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Non-invasive Method of Identification of *SAG*-1 Transgenic Mice by PCR Analysis of Oral Wash Cells

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ABSTRACT

We have simplified a non-surgical procedure as an alternative to surgically obtained samples to identify transgenic mice harboring *SAG*-1 transgene. The protocol involves the use of a small amount of oral wash containing enough oral different cells including epithelial cells serving as a sufficient source of DNA for two-step polymerase chain reaction (PCR) analysis. Oral wash is boiled and aqueous phase is applied directly to PCR cocktail containing an outer pair of primers for the first round amplification. Afterward, a small amount of the first round product is added to PCR cocktail containing an inner pair of primers for the second round reaction. The procedure provides outcomes consistantly matching those of tissue biopsy one. This protocol can be repeated many times with minimal stress to mice. This technique is reliable, does not require protease digestion and phenol-chloroform extraction, is more humane, and can be completed within 4 hr. This technique is an alternative to the tissue biopsy procedure to determine the *SAG*-1 transgenic status in mice.

INTRODUCTION

Transgenesis is the use of genetic engineering technology to introduce foreign gene into the genetic make-up of an organism so that all the cells, including the germ line, are genetically altered. Genetically engineered mice have become useful for studies of gene regulation and development, and some human diseases. A critical step in establishing transgenic mouse line is the identification of animals harboring the transgene. The mice may be initially identified by polymerase chain reaction (PCR) or by restriction enzyme digestion and Southern blot analysis. Although, gene integration analysis by PCR is subject to artifacts due to high sensitivity, the PCR is the first of choice for initial analysis, because it is fast, easy and can be completed within 1 day, while the others are laborious, time-consuming and costly, and may take 3-7 days to complete. On the other hand, identification of transgenic pups in an established and well-characterized line, screening by PCR alone may be sufficient. PCR technique has been applied to transgenic screening using DNA from variety of tissues including tail biopsy (Lin et al. 1989, Hanley et al. 1991), ear clippings (Chen et al. 1990), and whole blood (Chen et al. 1990, Skalnik et al. 1990). Recently, Irwin et al reported that saliva that contains oral epithelial cells and lymphocytes was used as a source of DNA for PCR (Irwi et al. 1996). The saliva was applied to sample collection paper and then purified using a solid phase DNA purification system. The paper was added directly to PCR cocktail for the first round of amplification; and a small amount of product from this amplification was removed and added to PCR cocktail for second round amplification.

In the present study, we describe a further simplified non-surgical procedure as an alternative to surgically obtaining samples serving as a source of DNA for screening transgenic mice carrying protozoan gene encoding a major surface antigen, *SAG*-1, of *Toxoplasma gondii* (Seng et al. 1996).

MATERIALS AND METHODS

Mice

SAG-1 transgenic mice, line 1-54 and 3-57 harboring a single and several copies of the *SAG*-1 transgene, respectively, were generated by microinjecting the cloned *SAG*-1 gene (Maki et al. 1996) into one of pronuclei of embryos of mice (Seng et al. 1996; 1999). All of these mice were maintained in conventional facilities at 21-22°C and with a relative humidity of 50-60%. The automatized dark/light cycles was of 12 hr. Five mice were kept in a cage (30×20 cm), and autoclaved tap water and food pellets (CLEA Japan, Inc.) were provided. The offspring at age of 3-4 weeks were subject to the transgene analysis.

Oral cavity wash

Mice, 3-4 weeks of age, were manually restrained in a vertical position by grasping the loose skin around the neck with the thumb and forefinger. An aerosol resistant tip attached to an adjustable 100 μl-pipetor was loaded with 35 μl of sterile water, and was pipetted back and forth into the oral cavity a few times, and 30 μl of the oral washes containing approx. 1×10^3 of different types of cells (Figs. 1A and 1B) obtained was placed to a tube. Boiled for 5 min, the sample was chilled on ice, centrifuged at 3,000 rpm for 5 min, and then 5 μl supernatant was transferred to a tube containing 45 μl of the first PCR amplification mixture, and the thermal cycling was performed. One μl of the first amplified product was added to the second amplification mixture.

