

Quantitative Analysis of Herpesvirus Sequences from Normal Tissue and Fibropapillomas of Marine Turtles with Real-Time PCR

Sandra L. Quackenbush,* Rufina N. Casey,† Rebecca J. Murcek,† Thomas A. Paul,† Thierry M. Work,‡ Colin J. Limpus,§ Anny Chaves,[¶] Leslie duToit,[¶] Javier Vasconcelos Perez,|| A. Alonso Aguirre,** Terry R. Spraker,†† Julia A. Horrocks,‡‡ Lotus A. Vermeer,§§ George H. Balazs,^{¶¶} and James W. Casey[†]

*Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045-2106; †Cornell University, Department of Microbiology and Immunology, Ithaca, New York 14853; ‡United States Geological Survey, National Wildlife Health Center, Honolulu Field Station, Honolulu, Hawaii 96850; §Queensland Parks and Wildlife Service, P.O. Box 155, Brisbane, Albert St. Q4002, Australia; ¶San Pablo Heredia, Costa Rica; ||Instituto Nacional de la Pesca, Centro Mexicano de la Tortuga, Mazunte, Tonameca km 7 Carretera San Antonio-Puerto Angel Apdo., Postal 16, Puerto Angel, Oaxaca National; **Wildlife Trust, 1200 Lincoln Avenue, Suite 2, Prospect Park, Pennsylvania 19076; ††State Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, Colorado 80523; ‡‡Department of Biological and Chemical Sciences, University of the West Indies, Cave Hill, St. Michael, Barbados; §§Barbados Sea Turtle Project, Bellairs Research Institute, St. James, Barbados; and ¶¶National Marine Fisheries Service, Southwest Fisheries Science Center, Honolulu Laboratory, 2570 Dole St., Honolulu, Hawaii 96822

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Quantitative real-time PCR has been used to measure fibropapilloma-associated turtle herpesvirus (FPTHV) *pol* DNA loads in fibropapillomas, fibromas, and uninvolved tissues of green, loggerhead, and olive ridley turtles from Hawaii, Florida, Costa Rica, Australia, Mexico, and the West Indies. The viral DNA loads from tumors obtained from terminal animals were relatively homogenous (range 2–20 copies/cell), whereas DNA copy numbers from biopsied tumors and skin of otherwise healthy turtles displayed a wide variation (range 0.001–170 copies/cell) and may reflect the stage of tumor development. FPTHV DNA loads in tumors were 2.5–4.5 logs higher than in uninvolved skin from the same animal regardless of geographic location, further implying a role for FPTHV in the etiology of fibropapillomatosis. Although FPTHV *pol* sequences amplified from tumors are highly related to each other, single signature amino acid substitutions distinguish the Australia/Hawaii, Mexico/Costa Rica, and Florida/Caribbean groups. © 2001 Academic Press

Key Words: herpesvirus; fibropapilloma; real-time PCR; marine turtles.

INTRODUCTION

Fibropapillomatosis (FP) is a neoplastic disease of marine turtles characterized by the presence of epithelial fibropapillomas and internal fibromas. FP of marine turtles is a recently emerging disease and likely presents a new viral epizootic (Herbst, 1994; Lackovich *et al.*, 1999; Quackenbush *et al.*, 1998). FP has appeared worldwide with sporadic but generally increasing frequency in green (*Chelonia mydas*), loggerhead (*Caretta caretta*), and olive ridley (*Lepidochely olivacea*) turtles and may potentially pose a significant threat to the long-term survival of marine turtles (Balazs and Pooley, 1991).

The etiologic agent of FP is most likely a herpesvirus; however, environmental cofactors may be involved (Herbst and Klein, 1995; Landsberg *et al.*, 1999). Herpesvirus sequences have been detected in fibropapillomas in green, loggerhead, and olive ridley turtles from Hawaii,

Florida, and Costa Rica using consensus PCR primers that recognize conserved regions of the DNA polymerase gene of herpesviruses (Lackovich *et al.*, 1999; Quackenbush *et al.*, 1998; VanDevanter *et al.*, 1996). Herpesvirus-like particles have been visualized in tumors by electron microscopy, and experimental transmission of fibropapilloma to green turtles has been successful using filtered, cell-free tumor homogenates (Herbst *et al.*, 1995, 1996; Jacobson *et al.*, 1991). Herpesvirus sequences were not detected in skin samples from turtles without FP, which suggests this virus is not a common infectious agent of marine turtles (Lackovich *et al.*, 1999; Quackenbush *et al.*, 1998).

