DDRT-PCR approaches applied for preeminent results in the isolation of DETs from fish brain tissues

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Received: July 16, 2013 – Accepted: September 20, 2013 – Distributed: March 31, 2015
(With 2 figures)

Abstract

Differential Display (DD) is a technique widely used in studies of differential expression. Most of these analyses, especially those involving fish species, are restricted to species from North America and Europe or to commercial species, as salmonids. Studies related to South American fish species are underexplored. Thus, the present work aimed to describe DD technique modifications in order to improve outcomes related to the isolation of DETs (Differentially Expressed Transcripts), using *Leporinus macrocephalus*, a large commercially exploited South American species, as a fish design. Different DDRT-PCR approaches were applied to brain samples and the products of the reactions were analyzed on 6% polyacrylamide gels stained with 0.17% Silver Nitrate ($AgNO_3$). The use of PCR reactions under high stringency conditions and longer oligonucleotides based on VNTR (Variable Number of Tandem Repeats) core sequences led to better results when compared to low stringency PCR conditions and the use of decamer oligonucleotides. The improved approach led to the isolation of differentially expressed transcripts on adult males and females of *L. macrocephalus*. This study indicates that some modifications on the DDRT-PCR method can ensure isolation of DETs from different fish tissues and the development of robust data related to this approach.

Keywords: Leporinus macrocephalus, differentially expressed transcripts, VNTR.

Aplicação de abordagens de DDRT-PCR para aprimoramento de resultados no isolamento de DETs a partir de tecido cerebral de peixes

Resumo

Display Diferencial (DD) é uma técnica amplamente utilizada em estudos de expressão diferencial. A maioria desses estudos envolvendo espécies de peixes está restrita a espécies da América do Norte e Europa ou a espécies comerciais, como os salmoniformes. Estudos relacionados a peixes da América do Sul são ainda pouco explorados. Desse modo, o presente trabalho teve como objetivo descrever modificações na técnica de DD, a fim de melhorar os resultados relacionados ao isolamento de DETs (Transcritos Diferencialmente Expressos), utilizando *Leporinus macrocephalus*, peixe explorado comercialmente na América do Sul, como espécie para tal delineamento. Diferentes abordagens de DDRT-PCR foram desenvolvidas a partir de amostras de tecido cerebral e os produtos das reações foram analisados em gel de poliacrilamida 6% corados com 0,17% de nitrato de Prata ($AgNO_3$). A utilização de reações de PCR sob condições de elevada estringência e oligonucleotídeos mais longos, com base em sequências cerne de VNTR (Número Variável de Repetições em Tandem), mostrou melhores resultados quando comparada a condições de baixa estringência e ao uso de oligonucleotídeos decâmeros. A estratégia empregada permitiu o isolamento de transcritos diferencialmente expressos em machos e fêmeas adultos de *L. macrocephalus*. Este estudo evidencia que modificações no método de DDRT-PCR garantem o melhor isolamento de DETs a partir de diferentes tecidos de peixes e asseguram a obtenção de dados mais sólidos relacionados a essa abordagem.

Palavras-chave: Leporinus macrocephalus, transcritos diferencialmente expressos*, VNTR*.

1. Introduction

In the last years, diverse genetic studies have focused on the analysis of differentially expressed genes (DETs - *Differentially Expressed Transcripts*) or, in other words, genes expressed as messenger RNAs (mRNA) which differ in abundance among cell types or specific tissues, and which may be regulated by chemical, physiological,

and environmental mechanisms (Liang and Pardee, 1995). These studies allow gene characterization and evaluation of transcript expression levels in relation to extrinsic factors. Therefore, it is possible to compare samples isolated at different developing stages, collected from different environments, or from different tissues or genders (Cervigni et al., 2008; Elis et al., 2008; Kavar et al., 2008; Alves-Costa and Wasko, 2010; Woo and Yum, 2011; Kumar et al., 2013; Dhorne-Pollet et al., 2013).