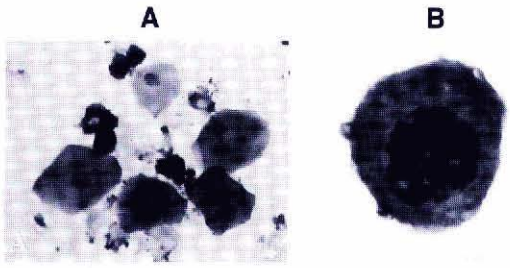

Figure 1. Different types of cells observed in oral washes were used as a source of DNA for PCR. Oral washes were stained with Giemsa's solution. Photos were taken by a light microscope with 10×10 (A) and 10×100 (B) magnifications.

Tissue biopsy

Distal 1 cm of tail from 4 weeks old mice under anesthetization with diethyl ether was excised with a sterile scissors. Tail biopsies were placed in 1.5 ml microcentrifuge tubes and digested for 4 hrs to overnight at 55°C with 700 μl of digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, and 1 % sodium dodecyl sulfate) and 35 μl of proteinase K (10 mg/ml). The digests were extracted with 700 μl of TE-saturated phenol, 700 μl of phenol/chloroform, and 700 μl of chloroform. DNA was precipitated from the aqueous phase with 2.5 volumes of absolute ethanol and 1/10 volumes of 3M sodium acetate. Precipitates were washed with 70% ethanol and air-dried. The pellets were then redissolved in 100 μl of sterile water. Amount of the DNA was quantitated by OD 260.

Polymerase chain reaction (PCR)

For the first amplification reaction, DNA isolated from epithelial cells and lymphocytes was amplified using an outer primer set consisting of 20 and 20 base pairs corresponding to nucleotides 5'CACACCGACGGAGAACCACT-3' and 5'TATCACTCGAAGCGTTACCC-3', respectively. A fragment of 555 bp was

amplified. For the nested amplification reaction, one μl of the first amplified product was amplified using an inner primer set consisting of 20 and 21 base pairs corresponding to nucleotides 5'-CACTCTCAAGTGCCCTAAAA-3' and 5'-GAACTTTGACTCCATCTTTCC-3', respectively. A 416 bp of the nested product was obtained. The first and second PCR were performed, in 50 μl of total reaction mixture containing a final concentration of 25 mM $MgCl_2$, 2 mM of each dNTP, 1.6 units of Taq polymerase (Perkin Elmer) and 50 pmol of each oligonucleotide primer, in an automated thermocycler (GeneAmp PCR system 2400, Perkin Elmer). The condition for temperature cycling were : 94°C for 5 min followed by 35 cycles of 94°C for 20 sec, 53°C for 30 sec, and 72°C for 1 min. Cycling was followed by a final extension step at 72°C for 10 min, and the reactions were held at 4°C. The genomic DNA purified from tail biopsy was also amplified with the same procedures using nested primer set. The amplified products were then electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination.

RESULTS AND DISCUSSION

The results of the *SAG*-1 transgene analysis by PCR are shown in Fig. 2. Using 250 ng of DNA isolated from tail tissue of transgenic mice carrying *SAG*-1 gene, the prominent fragments of 555 bp (Fig. 2A) and 416 bp (Fig. 2C) were successfully amplified with an outer and an inner pairs of oligonucleotide primers, respectively. A fragment of 555 bp amplified by the outer pair of primers using 5 μl of oral wash of the transgenic mice (Fig. 2B) was not able to be visualized on electrophoresed 1.5% agarose gel, however, the second step PCR of the first amplified product using the inner pair of primers amplified 416 bp as predicted size (Fig. 2D). In all cases, non of amplification of DNA and oral wash from normal mice generated any products.

PCR method has been known to be a powerful technique and based upon the highly specific and efficient enzymatic amplification of target DNA sequences of an entire genome, and widely used as a tool for diagnosing human and animal diseases, and characterizing transgene in animals, as well. Obtaining DNA, serving as a template by destroying nucleus membrane of cells by collagenase-digestion (Saiki et al. 1988) and boiling (Skalnik et al. 1990) is a critical step toward application of PCR technique. The crude DNA then is utilized directly (Hanley et al. 1991, Skalnik et al. 1990) or purified by phenol/chloroform extraction (Saiki et al. 1988) or collection paper (Irwin et al. 1996) for PCR.

PCR analysis of DNA obtained from trace amounts of human saliva has been successfully used for several years by forensic laboratories in DNA fingerprinting application (Hopkin et al. 1994, Walsh et al. 1991). PCR was

employed to detect the integration of HTLV-1 proviral DNA into lymphocytes in the saliva of a male ATLL patient (Taniguchi et al. 1993) and to identify transgenic status mice (Irwin et al. 1996). As described, all these techniques involved in the extraction of DNA from saliva for PCR analysis to detect pathogens of diseases, confirm transgenic status or were used for routine genetic monitoring.

