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Evaluating the specificity of primers employed for PCR-based diagnostics of Leishmaniases using multiple alignment analysis

Avaliando a especificidade de oligonucleotídeos iniciadores empregados no diagnóstico molecular de Leishmanioses por análises de alinhamentos múltiplos

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Abstract

The purpose of this study was to assess the specificity of distinct primers used in the molecular diagnosis for *Leishmania* detection by using biological sequence search and alignment tools. Four primer pairs routinely employed in the PCR-based diagnostics for Leishmaniasis detection were evaluated through the software Primer-BLAST, which compares nucleotide sequences against user-selected database to avoid primer pairs cause non-specific amplifications. The LU5A-LB3C primer pair showed good specificity among all primers analyzed, generating alignments exclusively against distinct sequences of *Leishmania* species and no matches were found with other parasite species. Whereas the primer pairs designated MP3H--MP1L, B1-B2 and 13A-13B demonstrated matches against distinct sequences of *Leishmania* strains and other species, such as Rhesus monkey (*Macaca mulatta*) and starlet sea anemone (*Nematostella vectensis*). These findings emphasize the importance of selecting suitable primers for diagnosis of molecular diseases by conducting previous screenings in order to infer their specificity and identity against target templates within biological sequence annotation database.

Keywords: Computational biology. Molecular diagnosis. Oligonucleotide identity.

Resumo

O objetivo deste estudo foi avaliar o grau de especificidade de oligonucleotídeos iniciadores empregados rotineiramente no diagnóstico molecular de Leishmania *a partir de ferramentas de bioinformática. Quatro pares de oligonucleotídeos iniciadores foram analisados pelo programa Primer-BLAST, o qual permite comparar a especificidade dos iniciadores a partir de um banco de dados de sequências de DNA e, assim, evitar a possível presença de fragmentos não específicos nas reações de amplificação. O par de iniciadores LU5A-LB3C demonstrou boa especificidade em comparação aos demais iniciadores analisados, gerando alinhamentos que reconheceram exclusivamente fragmentos genômicos de Leishmania. Por outro lado, os pares de iniciadores MP3H-MP1L, B1-B2 e 13A-13B apresentaram alinhamentos compatíveis a fragmentos genômicos de distintas linhagens de* Leishmania*, além de reconhecer fragmentos genômicos não específicos, tais como de macaco Rhesus (*Macaca mulatta*) e uma espécie de anêmona (*Nematostella vectensis*). Esses resultados enfatizam a importância de efetuar análises prévias da especificidade de oligonucleotídeos iniciadores a fim de promover melhor desempenho e sensibilidade acurada nos métodos de diagnóstico molecular de doenças.*

***Palavras-chave**: Biologia computacional. Diagnóstico molecular. Identidade de oligonucleotídeos.*

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Introduction

Leishmaniases are infectious-parasitic diseases caused by protozoa of the genus *Leishmania*, which are considered of increasing importance to public health in Brazil due to their high incidence and wide geographic distribution (Dantas-Torres & Brandão-Filho, 2006). The detection of seropositivity from multiple samples of distinct species of *Leishmania* has been extensively carried out using enzyme-linked immunosorbent assays (ELISA) and immunofluorescence antibody tests. However, it is well known that these techniques present limitations, such as low sensitivity of antibody detection in early or asymptomatic infections (Leontides, Michelakis, Billinis, Kontos, Koutinas et al., 2002) and the presence of false positive due to cross-reactions with other diseases (Barbosa de Deus, Mares-Guia, Nunes, Costa, Junqueira et al., 2002).

The advent of the molecular diagnosis for detection of numerous diseases by using polymerase chain reactions (PCRs) has provided new strategies to minimize eventual technical limitations. Within this context, PCRs have been extensively employed as a quick, specific and sensitive technical approach for detection of *Leishmania* (Reithinger, Lambson, Barker, Davis, 2000; Bensoussan, Nasereddin, Jonas, Schnur, 2006; JaffeMaia & Campino, 2008; Queiroz, Assis, Oliveira, Machado, Nunes et al., 2010; Leite, Mendes, Ferreira, de Andrade, 2011). According to Ye et al. (2012), specific primers are designed for detection of organismal DNA and the homology between such primers and target DNA confers high specificity to PCR-based diagnosis, since the identification of the amplification products under given reaction conditions reveals the presence of an infectious organism. Selecting appropriate primers is probably the most important factor for the PCR and specific amplification of the target fragment requires that there are no other primer binding sites in DNA, which could cause amplification of non-specific products (Ye, Coulouris, Zaretskaya, Cutcutache, Rozen et al., 2012).