A large number of methods have been used to isolate and characterize DETs, and DD (*Differential Display*) (Liang and Pardee, 1992) represents one of the most popular techniques concerning this approach. The DD methodology has been widely used in differential expression studies since it shows high efficiency and is characterized for its simplicity, reduced cost and for not requiring any previous knowledge about the target genome (Liang and Pardee, 1992). The large applicability of this technique may be proven by more than 16,500 published articles, describing the use of this method (Medline 2013, January - PubMed, NCBI Web site http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). Moreover, the differential display can simultaneously visualize increased or decreased expression of numerous mRNAs from many samples, and requires relatively small amounts of starting material (Pardee and McClelland, 1999).

Although DD also has some disadvantages, as the possibility of isolation "false-positive" transcripts (PCR products that appear to be differentially expressed on the gel but that cannot be verified when subsequent expression analyses are performed) (McClelland et al., 1995; Liang, 1998; Pardee and McClelland, 1999), technical improvements can increase its successful application. For that reason, modifications like the use of tailored primers to amplify members of a particular gene family (Jurecic et al., 1996), optimization of annealing temperature (Malhotra et al., 1998), and primer design, including the use of single-primer correction and an annealing control primer (ACP) system (Graf et al., 1997; Kim et al., 2008, Kim et al., 2010; Ma et al., 2011), have been suggested.

Despite polymerase chain reaction (PCR)-based mRNA differential display has been widely used for identifying differentially expressed transcripts in a variety of species (e.g. Liang, 2002), some animals, as fishes, generally show a few data related to this approach. The majority of differential gene expression studies in fish species, through the use of DD, are restricted to North American and European species, or to commercial fishes, as Salmoniformes, which claim to generate important results to fish farming (Parrington and Coward, 2002). In this way, studies related to South American species are poorly exploited. Besides, works describing conditions to improve the results of fish DD are still required in this animal group. Dakis and Kouretas (2002), for example, have described some modifications in the DD technique applied to fish samples, which were characterized using a combination of longer arbitrary primers (25 and 26-mer) together with 30-mer anchored primer for PCR reactions, and the use of a nondenaturing polyacrylamide gel to analyze PCR products.

Thereby, the present work describes some different modifications of the DD technique in order to improve the results of this method in relation to DETs isolation in fish species. In an attempt to achieve this goal, we applied a DDRT-PCR approach for mRNA screening in brain tissues of *Leporinus macrocephalus* (piauçu), a large commercially exploited South American fish species. The improved approach permitted the isolation of differentially expressed transcripts in adult males and females of this species.

2. Material and Methods

2.1. Animal samples and RNA isolation

Adult specimens of *Leporinus macrocephalus* (Characiformes, Anostomidae) were obtained from a private fishery station in São Paulo State, Brazil (Kabeya Fishery Station, municipality of Glicério), where they were maintained at 25 °C, and with constant aeration, until collection of brain tissues. As there is no genomic data for *L. macrocephalus*, the particular choice of this fish species was mainly due to the principle that the DD methodology does not require a previous knowledge on the mRNA sequences of the target biological samples. Brain tissue samples (including medulla, cerebellum, optic lobes, pineal gland, pituitary gland, cerebral hemispheres, and olfactory lobes) of 5 males and 5 females were collected and immediately stored at –80 °C until RNA extraction.

Approximately 100 mg of the brain samples were mechanically homogenized with 1 mL of TRizol Reagent (Invitrogen™) and total RNA extraction followed the manufacturer's protocol. RNA samples were eluted in RNase-free water and quantified (NanoDrop 1000 Spectrophotometer) by measuring the optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio $\geq$1.80, and its integrity was ensured by agarose gels electrophoresis.

2.2. DDRT-PCR approaches

Total RNA samples obtained from each male and female were incubated with DNase I to remove DNA contamination, which represents a further source of false-positive bands that can be observed on differential display gels. After DNase treatment, small aliquots of each sample were loaded onto an agarose gel to check for RNA integrity. Subsequently, 2 µg high-quality RNA samples were used to prepare RNA pools of males and females, separately, in order to avoid the detection of interindividual variations not related to differences between sexes. The RNA pools were reverse transcribed (RT-PCR), separately, with the commercial kit SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen™) using an oligonucleotide $(dT)_{12\text{-}18}$ (Invitrogen™) as an anchor primer, according to the manufacturer's instructions.

