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Full Length Research Paper

The use of degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) technique to detect and isolate cytochrome P450 2E1 and 2A genes

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Cytochrome P450 (CYP, P450) enzymes are widely studied for their involvement in the metabolism of drugs as well as endogenous substrates. Assessing some forms of P450 such as P450 2E1 and 2A phenotype may be of great importance to predict organism's susceptibility to xenobiotic and environmental pollutants which are metabolically activated by these isoenzymes. In the present study, we designed universal degenerate oligonucleotide primers sets based on the highly conserved nucleotide sequences at the 5' and 3' cDNA ends of P450 2E1 and 2A gene family from *Camelus dromedaries* (Arabian camel), *Bos Taurus* (cow), *Capra hircus* (domestic goat), *Ovis aries* (domestic sheep), *Oryctolagus* cuniculus (European rabbit), *Xenopus tropicalis* (Western clawed frog), *Rattus norvegicus* (Norway rat), *Mus musculus* (house mouse) and *Mesocricetus auratus* (golden hamster). We successfully demonstrated that these primers sets were able to detect the expression of P450 2E1 and 2A genes from these eukaryotic organisms. Moreover, we amplified 1070 and 1200 nucleotides of both P450 2E1 and 2A from these organisms and this represent two third of the actual size of these P450 isoforms. The nucleotide sequences of these genes showed percent identities ranging from 80 to 85%. The degenerate primers designed in this study allowed the detection and amplification of known and unknown P450s belonging to the gene families 2E1 and 2A from different organisms.

Key words: Cytochrome P450, xenobiotics, degenerate primers, mixed function oxidases.

INTRODUCTION

Cytochrome P450s (P450s) are superfamily of heme-containing proteins distributed widely throughout nature, involved in the metabolism of a broad range of substrates and catalyzes a variety of interesting chemicals reactions (Hasler et al., 1999). They catalyze both endogenous and exogenous compounds, converting them to more soluble hydrophilic metabolites that can be removed readily from the body (Guengerich, 2001). However, in some situations, the metabolism of chemicals by the P450 enzymes may be undesirable or detrimental to the body and can lead to toxic or reactive intermediates resulting in target organ toxicity and/or carcinogenic insult (Nebert and Dalton, 2006; Stiborova and Rupertova, 2011; Rooney et al., 2004). The expression levels of P450 proteins is known to be highly expressed in a wide range of human cancers, these include P4501A, 1B, 2C, 3A and 2D P450 subfamily members (Lamb et al., 2007). Cytochrome P450 are proteins expressed in a variety of mammalian tissues including liver, kidney, lung, adrenal,

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gonads, brain, skin and others. These forms of P450 proteins are membrane bound and reside in the endoplasmic reticulum and in the mitochondrial membrane. Some other forms of P450 proteins are also observedin the outer nuclear membrane, different Golgi compartments, peroxisomes and other plasma membrane (Seliskar and Rozman, 2007; Hannon-Fletcher et al., 2001). While most of the P450 enzymes have shown to be constitutively expressed, many are increased markedly in expression upon exposure to various inducers such as ethanol (Khan et al., 2011), polychlorinated biphenyls and polychlorinated dibenzo-ρ-dioxins (PCDDs) (Murtomaa et al., 2010; Uppstad et al., 2010). The nomenclature of P450s is based on their amino acid sequence identity.

The letters CYP are followed by a number indicating the P450 gene family (more than 40% identity), then a letter for the subfamily (more than 55% identity) and finally a number for each individual protein. Although, the amino acid sequence identity between different P450s may be very low, their secondary structure is highly conserved (Creshar and Petric, 2011). Humans have 57 P450 genes and 58 pseudogenes distributed in 18 P450 families, one fourth of the human P450s are not well-characterized and therefore considered 'orphans' (Guengerich et al., 2011). The mouse genome has 102 P450 genes and nearly 90 pseudogenes (Nelson, 2011)