In the last decades, bioinformatics has contributed to the development of computational biology tools and their application for several research fields in biology, furthering the understanding of living systems at all scales through the application of computational methods (Schatz, Delcher, Salzberg, 2010; Gabriel, 2012; Gabriel, de Figueiredo, de Farias, 2013). One of these applications is to improve the performance of molecular diagnosis for several pathogenic diseases by selecting highly specific PCR primers. For that reason, it is relevant emphasize the importance of selecting suitable primers for PCR-based diagnostics, conducting previous analysis by using bioinformatics tools in order to infer their specificity against target DNA sequences. The construction of specific primers involves typically the initial identification of the fragment of interest to be flanked generated either manually or using software tools, followed by searching against an appropriate nucleotide sequence database to examine their binding with non-specific target sequences. Nevertheless, the latter is not an easy process as one needs to examine many details between primers and targets, such as the number and the positions of matched bases, the primer orientations and distance between forward and reverse primers (Qu, Shen, Zhao, Yang, Zhang, 2009; Ye, Coulouris, Zaretskaya, Cutcutache, Rozen et al., 2012). Thus, this study aimed to assess the specificity of primer pairs routinely employed in PCR-based diagnostics for detection of Leishmaniasis using search and alignment tools from bioinformatics software.

Materials and methods

Four pairs of primers were selected randomly for comparative analyses of multiple alignments: MP3H (5'-GAACGGGGTTTCTGTATGC-3') and MP1L (5'-TACTCCCCGACATGCCTCTG-3') (Lopez, Inga, Cangalaya, Echevarria, Llanos-Cuentas et al., 1993); B1 (5'-GGGGTTGGTGTAATATAGTGG-3') and B2 (5'-CTAATTG TGCACGGGGAGG-3') (de Bruijn & Barker, 1992); LU5A (5'-TTTATTGGTATGCGAAACTTC-3') and LB3C (5'-CGT(C/G)CCGAACCCCGTGTC-3') (Harris, Kropp, Belli, Rodriguez, Agabian, 1998); 13A (5'-GTGGGG GAGGGGCGTTCT-3') and 13B (5'-ATTTTACACCAAC CCCCAGTT-3') (Rodgers, Popper, Wirth, 1990). These primers were chosen since they have been routinely employed successfully in the molecular diagnosis of Leishmaniasis (Oliveira, Lonardoni, Teodoro, Silveira, 2011).

Three pairs of primers (MP3H-MP1L, B1-B2 and 13A-13B) are derived from kDNA sequences of *Leishmania*, whereas the primers LU5A-LB3C were designed from the SL RNA (mini-exon) gene repeat (Oliveira, Lonardoni, Teodoro, Silveira, 2011). Originally, these primers were designed to amplify specific genomic regions of *Leishmania* spp. The expected length for amplicons obtained with pairs of primers

MP3H-MP1L, 13A-13B, B1-B2 and LU5A-LB3C are 70bp, 120bp, 750bp and 146-149bp, respectively.

The comparative analysis of the specificity of these primers was carried out by using Primer-BLAST (primer-basic local alignment search tool), an open access computational software that compares fragments of nucleotide or protein sequences against sequences database, providing statistical scores for matches which can be used to infer functional and evolutionary relationships between sequences. Primer-BLAST was specially developed to screen primers against user-selected database in order to avoid primers that could cause non-specific amplifications (Ye, Coulouris, Zaretskaya, Cutcutache, Rozen et al., 2012).

Initially, the sequences of the primer pairs mentioned above were inserted in the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), as enter query sequences, followed by selecting specificity and checking parameters from two distinct databases. First, the primer pairs were compared against sequences from several chromosomal loci identified within genome of a large spectrum of organisms which have been continuously deposited in the National Center of Biotechnology Information (NCBI) database, except the sequences whose accessions start with "AC" (alternate assemblies), which are automatically excluded of these parameters to reduce redundancy. Second, the primer pairs were compared against only all sequences of distinct *Leishmania* species also available in the biological sequences database NCBI. It is necessary to emphasize that the evaluation parameters were automatically adjusted by the Primer-BLAST during searching and alignment of the input sequences.