Study of fibropapilloma-associated turtle herpesviruses (FPTHV) has been limited to *in vivo* assessment of viral DNA, mostly by PCR, and further constrained by the failure of all attempts at *in vitro* cultivation to date (Lackovich *et al.*, 1999). Standard PCR demonstrates a strong correlation between the presence of the turtle herpesvirus DNA polymerase gene and FP, but does not provide a measure of the levels of FPTHV DNA in tumors and other infected tissues. To further implicate FPTHV as the agent of FP, we quantified viral DNA loads in tissues from green, olive ridley, and loggerhead turtles afflicted with

¹ To whom correspondence and reprint requests should be addressed at Department of Microbiology and Immunology, C5-153 Veterinary Medical Center, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853-6401. E-mail: jwc3@cornell.edu.



TABLE 1

Detection of Turtle Herpesvirus Sequences in Sea Turtles

	<i>n</i>	No. positive (%)
Green turtles—Australia		
Fibropapillomas	13	12 (92)
Skin from tumor positive turtles	11	5 (45)
Skin from tumor free turtles	14	3 (21)
Loggerhead turtles—Australia		
Fibropapillomas	2	2 (100)
Skin from tumor positive turtles	2	0 (0)
Skin from tumor free turtles	13	2 (15)
Green turtles—Barbados		
Fibropapillomas	1	1 (100)
Skin from tumor positive turtle	1	1 (100)
Olive Ridley turtles—Mexico		
Fibropapillomas	4	4 (100)
Skin from tumor free turtles	4	0 (0)

FP from the Pacific and Atlantic Ocean and evaluated the genetic relatedness of the FPTHV *pol* gene from these areas.

RESULTS

Detection of FPTHV *pol* sequences in turtles from Australia, Barbados, and Mexico

To determine whether fibropapillomas from green and loggerhead turtles from Moreton Bay, Australia contain a herpesvirus, DNA was extracted from tumors and subjected to PCR using turtle herpesvirus-specific primers (GTHV1 and GTHV2). These primers were designed from turtle herpesvirus DNA polymerase sequences previously found to be associated with fibropapillomas from marine turtles and amplify a 165-bp fragment of the DNA polymerase gene (Quackenbush *et al.*, 1998). A total of 20 tumors were assayed from 14 green, 2 loggerhead, and 4 olive ridley turtles. DNA from uninvolved skin from 14 tumor-bearing turtles and skin from 31 tumor-free animals was also assayed (Table 1). With the exception of one tumor from a green turtle, herpesvirus *pol* sequences were detected in all tumors. Five of 11 histologically normal skin samples from green turtles with fibropapillomas also were found to harbor herpesvirus. Five of 27 tumor-free Australian turtles demonstrated the presence of THV *pol* sequences in skin samples.

A fibropapilloma and uninvolved skin were obtained from one green turtle collected in Barbados. Using the FPTHV-specific *pol* primers, both of these samples were found to be positive by PCR (Table 1). Four tumors collected from olive ridley turtles from Mexico were also found to contain FPTHV *pol* sequences. Skin samples from four olive ridley turtles without any presence of tumors were negative for FPTHV (Table 1).

These results are consistent with those from a previous study of green and loggerhead turtles from Hawaii

and Florida and olive ridley turtles from Costa Rica (Quackenbush *et al.*, 1998). However, in contrast to previous studies, herpesvirus sequences were detected in skin samples from both tumor-free green and loggerhead turtles from Moreton Bay, Australia (Quackenbush *et al.*, 1998).

Cloning and sequence analysis of herpesvirus DNA polymerase amplicons from tumors collected in Australia, Barbados, and Mexico

To further characterize the herpesvirus that was present in fibropapillomas collected from new geographic locations, a 483-bp fragment of the turtle herpesvirus DNA polymerase gene was cloned and sequenced. DNA isolated from tumors from green, loggerhead, and olive ridley turtles from Australia, Barbados, and Mexico was PCR amplified with the FPTHV *pol* primers, GTHV2 and GTHV3. The amino acid sequence derived from each of these fragments was aligned with the turtle herpesvirus sequences previously described (Fig. 1) (Quackenbush *et al.*, 1998). The Australian loggerhead sequence (LTHV-Aust) is identical to that of the Hawaiian green turtle sequence (GTHV-Ha). Two nucleotide changes resulting in one amino acid substitution [leucine (L) to serine (S)] are present in the Australian green turtle sequence (GTHV-Aust) compared to the GTHV-Ha sequence. The Barbados green turtle sequence (GTHV-Bar) is more closely related to the Florida green (GTHV-FI) and loggerhead sequences (LTHV-FI). The Barbados sequence differs from the Florida sequences by three nucleotide changes, which result in two amino acid substitutions. The olive ridley herpesvirus sequence from turtles collected in Mexico (ORTHV-Mex) differs from the olive ridley sequence (ORTHV-CR) previously identified in turtles from Costa Rica by three nucleotide changes, which result in one amino acid substitution [histidine (H) to asparagine (N)]. The H-to-N amino acid substitution is also found in the GTHV-Bar sequence.