The selected P450 genes used in the current study are 2E1 and 2A and both are of particular importance in most organisms under study. The P450 2E1 isoform is responsible for the metabolism of many small compounds and molecules including ethanol, carbon tetrachloride, chloroform, tetrachloroethylene, styrene and benzene (Piccoli et al., 2010; Prieto-Castello et al., 2010). Assessing the P450 2E1 phenotype in subject exposed to industrial chemicals and environmental pollutants may be useful either to predict their susceptibility to or to detect early effects from exposure to those chemicals. P450 2E1 metabolizes some endogenous physiological substrates; these include acetone and fatty acids such as linoleic and arachidonic acids (Prieto-Castello et al., 2010). P450 2E1 is involved in the metabolism of ethanol and generate reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and 1-hydroxyethyl free radicals. These ROS are known to react with cell membrane and initiate lipid peroxidation, resulting in the formation of protein adducts and increases collagen synthesis, which ultimately leads to alcoholic liver diseases including liver cirrhosis (Nebert and Dalton 2006). P450 2A subfamily plays an important role in xenobiotic disposition in the liver and in metabolic activation in extrahepatic tissues. P450 2A transcripts and enzymes are inducible by xenobiotic compounds and some of the P450 2A genes are influenced by physiological status such as circadian rhythm and pathological conditions such as inflammation, microbial infection and tumorgenesis (Su and Xinxin, 2004). Variability in the expression of the P450 2A genes, which differs by species, animal strain, gender and organ may alter the risks of chemical toxicity for numerous compounds that are P450 2A substrates (Su and Xinxin , 2004).

In the present study, the degenerate oligonucleotideprimed polymerase chain reaction (DOP-PCR) technique has been developed to allow rapid detection and amplification of P450 genes belonging to the families 2E1 and 2A from *Camelus dromedaries* (Arabian camel), *Capra hircus* (domestic goat), *Ovis aries* (domestic sheep), *Bos taurus* (cow), *Xenopus tropicalis* (frog), *Rattus norvegicus* (Norway rat), *Mus musculus* (house mouse), *Oryctolagus cuniculus* (European rabbit) and *Mesocricetus auratus* (golden hamster).

MATERIALS AND METHODS

Animals and tissues preparation

Liver tissues were collected from different animals under study. Arabian one-humped camel (*Camelus dromedarius*, 500 to 800 kg, 6 to 8 years old); *Bos taurus* (cow), *Capra hircus* (domestic goat), *Ovis aries* (domestic sheep), *Oryctolagus cuniculus* (European rabbit), were obtained from the local slaughterhouse, after the animals were killed under the observation of skilled veterinarian. *Xenopus tropicalis*, *Rattus norvegicus*, *Mus musculus* and *Mesocricetus auratus* were obtained from the animal house, Department of Zoology, Faculty of Science, King Saud University. Tissues samples to be used for RNA analysis were immediately submerged in RNAlater solution (Ambion, Courtabeuf, France) to avoid RNA degradation, stored at 4°C for 24 h and then at -20°C until needed.

RNA isolation and cDNA synthesis

Samples of 30 to 60 mg of the preserved tissues were homogenized in RTL lyses buffer (Qiagen) supplemented with 1% 2-mercaptoethanol, using a rotor-stator homogenizer. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), with a DNase digestion step, according to the manufacturer's instructions. Elution was performed with 50 µl nuclease-free water. Concentrations and integrity of RNA samples were assessed using NanoDrop-8000 (Thermo Scientific) and formaldehyde agarose gel (1%) electrophoresis. Total RNA in aliquots of 2 µg was retrotranscribed into single-stranded cDNA using the ImProm-II Reverse Transcription System (A3800, Promega). Complementary DNA was synthesized by reverse transcription and used as a template for the amplification of the CYP2E1 and CYP2A DNA at a concentration of 20 ng per reaction.

PCR

Degenerate PCR primers were designed utilizing DNASTAR programs. These primers were designed around two highly conserved sequences located near the N and C terminus of CYP2E1 and 2A cDNA from different identified eukaryotic cytochrome P450 mRNA deposited in the GenBank. Gradient PCR was carried out in a final volume of 50 µl containing 25 µl 2× high-fidelity PCR master mix (GE Healthcare, USA), 5 µl (20ng) c-DNA,

