Evaluation of an in vitro telomeric repeat amplification protocol assay to detect telomerase activity in canine urine

Angela L. McCleary-Wheeler, DVM; Laurel E. Williams, DVM; Paul R. Hess, DVM, PhD; Steven E. Suter, VMD, PhD

Objective—To evaluate the usefulness of a PCR-based telomeric repeat amplification protocol (TRAP) assay for detecting telomerase activity in cells from a canine transitional cell carcinoma (TCC) cell line and, ultimately, in the urine of dogs with TCC.

Animals—11 dogs with histologic or cytologic evidence of TCC, 10 dogs with benign lower urinary tract disease, and 9 healthy dogs.

Procedures—Telomerase activity was initially evaluated in cells from canine TCC (K9TCC) and telomerase-negative (WI-38) cell lines. Following assay optimization, telomerase stability was evaluated at various storage durations and temperatures. Urine samples were then obtained prospectively from study dogs.

Results—Telomerase activity was detected in the K9TCC cell line. The TRAP assay detected telomerase activity in as few as 10 K9TCC cells alone and as low as 2% of a total cell population in K9TCC and WI-38 mixing experiments. A loss of telomerase activity was detected with increasing urine storage durations at various temperatures. Telomerase activity was clearly detected in samples collected from 10 of 11 dogs with TCC, 2 of 10 dogs with benign lower urinary tract disease, and none of the 9 healthy dogs.

Conclusions and Clinical Relevance—The TRAP-based assay detected telomerase activity in the canine TCC cell line and revealed that the telomerase ribonucleoprotein complex was inherently unstable at various storage durations and conditions. Telomerase activity was also detectable in urine samples obtained from dogs with TCC, which suggested the TRAP assay may be useful in diagnosing TCC in dogs. (Am J Vet Res 2010;71:1468–1474)
a definitive diagnosis of TCC. Urinary bladder tumor antigen tests, which involve an antibody against canine bladder tumor–associated glycoprotein complexes, are > 80% sensitive. However, they are also only 41% specific, as dogs with benign lower urinary tract diseases that have proteinuria, glucosuria, and hematuria can have false-positive results.5

Telomeres, which are located at the ends of double-stranded DNA in vertebrates, are tandem repeats of the sequence TTAGGG that form a complex nucleoprotein cap on DNA7 that protects the chromosomes ends from undergoing recombination or fusion or being recognized by a cell as damaged DNA. Deoxyribonucleic acid polymerase is unable to replicate the 5’ ends of linear chromosomes, resulting in subsequent loss of 50 to 200 bp of telomeric DNA with each replication.7,8 Ongoing telomere shortening causes growth arrest and cell senescence.9–11 Many cells that proliferate indefinitely such as gastrointestinal crypt cells, hematopoietic stem cells, activated lymphocytes, and germline cells avoid telomere shortening through the use of telomerase.9,11 Telomerase is a ribonucleoprotein reverse transcriptase capable of synthesizing terminal TTAGGG repeats, thereby extending telomere length and compensating for telomeric attrition during replication. More than 85% of human cancer cells avoid cell senescence through an increase in telomerase expression, whereas telomerase activity is low or undetectable in healthy tissues.12 Telomerase activity is also detectable in various neoplasias in dogs.13–17

The TRAP assay is a sensitive test used to identify the presence of telomerase in various cells and tissues.18,19 This assay has been used to detect telomerase activity in 90% of tumor tissues in humans.20 When used to diagnose TCC in humans, the reported sensitivity and specificity for TRAP urine assays have been highly variable,21–39 whereas more recent, rigorous quantitative studies12,40–42 have revealed more consistent sensitivities and specificities of 90% and 88%, respectively, which compares favorably with findings for cytologic evaluation of urine (sensitivity, 48%; specificity, 94%).

The initial objectives of the study reported here were to evaluate telomerase activity in cells from a canine TCC cell line by use of a commercially available TRAP-based assay that has been validated in canine tissues13–17 and determine whether various cell concentrations and storage conditions affect the ability of this assay to detect telomerase activity. The final objective was to determine whether the TRAP assay could detect telomerase activity in urine obtained from dogs with TCC as an initial step in developing an in vitro telomerase assay that could be used as a sensitive, noninvasive test for urinary bladder TCC in dogs.

