in the first reaction. The products of the nested PCR were subjected to electrophoresis in a 2% agarose gel that contained ethidium bromide. The concentrations of the different anticoagulants in blood, plasma, and the PCR mixtures are detailed in Table 1.

The results obtained are shown in Figure 1. The validity of PCR can be appreciated in serum and plasma with EDTA, whereas interference leading to negative results was observed in plasma with citrate and plasma with heparin.

Several studies report on the inhibitory effect of different anticoagulants in different PCRs for viruses such as hepatitis C virus (5, 9, 12, 18), human immunodeficiency virus (3, 6, 7, 8, 13, 19), and hepatitis B virus (14); for protozoa such as *Plasmodium falciparum* (11); and for bacteria such as *Streptococcus pneumoniae* (4). However, only limited data are available for the detection of fungal DNA in plasma samples. Loeffler et al. (10) concluded that although plasma and whole blood spiked with *Aspergillus* conidia showed an identical lower detection limit, the sensitivity of PCR performed with plasma samples was lower than that of PCR performed with whole-blood samples.

On the other hand, there are studies that point out the absence of any inhibitory effect with some anticoagulants, such as the equivalence of results obtained in the amplification of human cytomegalovirus DNA from plasma samples with heparin and EDTA, respectively (15), or that of results obtained with plasma samples and serum (2). Similarly, some authors did not find differences between the results obtained in a PCR using heparinized plasma or serum to detect human immunodeficiency virus RNA (17). In the case of DNA amplification of *S. pneumoniae* from blood, Friedland et al. (4) observed inhibition of PCR in the presence of EDTA and citrate but not in the presence of sodium heparin.

In order to determine the reason for the inhibition caused by heparin in PCR assays, Yokota et al. (21) reached the conclusion that PCR is clearly affected when heparinized plasma is used as the DNA source, and that the degree of interference depends on three factors, as follows: (i) the type of *Taq* DNA polymerase used in the reaction, (ii) the number of leukocytes present in the blood (that is, the quantity of DNA at the beginning of the reaction), and (iii) the concentration of heparin in the samples.

In our PCR, we used *Taq* polymerase (Perkin Elmer, Bedford, Mass.), for which Yokota et al. (21) pointed out a maximum tolerable quantity of heparin of 0.1 U, starting from 100 ng of DNA in the reaction mixture. We had a concentration of 1,500 ng of DNA and 0.5 IU of heparin in the reaction mix. Thus, we can deduce that the quantity of heparin was sufficient to interfere with the reaction.

In conclusion, given the results obtained, we recommend not using plasma to carry out PCR in the diagnosis of invasive aspergillosis in order to ensure the absence of any inhibitory effect.

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TABLE 1. Concentrations of the different anticoagulants in blood and plasma samples, as well as the PCR mixture

Sample type or mixture	Anticoagulant concn[a] in:		
	Sodium citrate	Tripotassium EDTA	Heparin
Blood[b]	0.106	2	5 (500)
Plasma	0.2	4	10 (1,000)
PCR mixture	0.02	0.4	1 (100)

[a] The sodium citrate concentrations are molar concentrations, the tripotassium EDTA concentrations are in milligrams per milliliter, and the heparin concentrations are in milligrams per milliliter (values in parentheses are in international units per milliliter).

[b] In the blood centrifuge, hematocrit values close to 47% were obtained.

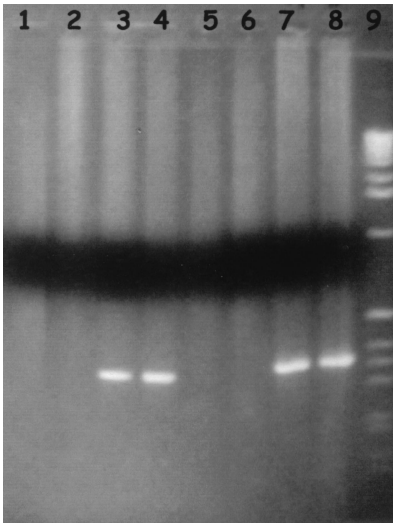

FIG. 1. Electrophoresis with a 2% agarose gel. Lanes: 1, plasma from rat A with citrate; 2, plasma from rat B with citrate; 3, plasma from rat A with EDTA; 4, plasma from rat B with EDTA; 5, plasma from rat A with heparin; 6, plasma from rat B with heparin; 7, serum from rat A; 8, serum from rat B; 9, size marker.

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Marta E. García
Jose L. Blanco*
Departamento Patología Animal I (Sanidad Animal)
Facultad de Veterinaria
Universidad Complutense
28040 Madrid, Spain

Jesús Caballero
Domingo Gargallo-Viola
Glaxo Smith Kline
28760 Tres Cantos, Madrid, Spain

*Phone: 34 91 394 3717
Fax: 34 91 3943908
E-mail: jlblanco@vet.ucm.es