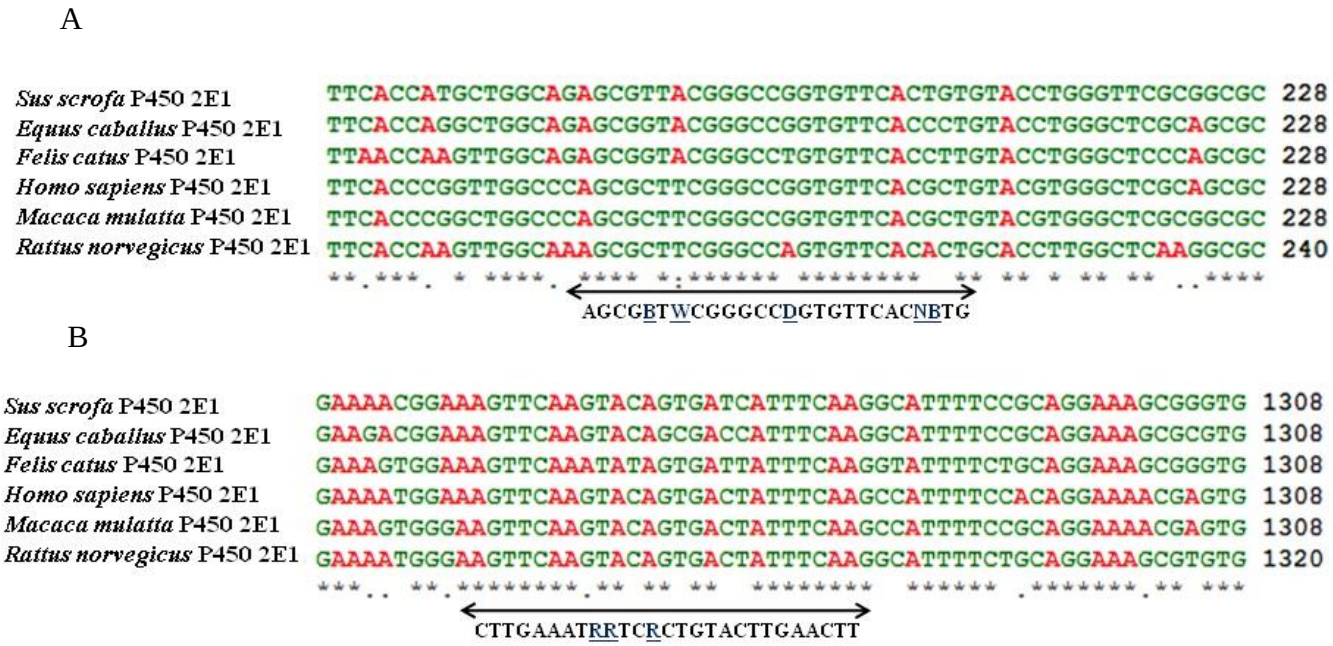

Figure 1. Alignment of the deduced nucleotide sequences for CYP2E1 from different organisms deposited in the GenBank and were used for designing forward (A) and reverse (B) degenerate primer. The underline section represents the location of the forward and reverse primers and the highly degenerate nucleotides in both primers.

3 µl (30 pmoles) of each P450 primers, P450 2A forward, 5'-GAGGCYCTSGTGGACCAVGSWGARGMBTT-3' and reverse, 5'-NRAAGYTCTGSAKRATGGYKGTGA-3'; P450 2E1 forward and reverse, 5'-AGCGBTWCGGGCCDGTGTTCACNBTG-3', 5'-CTT-GAAATRRTCRCTGTACTTGAACTT-3'. Where, R is either A or G; Y is C or T; K is G or T; M is A or C; S is G or C; W is A or T; B is G or C or T; D is A or G or T; V is A or C or G and N is A or G or C or T. The PCR condition was 1 cycle at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 50 to 60°C for 1 min, and 72°C for 1.5 min. The final extension step was carried out at 72°C for 5 min. PCR products were analyzed using 1.0% agarose gel electro-phoresis. The molecular weight of these PCR products was determined using Alpha Imager, Alpha Innotech Version 2.0.0.9.

Cloning, plasmid preparations and sequencing of the cloned PCR products

To ligate the generated PCR products onto pGEM-T- Easy vector (Promega Co.), 2 µl of each PCR products were taken in a clean 0.5 ml tube to which 1 µl pGEM-T- Easy vector (50 ng) and 1 µl of 10X ligase buffer were added followed by the addition of two units of ligase enzyme. The final volume of the ligation reaction was adjusted to 10 µl by the addition of nuclease free water. The tube was incubated at 16°C for 16 h. Transformation of *Escherichia coli* JM109 competent cells was carried out according to Sambrook et al. (1989). The recombinant *E. coli* JM109 harbouring pGEM-T-Easy vector was screened in selective LB/IPTG/X-gal/Ampicillin/ agar plates. Moreover, colonies PCR was conducted to screen recombinant bacteria for ligated DNA insert using T7/SP6 primers. A small part of each bacterial colony was transferred to a clean sterile Eppendorff tube to which the rest of the PCR reaction components was added as described earlier. The PCR condition was as follows: 1 cycle at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. The PCR products were analyzed through 1.5% agarose gel by electro-phoresis. Plasmids were prepared from all potential PCR positive clones using PureYield™ Plasmid Miniprep System Cat # A1222, Promega Corporation, USA. Sequencing of the PCR product cloned onto pGEM-TEasy vector was carried out according to Sanger et al. (1977) using MegaBACE 1000 DNA Sequencing System (Pharmacia/Amersham Co.). The chain termination sequencing reaction was conducted utilizing the DYEnamic ET terminator kit as an integral part of the MegaBACE 1000 DNA sequencing system. The sequencing reactions products were purified using DyeEx 2.0 Spin Kit Cat # 63206 (Qiagen Co.) and applied to MegaBace 1000 Sequencing machine. The cDNA sequences for P450 2E1 and 2A from all organisms under study were analyzed using the ClustalW and DNASTAR programs.

