Localized in Situ Amplification (LISA): A Novel Approach to in Situ PCR

Gregory J. Tsongalis,1,2 Althea H. McPhail,1,2 R. Daniel Lodge-Rigal,1 John F. Chapman,1 and Lawrence M. Silverman1

Amplification of specific gene target sequences has become a routine molecular procedure in a variety of laboratories. When coupled with either a direct or indirect method of detecting amplified product, in situ amplification offers an extremely powerful investigative tool. We describe a protocol for a localized in situ amplification (LISA) reaction that includes tissue-culture cloning rings and allows for the amplification of gene target sequences in specific regions of paraffin-embedded tissue sections. Digoxigenin-11-dUTP was added to the amplification reaction and thus incorporated into the amplified products, providing a mechanism by which direct nonisotopic detection could be performed. To demonstrate the approach, LISA was performed on known positive Pneumocystis carinii rat lung tissues, with primers specific for the P. carinii rRNA gene sequence.

Indexing Terms: Pneumocystis carinii/DNA hybridization/immuno-histochemistry

The polymerase chain reaction (PCR) is widely used for a variety of specific applications.4 PCR plays a crucial role in the determination of carrier status in numerous genetic disorders, such as cystic fibrosis, muscular dystrophy, hemophilia, and fragile X syndrome, whereby specific genomic DNA target sequences are amplified for mutation analysis by one of an increasing number of efficient molecular techniques (1). For both research and diagnostic purposes, PCR has been useful for amplifying DNA sequences of interest from various tissues, including archived formalin-fixed paraffin-embedded tissues (2).

An extremely powerful technique results from the combination of PCR and in situ hybridization (ISH). The coupling of these two techniques combines one methodology with extreme sensitivity and specificity (PCR) with the ability to determine cellular localization (ISH) (3–7). Single-copy DNA target sequences that were previously too scarce to detect by ISH because of limited sensitivity can now be amplified so that they are more easily detected. Several investigators have described protocols for using these two techniques to detect viral genomic DNA in tissue samples (3–10) and human VH3 immunoglobulin genes in cytocentrifuged cell preparations (10).

We describe a protocol by which the rRNA gene sequence of Pneumocystis carinii is amplified by using a direct in situ PCR protocol and is detected by a nonradioactive immunohistochemical detection system with formalin-fixed paraffin-embedded tissue sections. Furthermore, this approach allows amplification of different targets on (different regions of) the same tissue section by using tissue-culture cloning rings as vessels for each reaction. This localized in situ amplification (LISA) thus has advantages over previous protocols that included coverslips.

Materials and Methods

Tube Amplification

We determined the optimal conditions for our primer set with an Ericomp TwinBlock system. The primers were specific for a 378-base-pair (bp) rRNA sequence from P. carinii as described by Lipschik et al. (11). We isolated control DNA (purified P. carinii DNA) from rat lung tissue that was heavily infected with P. carinii. Negative control amplification reactions consisted of reactions without target DNA sequence and reactions with isolated human genomic DNA or noninfected rat lung DNA as a source of target sequence. The amplification reaction was performed in a total reaction volume of 100 μL and consisted of 300 ng of both the forward and reverse primer (CCAGATTAGCTTTGCTGATCGGG and ACTTCCAGTAATAGGCTTATCG, respectively) and 2.5 U of Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT) in a reaction solution containing, per liter, 200 mmol of each deoxynucleotide triphosphate, 1.5 mmol of MgCl₂, 67 mmol of Tris-HCl (pH 8.8), 10 mmol of 2-mercaptoethanol, 16.6 mmol of ammonium sulfate, and 6.7 μmol of EDTA. Control DNA (~500 ng) was denatured at 94°C for 6 min prior to amplification. Tube amplification was accomplished with 35 cycles consisting of 2 min of annealing at 55°C, 3 min of extension at 72°C, and 1 min of denaturation at 94°C. The final cycle included a 10-min extension step at 72°C. An aliquot of the amplified product was electrophoresed on a 2% agarose gel and detected by ethidium bromide staining with subsequent ultraviolet illumination. We determined the effects of digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) on PCR amplification by including 10 μmol/L labeled nucleotide in the above reaction. To confirm the incorporation of this nucleotide into the amplified product, the DNA was transferred to Immobilon-N (Millipore Corp.,

