

Reverse Transcriptase PCR Amplification of *Rickettsia typhi* from Infected Mammalian Cells and Insect Vectors

JAMES A. HIGGINS,† SUZANA RADULOVIC, BRUCE H. NODEN,
JILL M. TROYER, AND ABDU F. AZAD*

Department of Microbiology and Immunology, University of Maryland
Medical School, Baltimore, Maryland 21201

Received 14 October 1997/Returned for modification 15 January 1998/Accepted 19 March 1998

We developed a reverse transcriptase PCR assay to detect expression of 120- and 17-kDa antigen genes in *Rickettsia typhi*. Infected Vero cell and flea RNAs were reverse transcribed by using random hexamers. The cDNA was amplified by using high concentrations of primer and template in an inexpensive, nonradioactive assay.

Bacteria of the genera *Rickettsia* and *Orientia* are responsible for causing a number of serious diseases, such as Rocky Mountain spotted fever, endemic and epidemic typhus, scrub typhus, and African tick fever. All have complex epidemiologic cycles involving both vertebrate and invertebrate (vector) hosts. Rickettsiae are obligate intracellular parasites, reproducing either in the cytoplasm or the nucleus of the host cell. Extraction of pathogenic rickettsiae from host cells is a laborious procedure that requires appropriate biosafety level 3 facilities.

Rickettsiae are thought to be metabolically inert prior to contact with the host cell, at which time they are internalized by phagocytosis. Once within the host cell, doubling time is slow (approximately 10 h for *Rickettsia prowazekii*) (8). Half-lives for various housekeeping gene mRNAs (e.g., those for citrate synthase and ATP/ADP translocase) range from 8.4 to 20.5 min, as determined by RNase protection assays (3, 4, 9). Because rickettsial RNase protection assays (RPA) require large amounts of infected-cell RNA (80 to 100 µg for citrate synthase gene detection and 8 to 10 µg for 16S rRNA) (3) and involve radiolabelling, we were interested in utilizing reverse transcriptase PCR (RT-PCR) to examine infected Vero cells, and infected vector fleas (*Ctenocephalides felis*), for the presence of antigenically important *R. typhi* transcripts.

As targets, we chose the 120-kDa surface protein antigen (SPA) (recombinant *Omp B*), which is thought to mediate entry into the host cell, and the 17-kDa surface protein, which is recognized by convalescent-phase sera and can mediate, along with SPA, protective immunity in animal models (5). To investigate expression of these important antigen genes, we used primers for genomic-DNA (gDNA) amplification of a 434-bp segment of the *R. typhi* 17-kDa antigen gene (1) in an RT-PCR. For the 120-kDa SPA, we used the published sequence of the gene encoding this antigen (6) to design forward primer 311, 5' CTT GGA AGT ATT AAC GGT 3', and reverse primer 312, 5' ACC TGT ATC TGT AGC AAT 3', capable of amplifying a segment (nucleotides 3228 to 4061) coding for dominant epitope regions (5). To confirm the accuracy of the primer sequences, the gDNA amplicon from the *R. typhi* Wilmington strain was cloned by using the TA Cloning

Kit (Invitrogen, Carlsbad, Calif.) and cycle-sequenced (Perkin-Elmer/Applied Biosystems, Foster City, Calif.) and found to have 100% homology with the published sequence by NCBI BLAST comparison.

For RT-PCR of *R. typhi* from mammalian cells, 9×10^5 Vero cells (ATCC C1008) were inoculated with 2×10^6 PFU of *R. typhi* Wilmington strain and monitored by Gimenez staining until the infection level reached 90%, about 11 days post-infection (p.i.). Using a concurrently infected 6-well plate of Vero cells, examined for plaque formation at 11 days p.i., we estimate that at this time there were 6×10^6 PFU present in the 150-cm² flask. Cells were detached from the 150-cm² flask with a cell scraper, transferred to a 50-cm² tube, and centrifuged at 1,500 rpm for 10 min. Supernatant was discarded, and the cells were treated with 1 ml of Tri-Zol reagent (all reagents used were from Life Technologies, Gaithersburg, Md.). Subsequent RNA extraction was done by following the manufacturer's protocol. The RNA pellet was dissolved in DEPC water, quantified spectrophotometrically, and stored at -20°C .