RESULTS

Designing of the degenerate PCR primers sets and optimization of the PCR

Figure 1 shows the alignment of the deduced conserved nucleotide sequences of P450 2E1 from different organisms that are used to design the degenerate primer sets. Both of these primers were localized near the highly conserved sequence around the 5' and the 3' end of the gene from 196 to 1269 nucleotides. Optimization of the PCR for P450 2E1 and 2A genes at different annealing temperatures from 50 to 60°C using *C. dromedarius* liver cDNA is shown in Figure 2. It was found that both of these degenerate primers sets were able to amplify

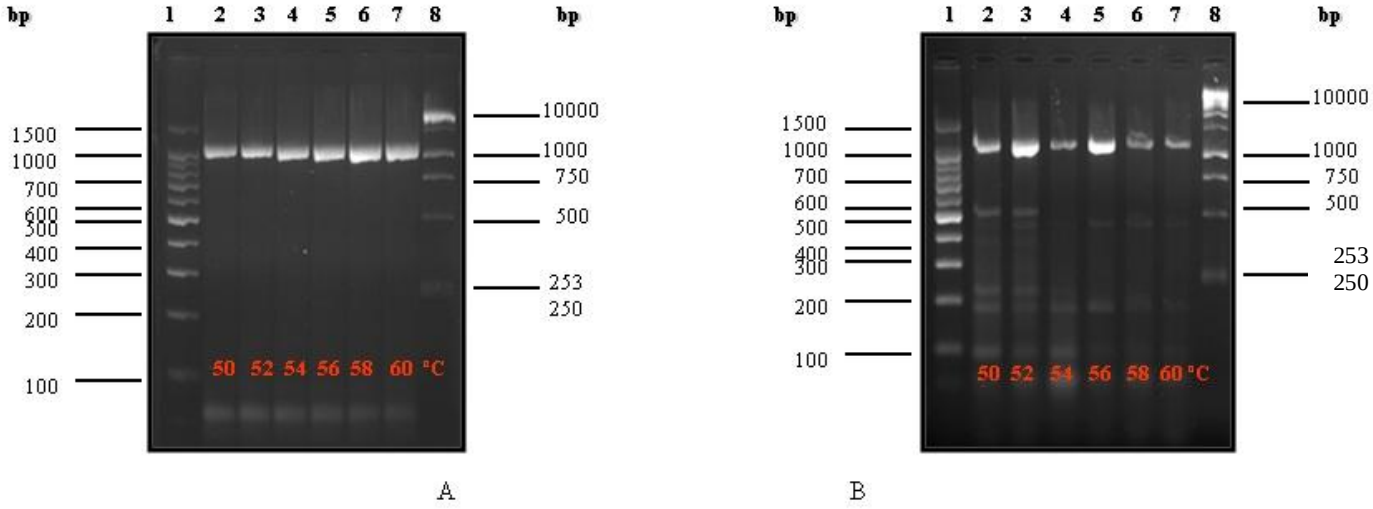

Figure 2. Agarose gel (1.0%) electrophoresis of PCR products for P450 2E1 (Panel A Lanes 2 to 7) and P450 2A (Panel B Lanes 2 to 7) from *C. dromedarius* at different annealing temperatures. Lanes 1 and 8 represent 100 bp and 1.0 kbp DNA molecular weight markers.