The cDNA samples were amplified using as single primers some oligonucleotides with 14-24 bp

that were designed based on VNTR core sequences (primers INS: 5'-ACAGGGGTGTGGGG-3', HBV3: 5'-GGTGAAGCACAGGTG-3', HBV5: 5'-GGTGTAGAGAGGGGT-3', EMBL: 5'-AGAGCTTCAGGCTGGGCAGCTAAG-3', YNZ22: 5'-CTCTGGGTGTCGTGC-3', and FvIIex8: 5'-ATGCACACACACAGG-3'), and also RAPD (Randomly Amplified Polymorphic DNA) decamer primers (primers 1, 2, 3, 4, and 5 of the OPP series - Operon Technologies Inc.) (Alves-Costa and Wasko, 2010; Alves-Costa et al., 2012). Each cDNA amplification reaction consisted of 2 µL of cDNA (10%), 0.2 mM of primer, 1 x 25 mM $MgCl_2$ PCR buffer, 0.2 mM of dNTPs, and 0.2 U of Platinum *Taq* DNA polymerase (Invitrogen™), in a final volume of 50 µL. Reactions using the 14-24 bp primers were carried out with an initial denaturation step at 94 °C for 2 minutes, followed by 40 cycles at 94 °C for 50 seconds, 55 °C for 2 minutes, and 72 °C for 50 seconds, with an additional extension step at 72 °C for 5 minutes. Reactions using the 10-mer primers were carried out with an initial denaturation step at 95 °C for 5 minutes, followed by 45 cycles at 94 °C for 1 minute, 36 °C for 1 minute, and 72 °C for 2 minutes, following the manufacturer's instructions of the Kit Ready-To-Go RAPD Analysis Beads (GE Healthcare Biosciences).

DDRT-PCR products (10 µL) were fractionated on 6% polyacrylamide gels stained with 0.17% Silver Nitrate ($AgNO_3$) (Sambrook and Russel, 2001), and visualized under white light. The molecular weigh of the amplified fragments was assigned through comparison with a 1 Kb DNA ladder (Invitrogen™).

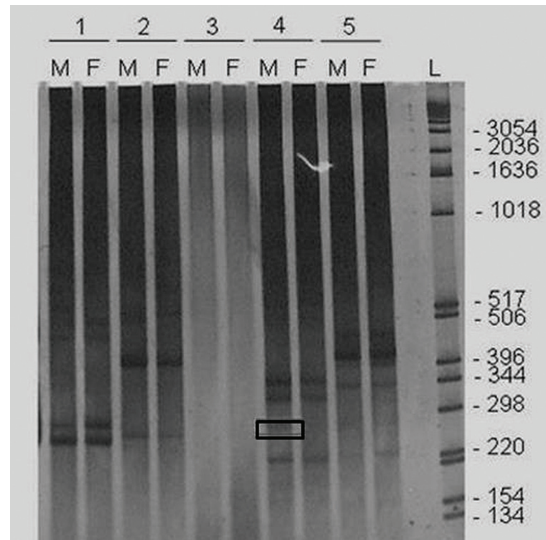

Figure 1. DDRT-PCR fingerprints on a 6% polyacrylamide gel stained with $AgNO_3$. The molecular weight of the amplified fragments (using decamer primers) was assigned through comparison with a 1 Kb DNA Ladder (Invitrogen™), indicated on the right side (**L**). **(1)** primer 1, **(2)** primer 2, **(3)** primer 3, **(4)** primer 4, and **(5)** primer 5 of the OPP series (Operon Technologies Inc.). **M=** pool of RNA male samples; **F=** pool of RNA female samples. A single amplified fragment that may correspond to a differentially expressed transcript is highlighted (box).

3. Results and Discussion

Preliminary analyses were performed to determine primers that provide superior and reproducible amplification results. As so, cDNA amplification was achieved using decamer oligonucleotides commonly used in RAPD analyses and also oligonucleotides that were designed based in VNTR core sequences that correspond to minisatellite short and highly conserved regions (Jeffreys et al., 1985). The use of the 10-mer primers, that commonly generate multiple bands for different organisms (Lee et al., 2001; Yang et al., 2004; Zhang et al., 2008), did not produce this type of amplification pattern for brain tissues of *L. macrocephalus*. Moreover, the amplification reactions usually resulted on several faint and diffuse bands, and the results were generally not reproducible (Figure 1). These faint bands often lead to a misinterpretation of the data. In addition, just a reduced number of candidate differentially expressed transcripts could be identified when comparing males and females of *L. macrocephalus* through the use of RAPD primers (Figure 1).

