Technical Brief

DDRT-PCR: Use of agarose gels for detection of amplified products

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The present study focuses on the detection of differentially expressed genes in migrating (healing) and nonmigrating (normal) corneal epithelium on agarose gel using a modified procedure of differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR). Rabbit corneal epithelial organ cultures were used to obtain nonmigrating and migrating samples. RNA was extracted using Trizol LS reagent. PCR was modified in order to allow detection of amplified products on 3% agarose gel with ethidium bromide staining. Products were also resolved on 6% denaturing polyacrylamide-urea gels and observed by silver staining. Agarose gels showed two prominent bands that were heavily expressed in the 458 bp and 587 bp region of the nonmigrating samples. In addition light bands were visible in the region corresponding to 234 bp and 450 bp. In the migrating samples, two light bands were visible in the region of 267 bp and 300 bp. Eight amplicons, six in the nonmigrating corneal epithelial sample and two in the migrating corneal epithelial samples, were also found to be differentially expressed when products were run on 6% denaturing polyacrylamide-urea gels. Thus, DDRT-PCR products can be detected on agarose gels and prove very helpful and economical in the initial studies of DDRT-PCR.

Corneal trauma has been documented to be the major cause of blindness in the developing countries of the world [1,2]. In Pakistan, it is reported to be the leading predisposing factor (42.26%) for the development of corneal ulceration, which constitutes 9% of the total 2 million incidences of blindness [3]. Corneal trauma is, therefore, a severe clinical problem and requires rapid recovery of the wounded area, failure of which may result in persistent epithelial defects and corneal ulceration, sometimes leading to loss of vision within 24 h.

Primarily, the wound repair process takes place by re-epithelialization. The initial phase of re-epithelialization involves migration of the epithelial sheet over the denuded basement membrane to cover the wound [4]. The current understanding of the molecular and cellular events of the migratory phase in corneal epithelial cells involves analysis of gene expression [2,5,6]. Using subtractive screening of cDNA library prepared from corneoscleral rims after cauterizing rat corneas, 76 clones whose corresponding mRNA increased during the wound healing process in an in vivo model were identified. A comparison with the Genbank/EMBL databases further identified 45 clones with novel sequences [6]. Haseba et al. [5] isolated five cDNA clones from mRNA of alkali-burned rabbit cornea. Zhu et al. [7] demonstrated that the expression of one of these genes, cytokeratin 12, is altered in the wounded cornea. Gene expression profile studies of human corneal epithelial cells showed a rich variety of uniquely active genes for surface membrane proteins located in the apical membrane, cell-cell junctions, or cell matrix junctions of the normal cornea [8].

A recent approach for the comparison of migrating (healing) and nonmigrating (normal) epithelium is differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR) [9]. DDRT-PCR was first introduced by Liang and Pardee [10,11] and it enables identification of the total number of genes subject to up- or down-regulation in a particular setting. Yu et al. [9], using the original protocol of this technique [10,11], studied migrating and nonmigrating corneal epithelia and have been able to detect nine differentially expressed genes. Since then numerous refinements have been proposed for the initial description of the differential display method [12,13]. The fact that for the direct detection of amplification products silver staining could be used instead of autoradiography [13] has made the technique less expensive.

We describe here modifications and use of the mRNA differential display method for preliminary studies to examine changes in the gene expression during wound healing of corneal epithelium in the rabbit by allowing the detection of DDRT-PCR products first on agarose (3%) and then on polyacrylamide-urea gels.

METHODS

Preparation of rabbit corneal epithelial organ culture: Albino rabbit eyes were used to prepare migrating (n=14) and nonmigrating (n=6) corneal epithelia in organ culture as described by Panjwani et al. [14]. Briefly, the corneas were de-
marcated with a 7 mm trephine, and the epithelium within this region was removed with a #10 scalpel blade. The corneas were then excised along the limbus, rinsed in Hanks Balanced Salt Solution (HBSS, Sigma, St.Louis, MO) and disinfected for 5-7 min in antibiotic-antimycotic solution. Corneas were then excised along the limbus, rinsed in Hanks Balanced Salt Solution (HBSS, Sigma, St. Louis, MO) proposed by Jumblatt and Neufeld [15]. The cultures were then placed at 37 °C in a CO₂ incubator. After 48 h, the migrating and nonmigrating epithelia were collected under a dissecting microscope using scalpel blade and forceps and transferred immediately to a preweighed microtube and frozen in liquid nitrogen.

**RNA Extraction:** For extraction of total RNA, the frozen migrating and nonmigrating epithelia were homogenized in Trizol reagentLS (Gibco BRL 10296-010, Gaithersburg, MD). Simultaneous extraction of rabbit liver RNA was also carried out and later used as nonspecific tissue. The RNA was isolated according to the manufacturer’s instructions and stored in 80% ethanol at -70 °C. The integrity of total RNA was determined by analyzing on agarose gel.