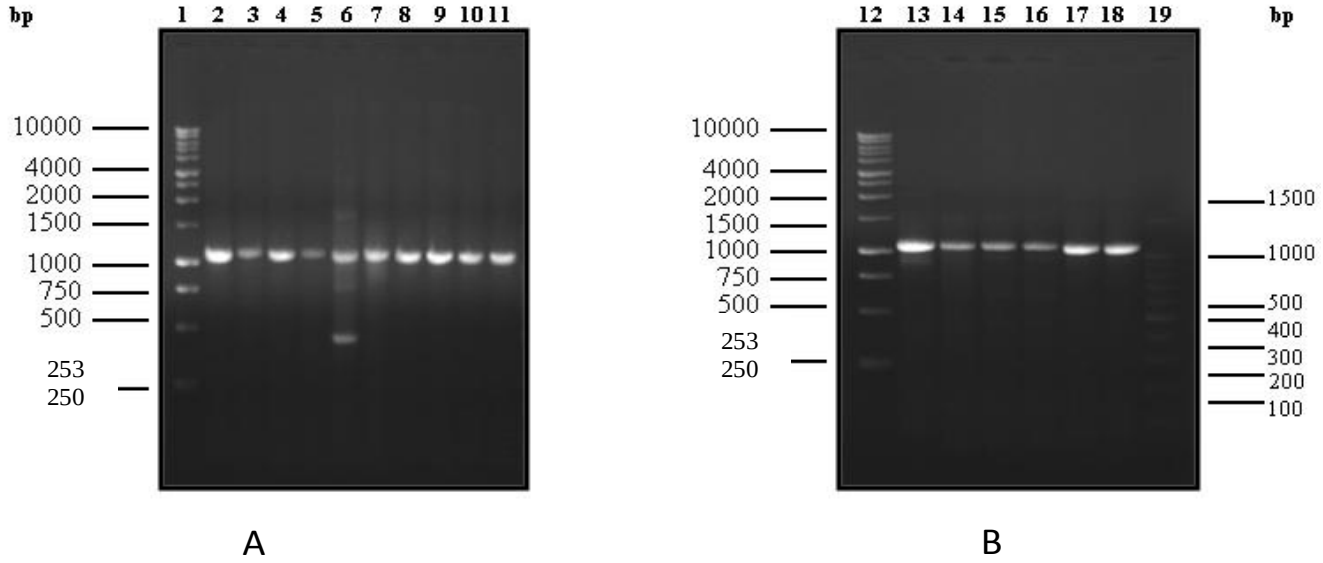

Figure 3. Agarose gel (1.0%) electrophoresis of PCR products of P450 2E1 and 2A genes, respectively from *Bos taurus* (Lanes 2 and 3); *Capra hircus* (Lanes 4 and 5); *Ovis aries* (Lanes 6 and 7); *Oryctolagus cuniculus* (Lanes 8 and 9); *Xenopus tropicalis* (Lanes 10 and 11) Panel A and *Rattus norvegicus* (Lanes 13 and 14); *Mus musculus* (Lanes 15 and 16) and *hamster* (Lanes 17 and 18) Panel B. Lanes 1 in Panel A and 19 in Panel B represent 1 kb and 100 bp DNA molecular weight markers.

exclusively specific PCR products corresponding to 1073 nucleotides for P450 2E1 (Figure 2, panel A, lanes 2 to 7) and 1200 nucleotides for P450 2A (Figure 2, panel B, lanes 2 to 7). Moreover, it was clear that no non-specific PCR products were obtained at different annealing temperatures for both P450 2E1 and 2A, indicating significant specificity for both of these primers sets. Figure 3 panels A and B shows the results of the PCR for P450 2E1 and 2A of the other organisms under investingation. Clearly reproducible specific PCR products were obtained for both P450 genes at 56°C annealing temperature with no non-specific amplification products.

Cloning and sequencing of the PCR products

Our strategy was initially based on the observation that sequences of the P450 2E1 and 2A genes from different