Results

Comparative alignments by using computational biology tool were performed to assess comparatively against a biological information database the specificity of sequences of primer pairs routinely employed in the molecular diagnosis of *Leishmania* (Table 1). The primer pair LU5A-LB3C showed good specificity in comparison to other primers analyzed, generating alignments exclusively against sequences of *Leishmania*, such as *Leishmania braziliensis* and *L. major*, but no matches were found for other parasites. Interestingly, the primer pairs MP3H-MP1L and B1-B2 demonstrated alignments against multiple strains of *Leishmania* (such as *L. braziliensis*, *L. major* and *L. donovani*) and other animal species, such as rhesus monkey *Macaca mulatta* in the chromosomes 2 and 10, respectively (Table 1). Similar results were observed for primer pair 13A-13B, which demonstrated additional matches against sequences of starlet sea anemone *Nematostella vectensis* and different strains of *Leishmania* (*L. braziliensis, L. major, L. infantum* and *L. donovani*) (Table 1).

Particularly, target templates with variable lengths were detected for some primer pairs aligned against multiple genomic sequences into a unique *Leishmania* species. For example, primer pair 13A-13B may anneal to 10 distinct regions within *Leishmania infantum* genome, amplifying products ranging from 420 to 4,300bp (see footnote in the Table 1), including non-specific amplicons. In contrast, the primer pair LU5A-LB3C produced amplicons of interest (146--149bp) and few non-specific products for *Leishmania* (Table 1), which could represent a better specificity of this sequence of primer pair for molecular Leishmaniasis detection.

In these comparative analyses, expectation-value (E-value) corresponding to 30,000 was observed for matches of all primer pairs and target sequence templates. E-value is an important statistical parameter calculated according to the quality of alignment (the score) and the size of the database (Lesk, 2002). Under this context, E-value close to zero indicates that the match is highly significant and represents the probability of the alignment has occurred by chance (Lesk, 2002). Although E-value found in this study is far from zero, such data is expected in basic local alignments generated from short query sequences, considering that the primer pairs tested contain between 19 and 25 nucleotides each primer (Table 1).

Discussion

The findings reported in this study emphasize differentiated degrees of specificity of distinct primer pair sequences employed for molecular Leishmaniasis detection by using comparative search and alignment analyses. The performance of these primer pairs has been tested under experimental approaches, demonstrating their variable sensitivity in PCR-based diagnostics for *Leishmania* (Oliveira, Lonardoni, Teodoro, Silveira, 2011). According to Oliveira et al. (2011), the primers MP3H-MP1L and B1-B2 showed good sensitivity with amplified DNA fragments for strains of the

L. braziliensis complex. Although low sensitivity of the primer pair LU5A-LB3C was observed in amplification reactions by Oliveira et al. (2011), the findings described herein demonstrated that this primer pair showed high specificity in comparison to other primer pairs, revealing alignments exclusively against sequences of *Leishmania* (Table 1). In addition, Harris et al. (1998) and Gomes et al. (2007) also confirm that primers derived from the mini-exon gene, such as oligonucleotides LU5A and LB3C, represent successful tools to detect and characterize DNA in PCR-based diagnosis of distinct species of *Leishmania*.

Table 1 - Target templates aligned with primer pairs routinely used in molecular diagnostics of *Leishmania*

Sequences of primer pairs (5'-3')	Target sequence templates	Blast hits Analyzed
MP3H - GGGGTTGGTGTAATATAGTGG(f)	*Leishmania braziliensis*[1]	9406
MP1L - CTAATTGTGCACGGGGAGG(r)	*Leishmania major* [2]	
	Leishmania donovani [3]	
	NC_007859.1 *Macaca mulatta* chromosome 2 (product length 1041bp)	4098
B1 - GAACGGGGTTTCTGTATGC(f)	*Leishmania braziliensis* [4]	10365
B2 - TACTCCCCGACATGCCTCTG(r)	*Leishmania major* [5]	
	NC_007867.1 *Macaca mulatta* chromosome 10 (product length 507bp)	2650
LU5A - TTTATTGGTATGCGAAACTTC(f)	*Leishmania braziliensis* [6]	4464
LB3C - CGT(C/G)CCGAACCCCGTGTC(r)	*Leishmania major* [7]	
13A - GTGGGGGAGGGGCGTTCT(f)	*Leishmania donovani* [8]	44312
13B - ATTTTACACCAACCCCCAGTT(r)	*Leishmania infantum* [9]	
	Leishmania major [10]	
	Leishmania braziliensis [11]	
	NW_001834350.1 *Nematostella vectensis* (product length 2517bp)	11537

