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Validation of commercial real-time PCR-arrays for environmental risk assessment: Application to the study of *p,p′*-DDE toxicity in *Mus spretus* mice liver☆

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ABSTRACT

Data on gene transcription profiles provide a comprehensive assessment of the toxic and defensive mechanisms that are triggered by pollutants. PCR-arrays have emerged as a reliable tool for analyzing the expression of a panel of relevant, pathway- or disease-focused genes under uniform cycling conditions. By using SYBR Green-optimized primer assays, it is possible to simultaneously amplify a sample with high specificity and amplification efficiencies. However, commercial PCR-arrays target a limited group of organisms, excluding most of those with environmental relevance, as is the case with *Mus spretus* mice. Our previous works with *M. spretus* showed a high sequence similarity between *M. spretus* and the model organism *M. musculus* allowing the use of commercial platforms with *M. spretus*. This work demonstrates the successful application of a commercial PCR-array designed for the model organism *M. musculus* to assess the biological effects caused by the organochlorine pesticide *p,p′*-DDE in a focused panel of stress-related genes in *M. spretus* mice. This cost-effective, easy-to-use platform detected quantitative gene profiling differences between *M. spretus* hepatic RNA samples and generated data highly concordant with those obtained by absolute qRT-PCR, the most sensitive method to quantify transcripts. This platform is also suitable for use in field studies with free-living *M. spretus* mice for routine environmental risk assessment. Our results provide a broad impression of the biological consequences of *p,p′*-DDE on the hepatic health of mice.

1. Introduction

Environmental transcriptomic studies provide a more comprehensive assessment of pollutants toxicity mechanisms and the defensive cellular responses that they trigger than do traditional biomarker studies (Abril et al., 2011; Gonzalez-Fernandez et al., 2008). These studies usually rely on the use of bioindicators, which are non-model organisms living in the site and used for the ecosystem health risk biomonitoring. However, the genomes of most ecologically important, non-model organisms have not been (fully) sequenced. Therefore, applying transcriptomic methodologies to environmental studies is often challenging because of the limited information contained in the available gene/protein sequence databases.

The transcriptome, defined as the entire complement of transcripts in a cell, tissue or organism, under certain conditions, can be analyzed by several technologies. With recent advancements and a radical decline in costs, the next generation sequencing (NGS) methodology has become a mainstream option for many laboratories. However, the microarray-based methodology is generally considered easier to use, with less complicated and less labor-intensive sample preparation and data analysis than NGS. Microarray technology simultaneously monitors thousands of gene transcripts, based on the sequence complementarity between probes and tested samples. Commercial microarrays are not usually applicable for environmental studies, as they target a limited number of organisms and exclude most species. Developing a self-designed for a species of interest is a laborious, time consuming and expensive since it requires previously obtaining expressed

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sequence tags, cDNA libraries or genome sequence data. Different strategies allow for overcoming these inconveniences and application of the heterologous microarray has gained wide interest to study the response to pollutants (Bar-Or et al., 2007; Buckley, 2007; Osuna-Jimenez et al., 2009). We have previously reported the successful application of a commercial *Mus musculus* oligonucleotide microarray to evaluate the biological effects of polluted ecosystems on the phylogenetically related *Mus spretus* mice by means of heterologous hybridization (Abril et al., 2014; Ruiz-Laguna et al., 2016). . NGS and microarrays have been widely used in discovery-based environmental research; unquestionably, this methodology is making a huge contribution to the identification of pollution exposure biomarkers at the transcript level. However, routine biomonitoring programs require the use of easy, fast, cheap and reliable tools. Real-time PCR is widely considered the gold standard for transcript quantification, adequate for studies focused on a small group of selected transcripts because it is rapid, easy and low-cost.

PCR-arrays have emerged as a reliable tool for analyzing the expression of a panel of 96 or 384 relevant, pathway- or disease-focused genes under uniform cycling conditions. By using SYBR Green-optimized primer assays, it is possible to simultaneously amplify a sample with the specificity and high amplification efficiencies needed for accurate real-time SYBR Green results. The flexibility, simplicity, and convenience of the PCR-arrays makes them accessible for routine use (Schmittgen et al., 2008). As in the case of the microarrays, commercial PCR-arrays target a limited group of organisms, excluding most of those with environmental relevance. Our previous works with the non-model, non-sequenced mouse *M. spretus* have sequenced many genic fragments in both coding and non-coding regions. These works demonstrated that the coding sequences were highly similar between *M. spretus* and *M. musculus* species and that the sequence divergences are mainly in the regulatory gene sequences (Abril et al., 2014, 2015; Pueyo et al., 2011; Ruiz-Laguna et al., 2006, 2005, 2016). With these data in mind, we addressed the analysis of the transcriptional changes caused by the organochlorine pesticide *p,p′*-DDE in a focused panel of stress-related genes in *M. spretus* mice by using a commercial PCR-array designed for the model organism *M. musculus*.