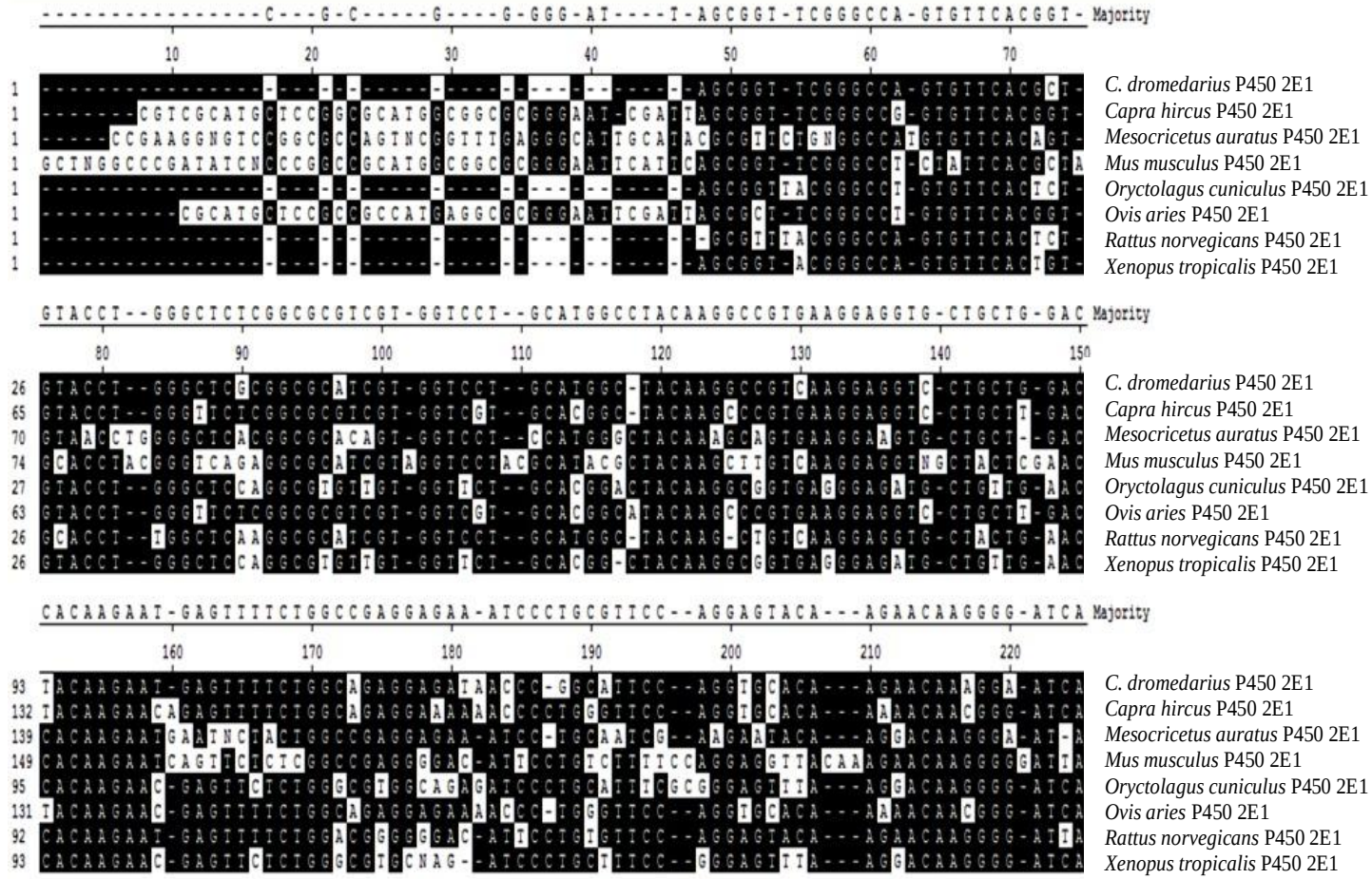

Figure 4. Alignment of the nucleotides sequences of P450 2E1 from different organisms under study. Shaded boxes indicate highly conserved nucleotides. Alignment was carried out using DNASTAR program.

organisms share some highly conserved stretches of nucleotides (Figure 1 for P450 2E1) that were used to design both degenerate primers sets. This observation opened the possibility that a combination of two degenerate primers would allow the amplification of all P450s genes that also possessed these two regions of very high sequence conservation. Each amplified cDNA fragment generated from different organisms might contain several different P450 gene fragments;n direct sequencing of the PCR products was not contemplated. Instead, the amplification products were cloned into the pGEM-T-Easy cloning vector (Zhou et al., 1995). Plasmid DNA from putative clones were then isolated and purified and the presence of a cDNA inserts were tested and verified by PCR amplification of the plasmids with T7/SP6 primers which anneal to the vector region flanking the cDNA inserts. Clones containing inserts were then sequenced to ensure the uniqueness of the inserts. Sequencing of large numbers of clones revealed that most of the amplified products corresponded to either P450 2E1 or 2A. Nucleotide sequences were compared with the nucleotide sequences deposited in the GenBank database using the BLASTn program on the NCBI BLAST Server. Only those clones that had significant similarity scores to numerous P450s and to no other genes were considered to be P450 genes. Some of these partial sequences, especially for the *C. dromedaries* P450 2E1 and 2A were deposited in the GenBank data base and given the accession numbers HQ998962 and HM047299. Sequence analysis using BLAST program revealed that most of the generated PCR fragments represent P450 genes, either 2E1 or P450 2A and in all cases, the similarity percents were found to be about 80 to 85% to several other P450 2E1 and 2A genes that were deposited in the GenBank. Figures 4 and 5 show the alignment of the nucleotides sequence for P450 2E1 and 2A, respectively from different animals under study. The percent identities between these partial sequences was found to be about 85% and this means that most of these sequences belonged to the same gene family and