FPTHV DNA levels in tumors and tissues of turtles with fibropapillomatosis

Standard PCR analysis does not provide a quantitative measure of the levels of FPTHV DNA present in tumors and infected tissues. To accurately assess the herpesvirus DNA load in different tissues, real-time quantitative PCR was employed. The number of copies of FPTHV DNA *pol* gene present in tumors and other tissues from turtles sampled from Hawaii and Florida are displayed in Fig. 2. All assays contained 100 ng DNA, which corresponds to approximately 20,000 cells, assuming a value of 6 pg of DNA per cell. As a reference point, a copy number of 2×10^4 FPTHV genomes would be equivalent to one copy of FPTHV per tumor cell under these assay conditions; however, no assumption can be made re-

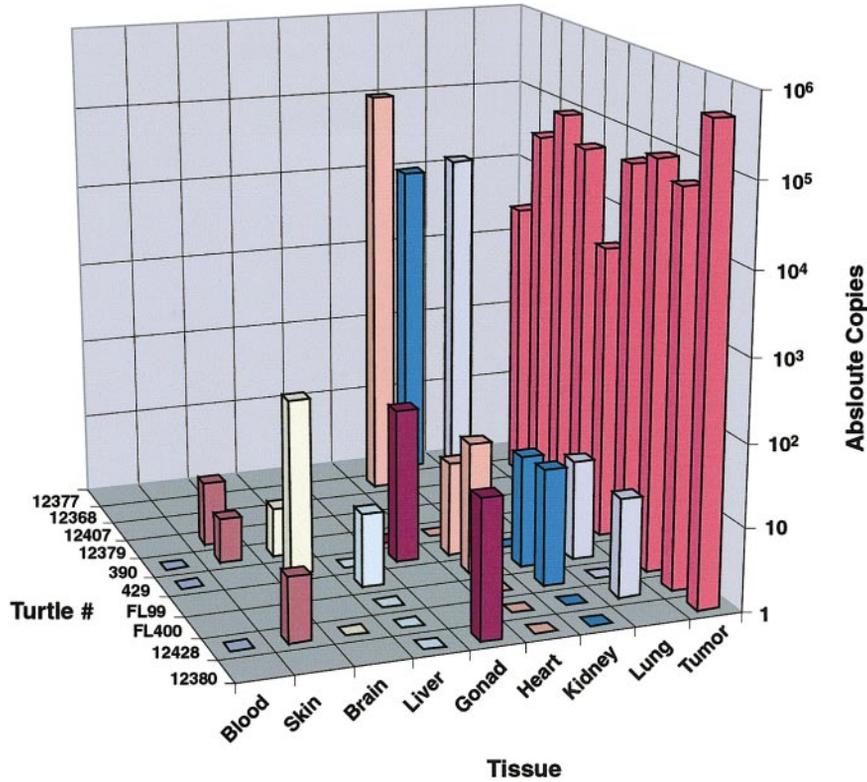


FIG. 2. Quantitative PCR measurement of FPTHV DNA *po/* copy number in tumor and normal appearing tissues from turtles sampled from Hawaii and Florida.