The use of DDT [1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane] in agriculture was prohibited in the early 1970's. Forty years later, *p,p'*-DDE [(1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene)], the main metabolite of DDT, continues to be detected in serum samples and fatty substances in many human and animal populations worldwide (Mangum et al., 2015b), especially after the outbreak of Zika virus and its damaging consequences (e.g., microcephaly). Individuals can be exposed to DDT/DDE via direct exposure or consumption of foods contaminated with residues. Because of their lipophilicity, persistence and slow elimination from the body, DDT/DDE are bioaccumulated and biomagnified through food webs. Exposure to DDT and its metabolites is considered a human health risk factor, as accumulated evidence indicates the association of *p,p′*-DDE exposure with neurotoxicity, endocrine disruption and reproductive system changes ((Song et al., 2014) and references herein). However, the mechanisms underlying *p,p′*-DDE are poorly understood. Thus, investigations involving the adverse effects of *p,p'*-DDE on human health, especially its involvement in cancer, metabolic syndromes and reproduction, are receiving more and more attention. These studies are usually carried out with *M. musculus*, the best-known vertebrate model organism. However, the standard *M. musculus* laboratory strains have serious limitations because of their reduced, natural genetic polymorphisms (Guenet and Bonhomme, 2003). In contrast, *M. spretus* provides a diversity of novel allelic variants and phenotypes unknown in commonly used laboratory mice (Dejager et al., 2009, 2010). *M. spretus* is an unprotected rodent widely distributed in Southern Europe (France, Spain and Portugal) and North Africa. This small mammal species typically inhabits marshlands where it attains high population densities. Within the last fifteen years, several laboratories have begun to use *M. spretus* as a bioindicator species in several environmental monitoring programs (Abril et al., 2011, 2012, 2014, 2015Marques et al., 2008; Montes-Nieto et al., 2007; Nunes et al., 2001; Ruiz-Laguna et al., 2006; Viegas-Crespo et al., 2003).

The aims of this study were to validate the use of commercial heterologous SYBR Green real-time PCR-arrays in *M. spretus* mice and contribute to the understanding of the *p,p'*-DDE toxicity mechanisms in mice livers.

2. Experimental procedures

2.1. Animal studies

Mus spretus (inbred SPRET/EiJ strain) mice were obtained from the Jackson Laboratory (www.jax.org). A total of 10 male mice (7 weeks and ~12 g) were allowed to acclimate for 3 days with free access to food (Harlan diet 2014) and water under controlled conditions (25/30 °C, 12 h light/dark photoperiod) prior to exposure.

The mice were distributed into two groups (n = 5 mice per group, 1–2 mice per cage). The diet for the experimental group contained *p,p′*-DDE, dissolved in corn oil at 150 mg/kg, which implied a daily average ingestion of 50 mg/kg bw of *p,p′*-DDE and 10 ml/kg bw of corn oil. The control mice received the same diet supplemented with only the corresponding quantity of corn oil (10 ml/kg/day). Fresh pre-weighed food and water were provided every other day, and leftovers were weighed when removed from the cage. Daily food and water intake was estimated by dividing the total food intake in a given cage by the number (1 or 2) of animals in that cage. After 30 days, mice were anesthetized, total body weight was measured, and the animals were then sacrificed by cervical dislocation and dissected. Individual organs were frozen in liquid N_2 and stored at −80 °C. After cryogenic homogenization of each individual liver in a 6770 Freezer/Mill apparatus (2 min, rate 15) from Spex SamplePrep (Metuchen, NJ, USA), samples were stored at −80 °C. The Animal Ethics Committee of the Cordoba University approved this investigation.

2.2. RNA isolation

Total RNA from individual livers was isolated by the RNeasy Mini kit (Qiagen) and the residual genomic DNA (gDNA) was removed by the Total RNA Cleanup with DNase Digestion kit using the Qiagen RNeasy Protocol (Qiagen). RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies(Schroeder et al., 2006)). RNA purity and concentrations were determined by spectrophotometry. gDNA contamination was tested by PCR amplification of RNA samples without a prior cDNA synthesis step. Only RNAs with RIN values > 8.5, 260/280 ratios above 2 and free of gDNA were used in qRT-PCR experiments.

The cDNAs were generated from 1 μg of total RNA from each sample using the QuantiTect Reverse Transcription Kit (Qiagen) per the manufacturer's protocol.

2.3. Real-time PCR-arrays

Samples (livers of control and *p,p′*-DDE exposed mice) were analyzed using Mouse Stress Response (cat. no. 00188191, StellARray™ Gene Expression System, Bar Harbor Biotechnology, USA) PCR-arrays. Each PCR-array is a 96-well plate containing gene-