The use of longer primers on differential display strategies may also lead to detection of DNA polymorphism through RAPD-like results. Some studies evidenced that primers with 13 bases or longer have better effects in the DD efficiency (Zhao et al., 1995; Liang, 1998; Motlik et al., 1998; Huang et al., 2001; Alves-Costa and Wasko, 2010; Alves-Costa et al., 2012). As the oligonucleotides of VNTR core sequences (14-24 bp), used as single primers to amplify the cDNA samples of *L. macrocephalus*, are longer than RAPD primers, the methodology could be effectively carried out at a relatively high stringency, thus yielding enhanced results - the generated bands were more distinct and the results of cDNA amplification were consistently repeatable (Figure 2). These data are consistent with the proposal that DDRT-PCR reactions need special adjustments for each species samples since its fingerprinting patterns may vary from one cell type to another (Blaes et al., 2007).

Despite this optimization in the DD approach in brain tissues of a fish species, our results led to the identification of a restricted number of amplification products, even using longer primers. The DD methodology generally leads to the visualization of 5 to 100 amplification products in polyacrylamide gel (McClelland et al., 1995). The unworkable achievement of a complex amplification pattern with a high number of bands can be due to the high PCR stringency conditions, through an annealing temperature of 55°C, or to the use of RNA sample pools that can avoid the detection of individual variations. Despite the relative reduced number of amplified fragments (around 5 to 15 bands) visualized on polyacrylamide gels, some of them could be detected only in males or females, which indicate that they correspond to presumptive differentially expressed transcripts between the two samples. Thirty

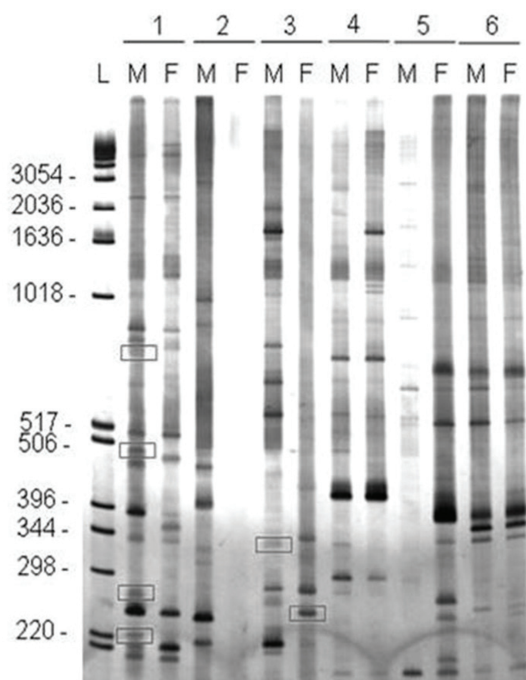

Figure 2. DDRT-PCR fingerprints on a 6% polyacrylamide gel stained with $AgNO_3$. The molecular weight of the amplified fragments (using longer primers, based on VNTR core sequences) was assigned through comparison with a 1 Kb DNA Ladder (Invitrogen™), indicated on the left side (**L**). **(1)** primer INS, **(2)** primer HBV3, **(3)** primer HBV5, **(4)** primer EMBL, **(5)** primer YNZ22, and **(6)** primer FvIIex8. **M=** pool of male RNA samples; **F=** pool of female RNA samples. The single amplified fragments that may correspond to differentially expressed transcripts are highlighted (boxes).

one cDNAs appeared to be differentially expressed on males and females of *L. macrocephalus*. Therefore, the improved DD approach using VNTR core sequences as primers in RT-PCR led to the identification of putative sex differentially expressed transcripts in this fish species and may be used as a strategy in future studies related to this approach in order to obtain consistent data.

Acknowledgements

The authors thank FAPESP (Fundação da Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for finnancial supports.

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