Materials and Methods

Dogs—To evaluate whether the TRAP assay could be used to detect telomerase activity in canine urine, urine (5 to 15 mL) was collected by midstream catch during natural voiding from 11 dogs with TCC evaluated by the Oncology Service at the North Carolina State University Veterinary Teaching Hospital. In 7 patients, a diagnosis of TCC was reached presumptively on the basis of ultrasonographic findings of a urinary bladder trigone or prostate mass and cytologic assessment of cells obtained from these masses via ultrasound-guided traumatic catheterization.43–45 In the remaining 4 dogs, a histologic diagnosis of TCC was reached after their tumors were incompletely resected. In 8 of 11 dogs, urine was collected during the time they were receiving systemic chemotherapy, a cyclooxygenase inhibitor, or both. In the remaining 3 dogs, urine was collected once during the initial abdominal ultrasonographic evaluation. These 3 dogs also presumptively received a diagnosis of TCC by means of ultrasonography and traumatic catheterization.

The same amount of urine was also collected by free catch from 10 dogs with benign LUTD (ie, disease of the urinary bladder) evaluated at the teaching hospital. The presence of an LUTD was established through urine dipstick analysis, complete urinalysis, and bacterial culture of urine. Urine was also collected by free catch from 9 clinically normal dogs. A dipstick test (n = 6) or complete urinalysis (3) had been performed to confirm no obvious LUTD existed.

To evaluate the effects of urine and storage conditions on telomerase detection in urine, urine was collected by midstream catch during natural voiding from one 3-year-old sexually intact male and one 3-year-old sexually intact female hound-type dog. Urine from all dogs was collected after institutional animal care and use committee approval and owner consent were obtained.

Cells—An adherent canine TCC cell line (K9TCC) and human lung fibroblasts that were free of telomerase (telomerase-negative WI-38 cells) were used to determine whether the TRAP assay could be used in dogs. Cells were grown in Dulbecco modified Eagle medium (K9TCC) or Eagle minimal essential medium (WI-38) plus 10% fetal bovine serum, 20mM L-glutamine, and 100 ng of antimicrobial/mL in a humidified, 5% CO2, 37°C atmosphere. A PCR-based Mycoplasma test kit was used to confirm both cell lines were free of Mycoplasma spp.

TRAP assay—Telomerase activity of all samples was measured by a PCR-based telomerase activity assay (TRAP) as described elsewhere.46 Although this kit was originally developed to measure telomerase activity in humans, the telomeric repeat sequence, (TTAGGG)n, is highly conserved in vertebrates and therefore can be applied to canine samples.15,16,47 The kit involves a 1-tube, 2-step procedure. Step 1 is a telomerase extension step where the telomerase protein isolated from cells is incubated with a substrate oligonucleotide (5’–AAATCCGTCGAGCAGAGTT–3’) and dNTPs (2.5mM each for adenosine, guanine, cytosine, and thymine), which results in the addition of TTAGGG repeats to the substrate oligonucleotide. Step 2 amplifies the oligonucleotide-telomerase product by PCR assay via supplied PCR primers (reverse primer and substrate oligonucleotide). The PCR amplification, primer-dimer, and telomerase quantitation controls are also supplied.

To isolate the telomerase protein, 1 X 10^6 cells were incubated for 30 minutes on ice in 200 µL of 1X CHAPS lysis buffer (10mM Tris-HCl, 20mM L-glutamine, 1mM MgCl2, 1mM ethylene glycol tetraacetic acid, 0.1mM ben-
zamidine, 0.5mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol) and 200 U of ribonuclease inhibitor/mL. After centrifugation at 12,000 × g at 4°C for 20 minutes, 160 µL of the supernatant was removed and divided into portions, quick frozen on dry ice, and stored at −80°C until analyzed. Protein quantification was performed by use of a modified Bradford assay on a reserved 20-µL aliquot of the lysate. Approximately 50 to 150 ng of protein was added to 50-µL reactions containing dNTPs, 1X reaction buffer (20mM Tris-HCl [pH, 8.3], 1.5mM MgCl2, 63mM KCl, 0.05% Tween 20, and 1mM ethylene glycol tetraacetic acid), and water devoid of ribonuclease and deoxyribonuclease. The mixture was incubated at 30°C, and then used in the PCR assay. A standard thermocycler and commercial DNA polymerase were used with the following conditions: 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 60 seconds. Twenty-five microliters of the PCR products were separated through 12.5% nondenaturing polyacrylamide gels for 90 minutes at 180 V. Gels were stained with SYBR green, exposed to UV light, and photographed with a gel imaging system.