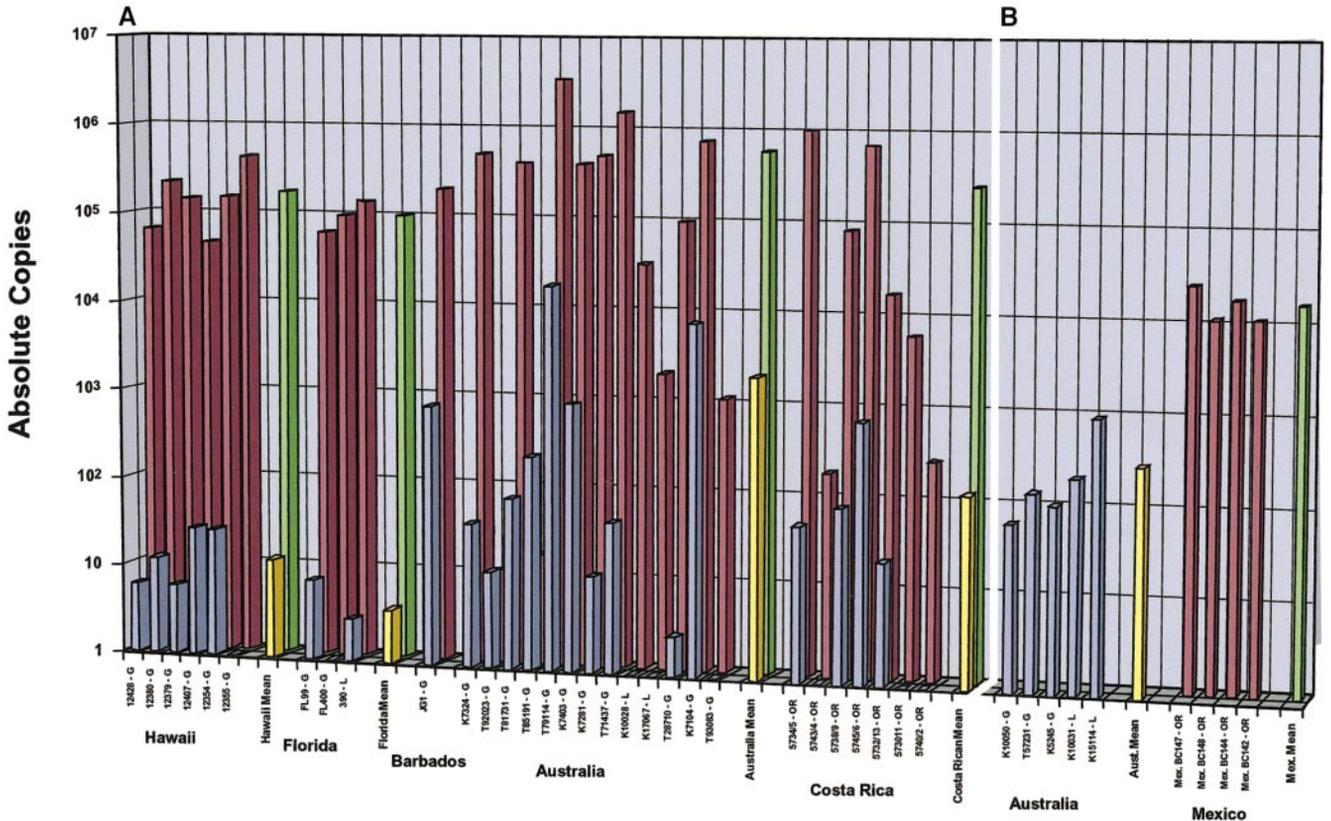


FIG. 3. Comparison of FPTHV DNA *po/* copy numbers from tumors and skin. (A) Matched tumor and uninvolved skin samples from Hawaii, Florida, Barbados, Australia, and Costa Rica. (B) Skin samples from tumor-free turtles and tumor samples from Mexico. Mean values in each set are shown to the right in lighter shades.

The olive ridley sequences from Mexico (Pacific) and Costa Rica (Pacific) also differ by only one amino acid substitution. Although GTHV-Bar contains unique amino acid substitutions, the sequences isolated from turtles in the Caribbean (Florida and Barbados) are highly related. The significance of these unique changes in the highly conserved DNA polymerase gene with regard to defined turtle populations may be substantiated by comparisons of sequences of less conserved herpesviral genes and inclusion of additional tumor samples.