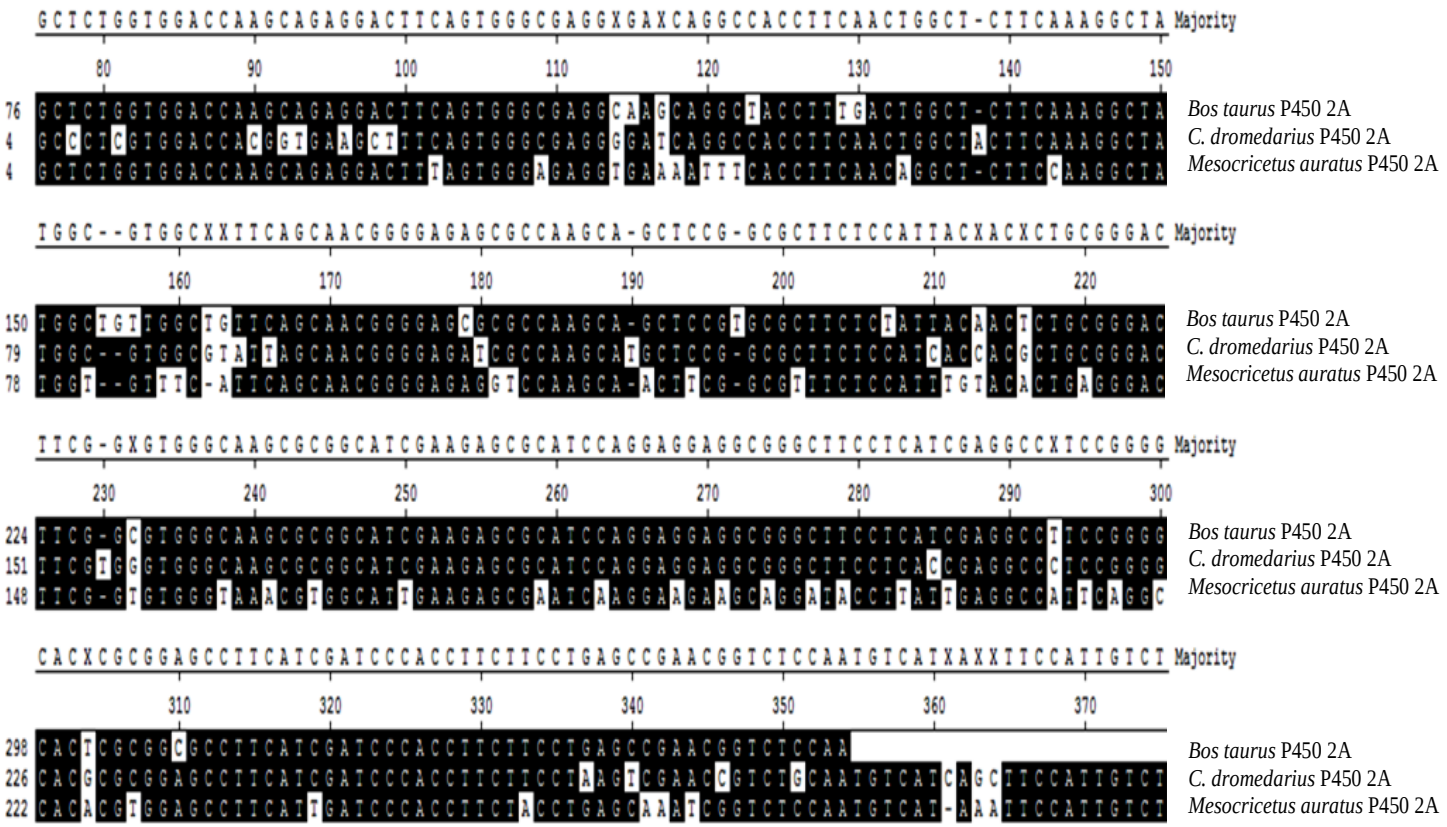

Figure 5. Alignment of the nucleotides sequences of P450 2A from different organisms. Shaded boxes indicate highly conserved nucleotides. Alignment was carried out using DNASTAR program.

subfamily, mainly P450 2E1 and 2A.