One person (ALMW) performed the TRAP assay on all samples to maintain consistency. Samples were considered positive for telomerase activity when they had a clear, consistent TRAP ladder of 6-bp increments beginning at 50 bp on a PAGE gel. Samples were classified as negative for telomerase activity when no clear, consistent 6-bp TRAP ladder was seen.

To determine the limit of detection (ie, the analytic sensitivity) of the TRAP assay in our laboratory, 10-fold serial dilutions of K9TCC cells were made with 10,000 to 10 cells added to the TRAP reaction mixture. Because many dogs with urinary bladder TCC have other urinary abnormalities such as cystitis or hematuria, the analytic sensitivity of this assay was evaluated through cell-mixing experiments involving serial dilutions of the telomerase-positive K9TCC cells in the telomerase-negative WI-38 cells prior to lysing (ratios of 90:10, 70:30, 50:50, 30:70, 10:90, and 2:98 K9TCC to WI-38 cells).

**Telomerase stability assessment**—Because the telomerase ribonucleoprotein complex has an inherent high degradation rate, particularly in an environment such as urine, the effects of urine sample storage time and temperature on telomerase activity were evaluated. To do so, urine samples were combined and measured into 12-mL aliquots. Then, 1 × 10^9 K9TCC cells were added to each aliquot, and these samples were stored at room temperature (approx 24°C), 4°C, or −20°C for 2, 4, 8, 12, and 24 hours. After incubation, the samples were centrifuged at 5,000 × g at 4°C for 10 minutes and washed twice with 10 mL of sterile PBS solution. After the final centrifugation, the supernatant was decanted, the cell pellet was lysed with 200 µL of CHAPS lysis buffer with ribonuclease inhibitor as described previously, and the product was stored at −80°C until analyzed. The modified Bradford protein assay was performed to ensure 50 to 150 ng of protein was added to each TRAP reaction. Telomerase activity was evaluated by use of the TRAP assay as described.

**Statistical analysis**—Sensitivity, specificity, and positive and negative predictive values for the TRAP assay were calculated by use of standard epidemiological methods. A veterinary bladder tumor antigen assay was used as the reference criterion test. Because of the small sample size, the 2-tailed Fisher exact test was used to compare proportions, with a value of P < 0.05 considered significant.

**Results**

**Validation of TRAP assay**—In the PAGE gel of K9TCC (TCC cell line) and WI-38 (telomerase-nega-

Figure 1—Photograph of a PAGE gel produced during an initial evaluation of K9TCC cells for telomerase activity by use of a TRAP assay. The gel shows the 6-bp TRAP ladder beginning at 50 bp in the positive control (+) and K9TCC (TCC) lanes, with no bands in the negative control (lysis buffer; LB) or heat-inactivated (a) lanes. M = 50-bp markers. WI38 = telomerase-negative WI-38 cells.

Figure 2—Photograph of a PAGE gel produced during evaluation of the analytic sensitivity of a TRAP assay for detection of telomerase activity in canine cells. The number of cells evaluated is displayed at the top of the third through sixth lanes. The characteristic TRAP ladder can be seen in as few as 10 K9TCC cells. See Figure 1 for remainder of key.
tive human cell line), telomerase activity appeared as ladders of 6-bp oligonucleotide repeats beginning with a 50-bp band (Figure 1). A 36-bp band, which served as an internal positive PCR control marker, was seen in all lanes. Evidence of telomerase activity was clearly visible in the telomerase-positive control cells supplied with the assay and the K9TCC cell line, whereas no telomerase activity was detectable in the WI-38 cell line, in samples in which telomerase activity had been inactivated by heating to 98°C, and in samples of lysis buffer.