A mean of 15.21 copies per cell (range = 0.001–170) of FPTHV DNA *pol* sequences were detected in tumors collected from turtles from six different locations throughout the world. This value would suggest that FPTHV plays a dominant role in maintenance of the tumor state. As stated earlier, the single copy number for FPTHV under these PCR conditions is 2×10^4 copies, the threshold single copy value if it is assumed that FPTHV is distributed equally among all cells in the tumor. The viral DNA load in individual tumors sampled from Hawaii and Florida were relatively homogeneous, ranging from 1.7 to 25 copies of FPTHV per tumor cell, a value in agreement with previous estimates by Southern blot analysis (Quackenbush *et al.*, 1998) and within the threshold value. In contrast, real-time PCR revealed that the copy numbers of viral DNA in tumors from Costa Rica and Australia varied by as much as 5 logs. Corresponding skin samples from the same turtles of these distinct geographical regions also showed the same trend in copy number variation compared to the Hawaii, Florida, and Barbados samples. Seven of 18 tumor samples were below the single copy threshold value. The hypothesis that FPTHV exerts a dominant role in tumor maintenance is challenged by these data, especially considering tumors where only 10 to a few hundred copies are present (Fig. 3A). This copy number variation may reflect the stage of tumor development. The Hawaii, Florida, and Barbados samples were obtained from moribund turtles while the Australia, Costa Rica, and Mexican samples were obtained from otherwise healthy freshly dead, turtles. Spontaneous regression of fibropapillomas has been documented photographically in some field studies and may also result in viral copy number variation (Balazs *et al.*, 2000; Bennett *et al.*, 2000). Further, the cell(s) targeted by FPTHV are unknown and the viral load may be different in the distinct cellular layers that comprise the tumor. Fibropapillomas consist of papillary epidermal hyperplasia supported on broad fibrovascular stromal stalks and the ratio of epidermal to dermal proliferation varies among lesions (Harshbarger, 1991; Herbst, 1994; Jacobson *et al.*, 1989; Lucke, 1938; Norton *et al.*, 1990). Eosinophilic intranuclear inclusions associated with herpesvirus particles have been observed in epidermal cells from some tumors by histopathology (Herbst

et al., 1999; Jacobson *et al.*, 1991). FPTHV expression and loads could also change during tumor progression. *In situ* hybridization analysis will be of value in addressing these questions by examining the distribution of viral DNA in tumors.

FPTHV sequences are also present in some normal tissues (in addition to skin) of animals with tumors, however, at significantly lower levels (mean = 0.0012 ± 0.0017 copies per cell, range = 0–0.002) than those found in tumors (Fig. 2). Some tissues in which FPTHV has been detected, heart, kidney, and lung, are associated with tumor development, suggesting that the low level of viral DNA detected may be indicative of early infection or likely tumor metastasis since FPTHV has not been found in blood.

To ascertain the requirement for FPTHV in tumor development, real-time PCR performed on samples from 38 animals determined that FPTHV DNA loads in tumors were 2.5–4.5 logs higher than in uninvolved skin from the same animal regardless of geographic location. The quantitative PCR data described here further substantiates FPTHV as the causative agent of fibropapillomatosis. Molecular cloning and DNA sequence analysis of this virus will allow for the generation of diagnostic reagents to monitor infected animals as well as aid in management decisions necessary to maintain survival of these endangered species.

MATERIALS AND METHODS

PCR amplification, cloning, and sequencing

Biopsies of tumors and nontumored skin were collected from live-captured immature and adult green and loggerhead turtles from Moreton Bay, Australia and adult female olive ridley turtles nesting in Oaxaca, Mexico (Pacific coast). Additionally, tumored tissues (skin and internal tumors) were obtained from freshly dead green turtles from Hawaii, Florida, and Barclays Park, Barbados that were stranded with severe fibropapillomatosis. DNA was isolated from tissues as previously described (Quackenbush *et al.*, 1998). One microgram of DNA in a total volume of 100 μ l was subjected to PCR amplification with turtle-specific herpesvirus primers: GTHV1 (5'TGTCTGGAGGTGGCGGCCACG3'), GTHV2 (5'GACACGCAGGCCAAAAAGCGA3'), or GTHV3 (5'AGCATCATCCAGGCCCAAA3'). The PCR mixture consisted of 20 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 50 mM KCl, 2.5% DMSO, 200 μ M of each dNTP, 10 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD). All samples were denatured at 94°C for 5 min and then amplified for 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C for 35 cycles, followed by 10 min at 72°C for one cycle. Fifteen microliters of each PCR amplification reaction was separated on a 2% agarose gel. PCR products were gel purified with a Qiaex II gel extraction kit (Qiagen Inc., Chatsworth, CA) and cloned