---

1 Department of Hospital Laboratories and Pathology, University of North Carolina, Chapel Hill, NC 27514.
2 Current address: Gwinnett Medical Center, Department of Pathology, Lawrenceville, GA.
3 Address correspondence to this author at: Division of Clinical Chemistry and Molecular Pathology, Department of Hospital Laboratories and Pathology, 1071 PST UNC Hospitals, Chapel Hill, NC 27514. Fax 919-966-4026.
4 Nonstandard abbreviations: PCR, polymerase chain reaction; ISH, in situ hybridization; LISA, localized in situ amplification; and bp, base pair.
5 Received June 18, 1993; accepted December 20, 1993.
Tissue Samples

We obtained formalin-fixed paraffin-embedded lung tissue sections from *P. carinii*-positive rat lungs. Sections from the same tissue block were stained with Gomori methenamine silver nitrate to verify the presence of *P. carinii* organisms. We placed 6-μm sections of tissue on organosilane-coated glass slides as per routine histological protocol. Tissue sections were deparaffinized in xylene for 5 min and rehydrated in graded alcohols and distilled water. The tissue was treated with proteinase K (1 mg/L) for 10 min in 10 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA at 37°C, and then dehydrated in graded alcohols.

LISA

Tissue sections prepared as described were placed on a slide thermal cycler (TempCycler II; COY Corp., Grass Lake, MI) with heating blocks designed to accommodate glass microscope slides. Slides were heated for 1 min at 94°C to inactivate the proteinase before addition of stainless steel or glass (Bellco Glass Inc., Vineland, NJ) tissue-culture cloning rings [0.8 cm (o.d.) × 0.8 cm (i.d.) × 1.0 cm] which were selectively placed on top of the tissue section (Fig. 1). We then used clear nail polish to seal the bottom of the ring to the tissue section, forming an amplification vessel for the LISA reaction. The amplification reaction consisted of a total volume of 25 μL per cloning ring, which consisted of 75 ng of both the forward and reverse primer (CCAGATTAGCTTTGCTGATCGCGGG and ACTTTCCAGTAATAGGCTTATCG, respectively) and 0.6 U of Taq polymerase in a reaction solution containing, per liter: 200 nmol of each deoxynucleotide triphosphate, 1.5 mmol of MgCl₂, 67 mmol of Tris-HCl (pH 8.8), 10 mmol of 2-mercaptoethanol, 16.6 mmol of ammonium sulfate, 6.7 μmol of EDTA, and 10 μmol of digoxigenin-11-dUTP. We added the reaction mixture to the center of the cloning ring and used mineral oil to prevent evaporation. We then placed the slides onto the slide thermal cycler. DNA was denatured in situ at 94°C for 2 min prior to amplification. LISA was accomplished by using 20 cycles, each consisting of a 1-min primer annealing step (55°C), a 1.5-min extension step (72°C), and a 1-min denaturation step (94°C). We altered these amplification cycles from those for tube amplification to preserve optimal tissue morphology. For negative controls, LISA was performed as described with the omission of primers or Taq polymerase from the amplification reaction, or with primers for target sequences known not to be present (i.e., cytomegalovirus primers).