**mRNA Differential Display:** The mRNA differential display was performed with a modified method of Liang and Pardee [10] and Yu et al. [9]. The RNA (0.2 µg) from the specific and nonspecific tissue was then preincubated at 65 °C for 5-min with 2 µl dithiothreitol (0.1 M stock solution) to denature RNA secondary structure. Reverse transcription (RT) was carried out for 60 min at 37 °C in a total RT mix of 20 µl, using 2.5 µM T₁₂ VA (where V may be A, G or C) as the degenerate primer, 20 µM dNTPs and 100 U MMLV reverse transcriptase. The reaction was stopped by incubation at 95 °C for 5 min.

The polymerase chain reaction was performed for the amplification of the RT reaction with a 10 mer oligonucleotide primer with the sequence 5’-AGGTGACCGT-3’ (Applied Biosystems 391 DNA synthesizer, Applied Biosystems, Foster City, CA) and the T₁₂ VA degenerate 3’-primer. This combination of primers has been previously used by Gipson et al. [9] who have successfully identified one differentially expressed gene in rat nonmigrating corneal epithelial tissue.

For each 100 µl of PCR mixture, 20 µl of the resultant cDNA was added to 80 µl of a solution containing 1.2 mM MgCl₂. 1X PCR buffer, 2 µM each of all four dNTPs, 1 µM 3’-primer, 0.2 µM 5’-primer and 5 U/µl Taq polymerase (Roche Molecular Systems, Inc., Branchburk, NJ). The samples were then subjected to 40 cycles of PCR using the following cycling parameters: 94 °C for 30 s, 42 °C for 1 min and 72 °C for 30 s; the last cycle was followed by a 5 min extension at 72 °C.

**Analysis of amplified products:** Amplified products (10 µl) of the control and corneal epithelial samples were first run on agarose gel (3%) and visualized using ethidium bromide dye. To visualize the shorter amplicons, 30 µl of the same products were subjected to denaturing polyacrylamide-urea DNA sequencing gel electrophoresis on a gel 6% in acrylamide and the bands were detected by silver staining [16].

**RESULTS & DISCUSSION**

The bands visualized on agarose gel (Figure 1) were found to be of high intensity when compared with the other bands of the same sample analyzed on polyacrylamide-urea DNA sequencing gel electrophoresis on a gel 6% in acrylamide and the bands were detected by silver staining [16].
In nonmigrating epithelial samples, two very prominent bands in the 587 bp and 458 bp region were seen on the agarose gel (Figure 1). In addition, light bands were visible in the region of 450 bp to 234 bp. Two light bands were also seen in the region of 300 bp and 267 bp in the migrating corneal epithelial samples (Figure 1, arrowheads). Previously, no report has shown the detection of DDRT-PCR products on agarose gels.

On polyacrylamide-urea gels (6%) two amplicons were found to be present in the migrating corneal epithelium only, whereas six products were expressed strongly in the nonmigrating samples (indicated by arrows in Figure 2).

Optimizing conditions for the detection of DDRT-PCR products by silver staining: In the original protocol [9], 2 µl of cDNA products were used for the 20 µl of PCR reaction (i.e. cDNA is in the ratio of 1:10). This modification brings in two major advantages of using 1:5 ratio of cDNA and PCR mix is that the (1) heavily expressed amplified products can easily be observed by ethidium bromide staining of agarose gels (3%) suggesting that further resolving of bands using acrylamide gels will be productive and (2) genes expressed can be detected on denaturing polyacrylamide-urea gel (6%) without the use of radioisotope, hence providing equally good results without unnecessary exposure to the hazards of radioactive material.

After optimizing the PCR conditions, amplified products of low molecular weights and low expressions could not be detected on the agarose gels. This appears to be the only drawback of the technique. However, the DDRT-PCR is likely to overcome this considering the relatively large number of amplified products in each run.

Validation of mRNA Differential Display: The following two lines of evidence suggest that the differentially expressed bands are not attributable to endogenous rRNA or to DNA contamination: (1) the pattern of banding changes during migration, and should not be the case if the bands are derived from endogenous rRNA, and (2) when the RT reaction was conducted in the absence of reverse transcriptase, essentially no bands were observed, which suggested that DNA contamination, which could result in generation of amplicons, was minimal.

In this procedure, due to the increased ratio of the resultant cDNA and PCR mix, nonspecific amplification may have increased. However, selection of bands has been made by running nonspecific liver RNA samples adjacent to specific corneal epithelial RNA samples to eliminate any background noise. Thus, only those bands that have not appeared in the nonspecific sample were identified to be differentially expressed (Figure 2).

Our study introduces a method of undergoing preliminary examination of DDRT-PCR products without the use of radioisotopes. This procedure is especially handy because it enables the amplification of differentially expressed products to be detected on agarose gel, proving less time consuming and painstaking. In addition, further identification can be carried out on silver stained denaturing polyacrylamide-urea gels. In the present study, this protocol has been employed and successful detection of eight differentially expressed genes, two in the migrating and six in the nonmigrating corneal epithelium, has been made. Whether they are playing a role in the healing process is yet to be established.

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REFERENCES


The typographical corrections below were made to the article on the date noted. These changes have been incorporated in the article and the details are documented here.

19 September 2000:

Institutional information for Dr. A. A. Siddiqui (Department of Biochemistry, The Agha Khan University, Karachi, Pakistan) was added; the existing institutional information was numbered.