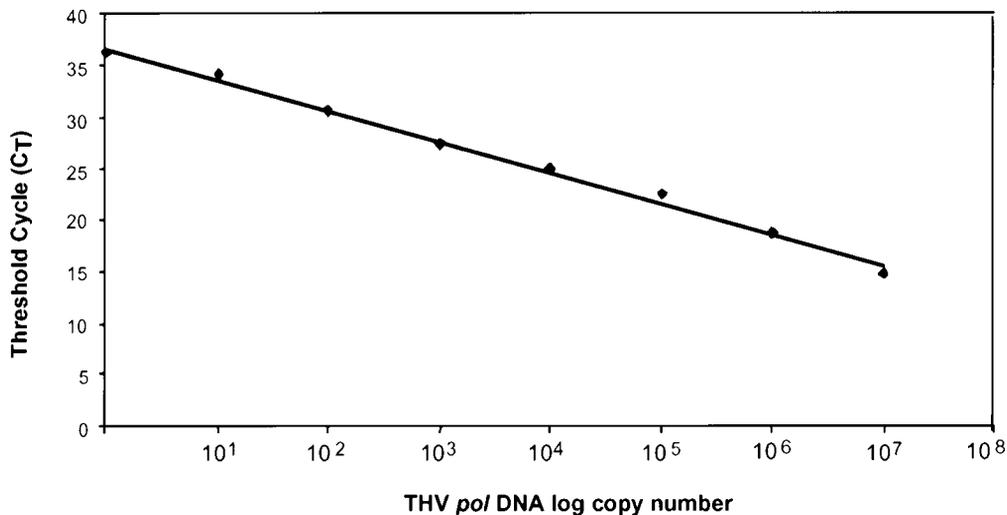


FIG. 4. Standard curve for real-time PCR. Serial log dilutions of pGTHV pol plasmid ranging from 1 to 10^7 copies per reaction were amplified. The C_T values, which correspond to the PCR cycle number in which the value is above the threshold limit, are plotted against the copy number of input plasmid DNA.

into pCR2.1 TOPO vector (Invitrogen Corp., Carlsbad, CA). Automated sequencing was done with an ABI 373A automated sequencer (Applied Biosystems, Inc., Foster City, CA) at the Biotechnology Resource Center at Cornell University.

Real-time quantitative PCR

An ABI 7700 PRISM quantitative PCR instrument was used to process and quantify levels of THV DNA. The primers and probe used to quantitate FPTHV were selected from the GTHV-Ha DNA polymerase gene. The primers used were turtle 5'- pol (5'-ACTGGCTGGCACT-CAGGAAA3') and turtle 3'- pol (5'-CAGCTGCTGCTTGTC-CAAAA3'), which generates a product of 86 bp. A fluorogenic probe, turtle pol -probe, which contained a FAM (6-carboxy-fluorescein) reporter molecule attached to the 5' end and a TAMRA (6-carboxy-tetramethyl-rhodamine) quencher linked at the 3' end (5'-[6FAM]-CGATGAAAAC-CGCACCGAGCGA-[TAMRA]-3') was synthesized by PE Biosystems. PCR amplification was performed in a 50 μ l reaction volume containing dNTPs at a concentration of 200 μ M for dATP, dCTP, dGTP, and 400 μ M for dUTP, 5 pmol of each primer, 20 pmol of probe, 0.5 U of AmpErase (PE Biosystems) uracil *N*-glycosylase (UNG), 0.25 μ l of AmpliTaq Gold (PE Biosystems), and 5 μ l of 10 \times TaqMan buffer. All assays contained 100 ng of genomic DNA. PCR mixtures were subjected to 2 min at 50°C to activate UNG (to degrade possible contaminating amplicons) and 10 min at 95°C to activate the *Taq* polymerase followed by 40 cycles of 15 s at 95°C and 1 min at 62°C with an ABI 7700 PRISM sequence detector (PE Biosystems).

For each reaction, the amount of fluorescence was measured as a function of the quantity of a reporter dye (FAM) that was released during amplification due to the

5' to 3' exonuclease activity of *Taq* polymerase. Serial log dilutions of the GTHV pol plasmid (Quackenbush *et al.*, 1998) were subjected to real-time PCR to establish standard curves. The threshold cycle (C_T) value for each sample was determined as the number of the cycle at which the measured fluorescence first exceeded the threshold limit (10 times the standard deviation of the baseline). The C_T values were plotted against the corresponding input DNA copy number and standard curves were fitted by linear regression with correlation coefficients of >0.95 (Fig. 4). The C_T values obtained from tissue DNA were used to calculate the viral genome copy number for each experimental sequence amplified. The limit of sensitivity of the real-time PCR was estimated as the highest plasmid dilution that yielded comparable C_T values in replicate samples.

Statistics

Student's *t* test and a 95% confidence interval based on a *t* distribution were used for all statistic analyses. A *P* value less than 0.05 was considered significant.

Nucleotide sequence accession numbers

The sequences described in this paper have been deposited with the GenBank database and assigned the following Accession Nos.: AF299107, AF299108, AF299109, and AF299110.

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