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

JAMES A. HIGGINS,‡ SUZANA RADULOCIVIC, 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. *Rickettsia* 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.

*Rickettsia* 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 120 kDa 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 mRNA, we published the 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 ACG 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 × 10⁵ Vero cells (ATCC C1008) were inoculated with 2 × 10⁶ 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 × 10⁵ 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 10⁶ 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 A₂₆₀/A₅₀₀ 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...
random hexamer-mediated cDNA synthesis was used. Briefly, DEPC water. the pellet was washed with 70% ethanol and resuspended in 1794 NOTES J. CLIN. MICROBIOL.

spike as indicated.

control, 120 kDa, infected fleas; lane 7, uninfected fleas; lane 8, infected fleas; lane 4, uninfected fleas; lane 5, infected fleas, 17 kDa (434/230 bp); lane 6, no-RT control; lane 3, no-RT control, infected Vero cells; lane 4, uninfected Vero cells; 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 μg (5 μl) of total RNA template (Vero cell or fleas) was added to 100 ng of random hexamers and 7 μl of DEPC water and the mixture was heated at 70°C for 10 min. Next, 4 μl of First Strand Buffer (25 mM Tris-HCl [pH 8.3], 75 mM KCl, 1 mM MgCl2), 10 μl of 0.05 M dithiothreitol, and 1 μl of 10 mM 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 PCR were performed in a 50-μl volume, with 6 μl of cDNA as the template, 8 μl each primer, 1× PCR buffer (20 mM Tris-HCl, pH 8.3; 50 mM KCl), 1.5 mM MgCl2, 200 μM 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 μl of each PCR product was electrophoresed on a 1% agarose gel and stained with ethidium bromide (0.3 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 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 μl of cDNA as template, 1 μM each primer) did not yield amplicons, we adapted reaction conditions from RT-PCR for another ART-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 × 106 PFU present in the Vero cell flask at day 11 p.i. contained 3.3 × 107 fg (35 μg) of RNA. Since the total yield of the flask was about 75 μg, this value seems high (constituting 44% of the total extracted RNA). However, based on light microscopic viewing of Gimennez-stained cells, we estimate that there are 700 to 800 rickettsiae per infected Vero cell at day 11 p.i. Since the per-rickettsial 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 rickettisal 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 quantify 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