When analytic sensitivity of the TRAP assay was evaluated through testing of serial dilutions of the K9TCC cells, telomerase activity was consistently detectable when as little as 10 K9TCC cells were used (Figure 2). When analytic specificity of the assay was evaluated through testing of serial dilutions of telomerase-positive K9TCC in telomerase-negative WI-38 cells, telomerase activity could be detected in as low as 2% TCC cells (Figure 3).

Telomerase stability—After K9TCC cells were added to freshly collected urine samples from healthy dogs and the samples were stored, those samples stored for >4 hours at room temperature consistently lacked detectable telomerase activity (Figure 4). Telomerase activity was detectable up to 8 hours after sample storage at 4°C and −20°C but began to cease by 12 hours at both of these storage temperatures.

Evaluation of clinical urine samples—Thirty clinical urine samples were collected from healthy dogs (n = 9), dogs with nonneoplastic LUTD (10), and dogs with TCC as diagnosed by use of ultrasonography of the urinary bladder and cytologic assessment of cells obtained via traumatic catheterization (7) or histologic methods (4). Seven of the dogs with nonneoplastic LUTD had positive bacterial culture results, whereas 3 had negative results (Table 1).

None of the urine samples collected from healthy dogs had evidence of telomerase activity when the TRAP assay was used (Figure 5). On the other hand, samples from 2 of the 10 dogs with benign LUTD had telomerase activity. These samples had varying amounts of blood, protein, bacteria, or WBC contamination (Table 1). Furthermore, samples from all 7 dogs that presumably had TCC on the basis of ultrasonographic and cytologic findings had obvious telomerase activity. Those dogs...
from all 4 dogs with a histologic diagnosis of urinary bladder TCC had evidence of activity as well, although 1 sample yielded only very faint bands on the PAGE gel.

Thus, the diagnostic sensitivity and specificity of the TRAP assay applied to clinical canine urine samples were 91% and 89%, respectively. Additional analyses revealed positive and negative predictive values of 83% and 94%, respectively, with a Fisher exact value of \( P < 0.001 \).

**Discussion**

Results of the present study of telomerase activity in the urine of dogs with TCC suggested that telomerase activity may be a useful diagnostic marker for urothelial carcinomas in dogs, which is similar to recently reported human findings. Importantly, our findings underscore the need for more experiments designed to optimize urine storage and processing protocols that would facilitate the development of an accurate, sensitive, noninvasive diagnostic test to aid in the diagnosis of TCC in dogs.

The main objective of our study was to determine whether telomerase activity could be detected in cells from a canine TCC cell line and in the urine of dogs in which TCC was diagnosed by use of a commercial TRAP assay. A sensitive, specific, and noninvasive test could be a valuable asset in the diagnosis of TCC in several clinical settings. First, this test could be used to diagnose TCC in dogs when urine cytologic results are equivocal or when only small numbers of neoplastic cells are present in the urine. Second, performance of cystoscopy can be challenging in small-breed dogs such as male Scottish Terriers and West Highland White Terriers, which are predisposed to urinary bladder TCC. A noninvasive urine test would also eliminate the risk of tumor seeding associated with fine-needle aspiration or surgical biopsy of the urinary bladder. Lastly, because the entire urinary tract is lined with urothelial cells, a urine TRAP assay could theoretically detect neoplasia in other areas of the urinary tract that are typically difficult areas from which to obtain cells for diagnosis (eg, ureter, renal pelvis, urethra, and renal parenchyma).