DISCUSSION

Molecular cloning of P450 genes and their heterologous expression and reconstitution of P450 activities has so far provided the best method for biochemical characterization of most of the P450 proteins. The combined availability of a huge P450 sequences from the GenBank and the new P450 database (http://drnelson.uthsc.edu/Cytochrome P450.html) and of the RT-PCR has now allowed the development of rapid accurate and efficient tools to detect and clone P450 fragments from any source and from any organism. The use of the PCR with gene specific primers to amplify P450 gene fragments of known identity is very useful in many situations such as to detect the expression levels of P450s in different tissues and in different developmental stages. But in some cases, if there are no data about the P450 genes in some organisms, the assumption is made that the conserved P450 sequences from number of species and representing one or more P450 subfamilies will be represented in the unknown P450s being targeted for cloning. In the case of P450 gene superfamily, one has to think about some conserved domain such as the heme-binding region consensus (FXXGXXXCXG), taken in consideration, this universal P450 signature is no longer a unique universal P450 filter since the phenylalanine is replaced by tryptophan in cytochrome P450 gene families 8 and 10 and by proline in cytochrome P450 74. Moreover, the second glycine is replaced by alanine in a number of P450s from various sources. Thus, highly degenerate PCR primers could offer a suitable solution to detect and to isolate P450 genes from any organism. In the present study, we focussed on P450 2E1 and 2A due to their importance in the metabolism of various endogenous and exogenous substrates. P450 2E1 has a wide spectrum of substrates and it oxidises a significant number of important industrial compounds such as alkanes, alkenes, aromatic and halogenated hydrocarbons and many related compounds (Bott et al., 2003). Cytochrome P450 2E1 is of great importance and in occupational and environmental medicine. P450 2E1 is highly expressed in the liver and it is also expressed in extrahepatic tissues such as kidney, lung, nasal mucosa and bone marrow (Botto et al., 2004). Moreover, P450 2E1 is also expressed in circulating lymphocytes, per-

mitting the quantitative determination of its protein and mRNA in peripheral blood (Haufroid et al., 2001). The other P450 used in this study was P450 2A. This P450 isoform is a complex having multiple genes which are highly similar in nucleotide sequences but are diverse in their modes of regulation in various species and organs. To date, 23 P450 2A genes and pseudogenes have been identified (Nelson, 2009). P450 2A proteins have also been detected in cows, hamsters, rabbits and pigs. Collectively, the P450 2A enzymes metabolizes numerous xenobiotic compounds such as coumarin, aflatoxin B1, N-nitrosodimethylamine, nicotine, cotinine, 1,3-butadiene and 2,6-dichlorobenzonitrile as well as endogenous compounds such as testosterone, progesterone and other steroid hormones (Ding and Kaminsk, 2003). Variations in the expression of the P450 2A genes probably alter the risks of chemical toxicity for numerous toxicants that are P450 2A substrates (Fernandez-Salguero and Gonzalez, 1995).

The main goal of this study was to develop a degenerate primer sets able to detect and amplify known and new unknown putative P450 genes belonging to the gene family 2E1 and 2A from any organism. The design of highly degenerate primers is of great importance for the identification of novel members of P450 genes superfamily. Optimally, highly conserved functional domains are used for design of degenerate primers as it has been the case for the detection of halocarboxylic acid dehydrogenase (Hill et al., 1999), ring hydroxylating dioxygenases (Yeates et al., 2000) and chloroethyene reductive dehalogenase (Christophe et al., 2004) genes using RTPCR. Herein, we were able to amplify 1073 and 1100 nucleotides from P450 2E1 and 2A, respectively which represent nearly two third of the actual full length cDNA of both genes. Our results show excellent specificity of the degenerate primer sets and these sets can be used to evaluate the changes in gene expression, the significant set of P450s that indicate the existence of toxicity may be obtained. Discovering these genes that are correlated with the substances class may indicate the mechanisms of toxicity and the affected pathways.

In conclusion, we successfully demonstrated that the universal degenerate oligonucleotides primed PCR were able to detect and to amplify P450 2E1 and 2A genes. Moreover, these sets of primers may also be used for the development of a diagnostic tool that can be applied to detect toxicity of a substance or for the early diagnosis of toxin or environmental exposure.

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