Legend: [1]NC_009323.2 (3751bp); NC_009320.2 (2786bp); NC_009295.2 (255bp); [2]NC_007264.2 (2485bp); NC_007287.2 (231bp); NC_007266.2 (720bp); [3]NC_018254.1 (3139bp); [4]NC_009323.2 (70bp); NC_009327.2 (70bp); NC_017948.1 (3072bp); NC_009324.2 (4427bp); [5]NC_007264.2 (3578bp); NC_007263.2 (3811bp); NC_007287.2 (1850bp, 104bp); NC_007284.2 (2265bp); NC_007271.2 (89bp). [6] NC_009308.2 (149bp); NC_009300.2 (150bp); NC_009295.2 (151bp); [7]NC_007273.2 (856bp); NC_007265.2 (2583bp); [8]NC_018254.1 (116bp); NC_018256.1 (3639bp); NC_018259.1 (3233bp and 3525bp); NC_018244.1 (4214bp); NC_018242.1 (3167bp); NC_018257.1 (4276bp); NC_018263.1 (2869bp); NC_018252.1 (1025bp); NC_018253.1 (571bp); [9]NC_009418.2 (1283bp and 1106bp); NC_009413.2 (3642bp and 915bp); NC_009416.2 (3233bp); NC_009401.2 (4206bp); NC_009399.2 (3161bp); NC_009414.2 (4278bp); NC_009420.2 (2874bp); NC_009409.2 (1025bp); NC_009419.2 (3320bp); NC_009410.2 (570bp); [10]NC_004916.2 (89bp); NC_007271.2 (3895bp); NC_007284.2 (4589bp); NC_007245.2 (4936bp); NC_007269.2 (3164bp); NC_007285.2 (4608bp); NC_007266.2 (1167bp); NC_007255.2 (4411bp) and [11]NC_017947.1 (3435bp and 3953bp); NC_009307.2 (3914bp).

Source: Research data.

Notes: * (f) forward primer; (r) reverse primer.

** Target sequences and their access numbers from GenBank database and product length generated by the flanking primers.

Sequences of target templates with variable lengths were detected for some primer pairs aligned against multiple genomic sequences into a unique *Leishmania* species, as observed in primer pair 13A-13B (Table 1), including non-specific amplicons. On the other hand, the primer pair LU5A-LB3C seems to be able to produce amplicons of interest (146-149bp) and few non-specific products for *Leishmania* (Table 1), which means this primer pair could demonstrate better specificity for PCR-based diagnostics of *Leishmania braziliensis*. Several authors report that conventional tests for detection of *Leishmania* present high false positive rate (Silva, Romero, Nogueira-Nascentes, Costa, Rodrigues et al., 2011, Srividya, Kulshrestha, Singh, Salotra, 2012). However, no matches were found between target

sequence templates and primer pairs for other parasites of the same family of *Leishmania*, such as *Trypanosoma cruzi*. Thus, it could be speculated that the use of such primer pairs may reduce, in part, the possibility of obtaining false positive results in PCR-based diagnosis in practical approaches. Additional experimental assays are required in order to confirm the data generated in the multiple alignment analyses concerning specificity of primer pairs employed routinely for PCR-based diagnostics of Leishmaniases.

Polymerase chain reactions have been characterized as the most important tool for basic and applied research in molecular biology and diagnostic methods for diseases (Yang & Rothman, 2004; Cancrini & Iori, 2004). The investigative approach conducted in this article from multiple comparative alignments against biological sequence database has been extensively employed in studies involving the design and the construction of specific primers for diagnostic of numerous diseases by PCR analyses (Qu, Shen, Zhao, Yang, Zhang, 2009). Although there are no reports in the literature describing only conclusive results of the specificity analyses of primer pairs through computational biology tools, these methodological strategies have been currently employed as a crucial prerequisite in the steps which precede the selection of primer sequences to be employed in molecular diagnosis of diseases based on the amplification reactions (Robertson & Phillips, 2008; Qu, Shen, Zhao, Yang, Zhang, 2009). In order to increase the robustness of molecular tests and to predict the potential PCR products by using similarity analyses of primers against target sequences, the PCR reliability, in terms of specificity on the detection and quantification of pathogen, may be sensibly improved by using computational biology tools.

Conclusion

Variable degrees of specificity against biological information databases were noticed in distinct primer pair sequences employed for molecular Leishmaniasis detection by using comparative search and alignment analyses. The findings reported in the present study reinforce relevant applications of the bioinformatics' tools by analyzing and selecting oligonucleotides constructed specially to improve the accuracy of the PCR-based diagnostics in a large spectrum of infectious diseases.

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