For extraction of *R. typhi* mRNA from infected cat fleas, fleas were fed at day 0 with 10^6 PFU of *R. typhi* Wilmington mixed with defibrinated human blood; control fleas were fed uninfected blood. Every 3 days thereafter, up to day 9 p.i., fleas received a maintenance meal of uninfected blood. Fleas were harvested on day 9 p.i.; based on our experience with immunofluorescent-antibody assays performed with flea guts, midgut infection is extensive at this time. The best yield of RNA was obtained by using the protocol of Noriega and Wells (7) (as opposed to phenol-based methods), with approximately 100 to 150 fleas that had been anesthetized by a 15-min exposure at -20°C . The RNA pellet was dissolved in DEPC water, quantified spectrophotometrically, and stored at -20°C . Both Vero cell and flea-derived RNAs were examined, by using formaldehyde agarose gel electrophoresis, to confirm that RNA had not become degraded during the extraction procedures. The yield of Vero cell RNA was in excess of 75 µg for a 150-cm² flask, and for 100 to 150 fleas the yield was approximately 30 µg; purity, as measured by A_{260}/A_{280} ratio (1.8), was satisfactory.

Prior to RT-PCR, it was essential to treat all RNA samples with DNase, by using 1 U of DNase/µg of RNA; otherwise, no-RT controls consistently yielded amplicons (we monitored the efficacy of DNase by subjecting 1 µg of 120-kDa SPA PCR product to treatment and examining the reaction products on an agarose gel to confirm degradation). Following DNase treatment, RNA was precipitated with 100% isopropanol and

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Maryland Medical School, 655 W. Baltimore St., Baltimore, MD 21201. Phone: (410) 706-3335. Fax: (410) 706-0282. E-mail: aazad@umaryland.edu.

† Present address: USDA Agricultural Research Service, LPSI, IDRL, Beltsville, MD 20705.

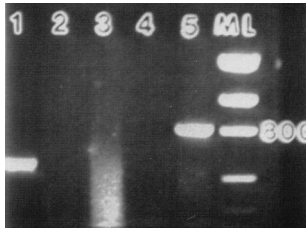


FIG. 1. RT-PCR of *R. typhi* 120-kDa antigen gene fragment from infected Vero cells. Lane 1, RT-PCR kit control (523 bp); lane 2, PCR contamination control; lane 3, no-RT control, infected Vero cells; lane 4, uninfected Vero cells; lane 5, infected Vero cells (833 bp). ML, low-DNA-mass ladder; the 800-bp rung is indicated.

the pellet was washed with 70% ethanol and resuspended in DEPC water.

For reverse transcription, the Superscript RT protocol for random hexamer-mediated cDNA synthesis was used. Briefly, 5 μ g (5 μ l) of total RNA template (Vero cell or fleas) was added to 100 ng of random hexamers and 7 μ l of DEPC water and the mixture was heated at 70°C for 10 min. Next, 4 μ l of First Strand Buffer (25 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl₂), 2 μ l of 0.1 M dithiothreitol, and 1 μ l of 10 mM deoxynucleoside triphosphate (dNTP) mix were added and the mixture was incubated at 42°C for 2 min, followed by the addition of 200 U of Superscript II RT. Following incubation at 42°C for 50 min, the reaction was inactivated by heating at 70°C for 15 min and then the mixture was subjected to RNase H treatment (2 U for 20 min at 37°C). The PCRs were performed in a 50- μ l volume, with 6 μ l of cDNA as the template, 8 μ M each primer, 1 \times PCR buffer (20 mM Tris-HCl, pH 8.3; 50 mM KCl), 1.5 mM MgCl₂, 200 μ M dNTP mix, and 1 U of *Taq* polymerase. Following denaturation of the initial cDNA-RNA hybrid (95°C for 4 min), an extra 1 U of *Taq* polymerase was added to each sample to improve the yield of amplicons. Amplification conditions were 95°C for 20 s, 55°C for 30 s, and 60°C for 1 min 30 s over 35 cycles. An aliquot of 20 μ l of each PCR product was electrophoresed on a 1% agarose gel and stained with ethidium bromide (0.3 μ g/ml).

Using this protocol, we have been able to amplify *R. typhi* cDNA segments for the 17-kDa surface protein and 120-kDa SPA genes from both infected Vero cells and vector fleas (Fig. 1 and 2). The band for the 120-kDa amplicon from the flea

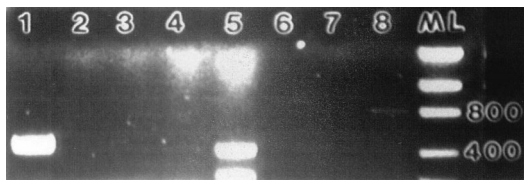


FIG. 2. RT-PCR of *R. typhi* 17-kDa surface protein and 120-kDa antigen gene fragments from infected fleas, *C. felis*. Lane 1, RT-PCR kit control (523 bp); lane 2, PCR contamination control; lane 3, no-RT control, 17 kDa, infected fleas; lane 4, uninfected fleas; lane 5, infected fleas, 17 kDa (434/230 bp); lane 6, no-RT control, 120 kDa, infected fleas; lane 7, uninfected fleas; lane 8, infected fleas, 120 kDa (833 bp). ML, low-DNA-mass ladder; the 400- and 800-bp rungs are indicated.