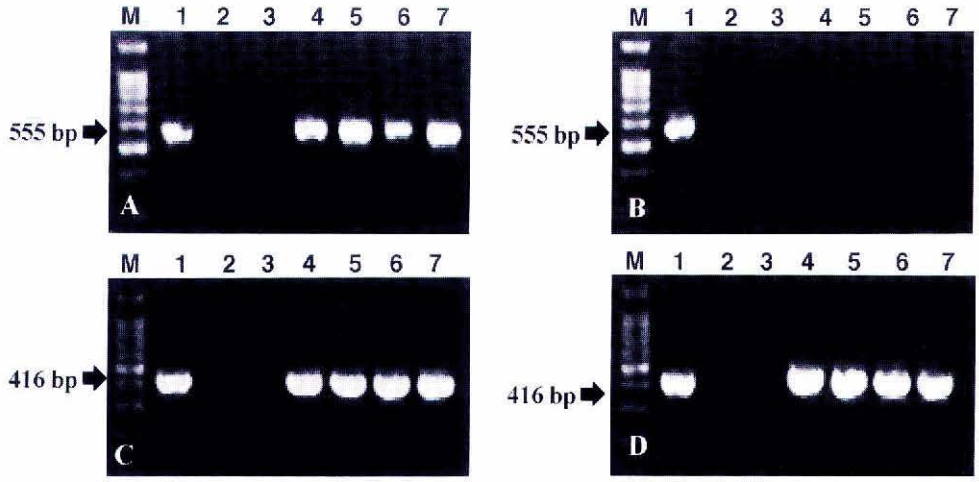

Figure 2. Identification of p30 transgene by PCR. First-step PCR of 250 ng of genomic DNA isolated from tail tissue (A) and of 5 μl of oral wash (B) using a pair of outer primers S-523 and AS-1078. A 555 bp product was detected only in (A), but not in (B). Second-step PCR of DNA obtained from tail tissue (C) and oral wash (D) using a pair of inner primers S-544 and AS-960 amplified a 416 bp product. Lane M, size marker; lane 1, DNA used to create the p30 transgenic mice as positive control; lane 2 and 3, normal genomic DNA as negative control; lanes 4 and 5, 1-54 transgenic line; 6 and 7, 3-57 transgenic line.

The present study offered a non-invasive procedure for identifying *SAG*-1 transgenic mice by PCR analysis of unpurified crude material of oral wash. It was demonstrated that DNA isolated from tail tissue amplified with the outer (Fig. 2A) and inner (Fig. 2B) pairs of primers generated the fragments, as predicted. On the contrary, the amplification of 5 μl of oral wash using the outer (Fig. 2C) or inner pair of primers solely did not generate any visible fragments (data not shown). However, when two-step amplification was performed, the expected fragment of 416 bp (Fig. 2D) was generated due to significant increase in sensitivity. The results obtained from two-step PCR of DNA extracted from oral wash cells consistently matched those obtained using DNA isolated from tail biopsy. The use of the inner primer PCR was found to be essential to generate sufficient amplification product from oral washes of mice aged 3-4 weeks. In some cases,

we observed that oral wash from mice of age of 8-10 weeks amplified with first step generated a specific visible band (unpublished data). It is considered, however, that the second step may be necessary, because the positive reaction may depend upon the ages of mice and copy number of DNA segment to be amplified. Compared to PCR utilizing tail DNA, the two-step PCR using oral wash as a source of DNA is more preferable, because it is simpler, much less traumatic to the mice. Furthermore, surgical intervention and local or general anesthesia are not required, and multiple samplings can be repeated as often as possible. This procedure is rapid and straightforward, involves boiling and cooling of saliva , and adding it to amplification mixture with specific sets of primers. This technique is a less-time consuming and more simplified one, as compared to those which involve the use of sample collection paper and solid phase DNA purification system (Irwin et al. 1996), cell digestion by organic solvents and phenol/ chloroform extraction and ethanol precipitation (Taniguchi et al. 1993).

Based on these advantages, we have found this non-surgically obtaining samples for analyzing the *SAG*-1 gene integration by two step PCR is consistent with our desire to safeguard the welfare of mice used in our laboratory.

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