Historically in human medicine, the mainstay of detection of urinary bladder cancer is cystoscopy, followed by a transurethral resection or biopsy if an abnormality is detected. Urine cytologic examination is also important in the detection and monitoring of urinary bladder cancer, although its diagnostic sensitivity is relatively low (highly variable, but approx 50% sensitive in general), particularly in humans with low-grade lesions. Additionally, urine cytologic assessment is subjective and has considerable interobserver variability. Cytologic examination also depends on an adequate number of cells being present in a specimen, and interpretation can be influenced by various collection methods and preservation techniques. For these reasons, human medical researchers have evaluated various urine biomarkers to aid in the noninvasive and early detection of urinary bladder TCC. There are many FDA-approved diagnostic tools for detecting and monitoring urinary bladder carcinoma in humans. Testing methods currently being evaluated include use of the TRAP assay, BCLA-4, hyaluronic acid-hyaluronidase, Lewis X antigen, microsatellite markers, and survivin. At this time, results suggest telomerase detection via TRAP assays may be a sensitive and specific test in humans.

In the present study, initial experiments involving telomerase-positive K9TCC cells and telomerase-negative WI-38 cells demonstrated the analytic sensitivity of the TRAP assay. We were able to consistently detect telomerase activity in only 10 K9TCC cells. We were also able to detect telomerase activity when as little as 2% of telomerase-positive cells were mixed with telomerase-negative cells, showing that under these conditions, the addition of large numbers of nonneoplastic cells does not influence TRAP assay outcomes. However, there are circumstances in which the presence of other nonneoplastic cells can yield a positive result. For example, urine samples from dogs with bacterial cystitis, which may contain activated lymphocytes known to be telomerase-positive cells, could yield false-positive results.

Our results regarding the effects of urine storage conditions are important because they show the potential value of the TRAP assay for evaluating clinical urine samples. The associated experiment, which was performed by adding a high number of the K9TCC cells to urine collected from dogs with unremarkable results of urine dipstick analysis (ie, no hematuria or proteinuria) and no clinical signs of urinary tract disease, was designed to evaluate the effects of urine storage time.
and temperature on telomerase activity. A significant loss of telomerase activity was detected in samples stored at 4°C for 8 hours, which is similar to findings in humans, suggesting that an increase in false-negative results occurs when urine samples are not stored appropriately. We speculate that the ability to detect telomerase activity in clinical urine samples depends on the number of TCC cells shed into the urine, the time of urine retention within the urinary bladder, and, as was demonstrated in the present study, the storage conditions once the urine has been collected.

In our study, telomerase activity was easily detected in urine samples from 10 of 11 dogs with TCC; in the remaining dog, there was less clear evidence of the characteristic TRAP 6-bp ladder (Figure 5). All 11 dogs were originally evaluated for clinical signs typically associated with a large urinary bladder or prostatic mass, and most were undergoing mitoxantrone and piroxim cam treatment when their urine samples were collected. This finding suggested that dogs with TCC and receiving chemotherapy continue to shed neoplastic cells into their urine. Telomerase activity was also detected in 2 of 10 dogs with benign LUTD. One of these dogs had severe pyuria with concurrent bacteriuria (> 500 WBCs/hp), whereas the other had antibodies against Leptospira spp, with 3+ proteinuria detected via urine dipstick test. However, more dogs with varying degrees of benign LUTD need to be evaluated to further accurately determine the specificity of this test. In the 9 dogs with no clinical evidence of LUTD, although urine samples from 3 yielded faint bands on the PAGE gel, these 3 dogs did not have the characteristic 6-bp ladder evident for the telomerase-positive urine samples and were thus deemed telomerase-negative in accordance with the instructions that accompanied the TRAP kit. Further evaluation with a more quantitative ELISA or real-time telomerase assay may yield more definitive results in situations such as these.

a. Cells supplied by Dr. Debbie Knapp, Purdue University, Lafayette, Ind.
b. ATCC CCL-75, American Type Culture Collection, Manassas, Va.
c. Primocin, InvivoGen, San Diego, Calif.
d. AppliChem, Cheshire, Conn.
e. TRAPeze Telomerase Detection Kit, Qbiogene, Irvine, Calif.
f. Eppendorf, Westbury, NY.
g. NanoDrop Technologies, Wilmington, Del.
h. Mastercycler epGradient S Thermocycler, Eppendorf, Westbury, NY.
i. Aflgene, Rockford, Ill.
j. Lonza, Rockland, Me.
k. VersaDoc 6000 Gel Imaging System, Bio-Rad Laboratories, Hercules, Calif.

References