sample was fainter than that of the 17-kDa amplicon, a phenomenon we have observed in gDNA PCR, but is still legible. When we observed that normal protocols (e.g., 2 μ l of cDNA as template, 1 μ M each primer) did not yield amplicons, we adapted reaction conditions from RT-PCR for another AT-rich genome, that of *Plasmodium* spp. (2). For these organisms, conventional protocols may have to be skewed to favor the amplification of low-abundance, parasite cDNAs.

We estimated the quantity of *R. typhi* RNA recovered from the Vero cells. *R. prowazekii*, with reproduction in Vero cells similar to that of *R. typhi*, has been calculated to contain 5.6 fg of stable RNA per cell (8). If we assume this figure is applicable to *R. typhi*, then the approximately 6×10^6 PFU present in the Vero cell flask at day 11 p.i. contained 3.3×10^7 fg (33 μ g) of RNA. Since the total yield of the flask was about 75 μ g, this value seems high (constituting 44% of the total extracted RNA). However, based on light microscopic viewing of Gimenez-stained cells, we estimate that there are 700 to 800 rickettsiae per infected Vero cell at day 11 p.i. Since the per-rickettsia RNA estimate we used for our calculations is derived from purified organisms and not actively replicating ones (8), it is reasonable to assume our yield of rickettsial RNA may be an underestimate. Obviously, without the ability to differentiate between rickettsial and host cell mRNAs and without information on the efficiency of reverse transcription of rickettsial message compared to that of the eukaryotic host cell, we are unable to quantitate the amount of rickettsial message that our RT-PCR assay is capable of detecting.

In conclusion, we have demonstrated a method for RT-PCR of antigenically important *R. typhi* transcripts from both mammalian and insect host cells. This procedure is quick, nonradioactive, and does not require large amounts of infected material. We have since used the procedure to detect other gene products in *R. typhi* (such as citrate synthase), indicating that the assay is capable of detecting a range of target genes.

We thank Rex Thomas of Heska Corp. for providing fleas.

This work was supported by National Institutes of Health grant R37A-I-17828.

REFERENCES

- Azad, A. F., J. B. Sacci, Jr., W. M. Nelson, G. A. Dasch, E. T. Schmidtman, and M. Carl. 1992. Genetic characterization and transovarial transmission of a typhus-like rickettsia found in cat fleas. *Proc. Natl. Acad. Sci. USA* **89**:43–46.
- Briones, M. R. S., M. Tsuji, and V. Nussenzeig. 1996. The large differences in infectivity for mice of *Plasmodium berghei* and *Plasmodium yoelli* sporozoites cannot be correlated with their ability to enter hepatocytes. *Mol. Biochem. Parasitol.* **77**:7–17.
- Cai, J., and H. H. Winkler. 1993. Identification of *tlc* and *gltA* mRNAs and determination of in situ RNA half-life in *Rickettsia prowazekii*. *J. Bacteriol.* **175**:5725–5727.
- Cai, J., and H. H. Winkler. 1996. Transcriptional regulation in the obligate intracytoplasmic bacterium *Rickettsia prowazekii*. *J. Bacteriol.* **178**:5543–5545.
- Ching, W.-M., H. Wang, B. Jan, and G. A. Dasch. 1996. Identification and characterization of epitopes on the 120-kilodalton surface protein antigen of *Rickettsia prowazekii* with synthetic peptides. *Infect. Immun.* **64**:1413–1419.
- Hahn, M.-J., K.-K. Kim, I. Kim, and W.-H. Chang. 1993. Cloning and sequence analysis of the gene encoding the crystalline surface layer protein of *Rickettsia typhi*. *Gene* **133**:129–133.
- Noriega, F. G., and M. A. Wells. 1993. A comparison of three methods for isolating RNA from mosquitoes. *Insect Mol. Biol.* **2**:21–24.
- Pang, H., and H. H. Winkler. 1994. The concentration of stable RNA and ribosomes in *Rickettsia prowazekii*. *Mol. Microbiol.* **12**:115–120.
- Pang, H., and H. H. Winkler. 1996. Transcriptional analysis of the 16S rRNA gene in *Rickettsia prowazekii*. *J. Bacteriol.* **178**:1750–1755.