Detection of Amplified Product

Amplified products containing incorporated digoxigenin-11-dUTP were detected with a modification of the protocol supplied with the Genius 1 kit (Boehringer Mannheim). Briefly, each reaction mixture was removed and the wells were rinsed with xylene. All solutions and reactions were at room temperature. The cloning rings were removed from the glass slides by soaking in acetone for 1–2 min. The slides were then washed three times with -500 μL of buffer 1 (100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) and then incubated for 30 min with 500 μL of buffer 2 (5 mL/L blocking reagent in buffer 1) in a humidified chamber. Subsequently, the slides were rinsed with 500 μL of buffer 1 and incubated for 1 h with a 1:100 dilution of antibody (alkaline phosphatase-conjugated anti-digoxigenin; Boehringer Mannheim) in a humidified chamber. Excess antibody was removed by three washes in buffer 3 (100 mmol/L Tris-HCl, 100 mmol/L NaCl, 50 mmol/L MgCl₂, pH 9.5) before the addition of the chromogen (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate). The detection reaction was monitored for optimal staining (~10–25 min) and stopped by rinsing three times in buffer 4 (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0). The tissues were then dehydrated in a series of graded alcohols and stained with eosin before coverslipping for microscopy. Negative control slides were also stained with eosin before coverslipping for microscopy.

Results

Tube Amplification

To determine optimal primer-specific amplification conditions, we tube-amplified the *P. carinii* rRNA sequence from purified *P. carinii* DNA. Agarose gel electrophoresis of the amplified product revealed a 378-bp fragment, which was consistent with the known sequence (Fig. 2A, lanes 5–7). Control reactions without target sequence DNA or with isolated human or rat genomic DNA as a source of target sequence resulted in no detectable amplified product. To determine whether the digoxigenin-11-dUTP would inhibit or alter our PCR product, we carried out tube amplification with the addition of 1 μmol of labeled nucleotide per 100 μL of reaction mixture. No detectable alterations in the amplified product were observed on gel electrophoresis (Fig. 2A, lanes 1–3). To demonstrate that the labeled nucleotide was indeed incorporated into the amplified product, we transferred the DNA from this gel to an

Fig. 1. The TempCycler II with tissue sections.
Stainless steel, glass, or acrylic cloning rings may be placed on top of the tissue section and sealed with clear nail polish to form an amplification chamber.
Fig. 2. (A) Gel electrophoresis of the 378-bp tube-amplified P. carinii rRNA sequence from control DNA; (B) immunochemical detection of digoxigenin-11-dUTP incorporated into amplified product: The gel from (A) was transferred to a nylon membrane, and labeled nucleotide was detected with anti-digoxigenin antibody. M, molecular weight markers; lanes 1–4, Mg titration of 1.5 mmol, 1.0 mmol, 0.5 mmol, and negative control (blank), respectively, with digoxigenin-11-dUTP; lanes 5–8, same as above except without digoxigenin-11-dUTP.

Immobilon-N nylon membrane and detected the digoxigenin-11-dUTP-labeled nucleotide with a digoxigenin-specific antibody (Fig. 2B, lanes 1–3). No antibody detection was observed in those samples that did not include the digoxigenin-11-dUTP in the PCR reaction (Fig. 2B, lanes 5 and 6).

LISA

LISA was performed on sections of rat lung known to be infected with P. carinii. Amplified P. carinii rRNA product localized to clustered regions of P. carinii organisms within the alveolar spaces (Fig. 3A). The area of amplification corresponded to intraalveolar P. carinii organisms visualized by silver stain (Fig. 3B). However, unlike the silver stain that detects the outer wall of the organism, LISA detects only the amplified product within the organism. In a simultaneously run control amplification reaction, with the primers omitted from the reaction mixture, no amplified product was detected (Fig. 3C). No nonspecific detection was evident in areas of tissue outside the region of the ring when the entire tissue section was exposed to antibody and chromogen.

Discussion

The coupling of in situ amplification with in situ detection protocols provides a sensitive method for detecting abnormalities at the molecular level and correlating them with microscopic pathology. Initial attempts at in situ PCR followed by ISH by Haase et al. (3) were limited by the prerequisite of using cell suspensions.

Although early in situ protocols were adopted for paraffin-embedded tissues, there have been technical problems with these procedures, such as destruction of morphology, optimization of preparative and amplification conditions for paraffin-embedded tissue sections, evaporation of reagents, and loss of localization of amplified product (3, 8, 9). The results we obtained by using a LISA reaction indicate that this technique may circumvent many of the previously encountered technical problems with in situ amplification.
LISA amplified a single gene target sequence in the alveolar spaces of the lung tissue within clusters of *P. carinii*, as demonstrated by silver stain. LISA does not detect the cell wall or other biochemical or structural components of the organism as do special stains, but instead detects amplified nucleic acid sequences. The amplified product detected within clusters of the organism is evident in the system described here.

LISA may be applied to a variety of tissue preparations, including paraffin-embedded tissues, touch preparations of tissue cells, and cytological specimens, thus serving many interests. We have found that the morphology of specimens remains good and that the addition or removal of the cloning rings does not alter this. We, as well as others, have noted that mounting tissue sections or cytocentrifuged preparations on organosilane-coated glass slides results in better adherence, thereby preventing loss of sample during the procedure (4, 7). The thermal cyclers contain a thermal block designed for standard histological glass slides, thus promoting an even distribution of heat throughout the slide. Decreased cycle and ramping times are promoted by the direct contact of the slide with the heating block. This system can also accommodate up to four slides per amplification, unlike the conventional heating blocks of more common thermal cyclers.

By using tissue-culture cloning rings, one can localize the amplification reaction to the area of interest on the tissue section. Because each ring provides a separate chamber for amplification, one can easily include several amplification reactions on the same tissue section. Thus, we can simultaneously perform several control reactions. This is unlike other methods of in situ amplification, in which a coverslip is used to form the amplification chamber and only one control reaction can be performed per section. The use of these rings to form an external chamber that is placed on top of the tissue section also allows one to optimize amplification conditions on genomic DNA in a more traditional fashion, with the exception of decreased cycle times. Reagent concentrations that optimize the tube amplification reaction can then be directly applied to LISA.

A major concern of in situ molecular procedures is the evaporation of reagents and the destruction of the tissue with manipulation of the coverslip. Although clear nail polish has been used successfully in decreasing the evaporation of the reaction mixture, it makes manipulation of the coverslip increasingly difficult. In LISA the cloning ring is easily positioned onto the tissue section and requires minimal nail polish to form a seal between the ring and glass slide. Removal of the ring is not required for the remainder of the amplification procedure; it is only removed during the detection process so that tissue outside the ring can function as a control for nonspecific binding of antibody or chromogen. Evaporation of reagents is eliminated by layering mineral oil over the reaction as in routine PCR amplification or by adding an AmpliWax bead (Perkin-Elmer/Cetus).

Nonspecific amplification is a possibility in any type of amplification protocol. Such is not the case when detecting the directly incorporated labeled nucleotide. However, one must be cautious of detecting labeled nucleotide that has nonspecifically incorporated into cellular genomic DNA (i.e., DNA gaps and breaks, etc.). We chose *P. carinii* as a preferred model to develop these protocols because it is an extracellular organism that forms clusters in the alveolar spaces of the lung. Thus, any detection in the nuclei of pulmonary epithelial or other cells can be easily recognized as background/nonspecific incorporation. This provides an excellent model for development of in situ amplification reactions. It is not intended that LISA replace traditional methods or methods such as ISH (12) that provide good sensitivity for detection of *P. carinii*.

We are currently further examining and optimizing both amplification and detection reactions for use with several tissue types and attempting to eliminate the possibility of nonspecific incorporation and (or) amplification. As shown in Fig. 3A, amplified products are clearly detectable. We are also evaluating several other methods of direct detection of amplified products that may prove to be more sensitive.

We thank C. Dykstra and S. Jones for providing control *P. carinii* DNA and tissues, D. G. Kaufman for the tissue-culture cloning rings, and C. R. Bagnell for photographic assistance.

References