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USE OF POLYMERASE CHAIN REACTION AMPLIFICATION FOR THE DETECTION OF PAPILLOMAVIRUS IN TUMOR TISSUEOF GREEN TURTLES WITH FIBROPAPILLOMAS

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PREFACE

This report is the result of cooperative research conducted by Dr. Yuanan Lu with support from the Southwest Fisheries Science Center Honolulu Laboratory, the U.S. Geological Survey (USGS) Honolulu Field Station of the National Wildlife Health Laboratory (NWHL), and the Joint Institute for Marine and Atmospheric Research (JIMAR). Dr. Lu's report describes the apparent detection of papillomavirus in freshly necropsied Hawaiian green turtles, *Chelonia mydas*, with fibropapillomas.

The cause of fibropapillomatosis, a debilitating and often fatal tumor disease, remains unknown although a viral etiology has been strongly implicated. The disease represents a potentially serious threat to the long-term recovery and healthy maintenance of green turtle populations at several locations worldwide, including Hawaii, Australia, Florida, and areas of the Caribbean. In addition, fibropapillomatosis has recently been histologically confirmed in Pacific populations of the olive ridley, *Lepidochelys olivacea*.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA, U.S. Department of Commerce.

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INTRODUCTION

The epidemic occurrence of fibropapillomatosis (FP) in the green turtle (*Chelonia mydas*) represents significant threat to the long-term survival of this marine species in the Hawaiian Islands. Although a substantial amount of circumstantial evidence suggests a viral etiology for green turtle FP, the causative viral agent or agents have not been identified.

Recent studies have focused on herpesviruses and retroviruses as possible tumor-causing agents. Much less attention has been paid to other viruses. One virus group, which we believe warrants intensive investigation, is the papillomavirus. Papillomaviruses are known to cause proliferative and neoplastic lesions and induce papillomas in mammals, as well as in other nonmammalian species. For example, papillomavirus infection in reptiles has been documented in side-necked turtles and green lizards.

Due to extreme difficulties in the propagation of papillomaviruses in vitro, an alternative technique in the identification of papillomavirus infection in FP is polymerase chain reaction (PCR). This technique has been widely applied in virus identification. Several animal papillomaviruses, such as hamster oral papillomavirus, deer papillomavirus, and bovine papillomavirus have been detected recently by PCR amplification. We are taking advantage of this rapid DNA amplification technique to analyze tumors and tissues from diseased green turtles in Hawaii for papillomaviral genomic sequences. The present report provides the results of our initial work.

MATERIALS AND METHODS

Sample Size and DNA Preparations

Three internal and 18 external tumor samples from nine green turtles stranded in a near-death condition in the Hawaiian Islands were collected during January-September 1997. Five healthy-appearing non-tumored tissues from these humanely euthanized affected animals were also included as controls in this analysis. Necropsies of these nine turtles were conducted by veterinary researchers of the NWHL and JIMAR. Tissues were also collected from two severely deformed green turtle hatchlings originating from a captive breeding program at Sea Life Park Hawaii on the island of Oahu.

Tumor tissues and other samples were minced into small pieces (1-2 mm in diameter) before storage at -70°C. For DNA extraction, 0.5-1.0 g of each tumor tissue was ground into a fine powder using a pre-chilled mortar/pestle and suspended in digestion buffer (100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 25 mM EDTA, pH 8.0; 0.5% sodium dodecyl sulfate and 0.1 mg/ml proteinase K) at a ratio of 100 mg tissue/1.2 ml buffer. Suspensions were transferred into tightly capped 50-ml polypropylene tubes and shaken for 12 to 18 hr at 50°C to degrade the cellular protein. Digests were then deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, and DNA was recovered by ethanol precipitation, dried and resuspended in 300-500 µl TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). Nucleic acid concentrations were quantitated by measurement at A_{260}, and DNA integrity was confirmed by electrophoresis on 1% agarose gels.

Detection of Papillomaviral Genome by PCR

To detect papillomaviral sequence, we employed three sets of oligonucleotide primers from the conserved L-1 gene, two from human papillomavirus 16 (HPV16), called L1C1/L1C2 and MY11/MY09, and one from hamster papillomavirus, designated HPV-F/R. Each PCR was conducted in a final volume of 50 µl containing 0.5-1 µg DNA, 1x PCR buffer, 200 µM each dNTP, 1 mM $MgCl_2$, 1.25 U *Taq*-DNA polymerase, and 50 nM each primer. Initial thermocycling conditions were as follows: denaturation at 94°C for 5 min; followed by cycling at 94°C for 1 min, 48°C for 1 min and 72°C for 1 min for a total of 45 cycles.

Following PCR amplification, amplified products were size-fractionated on 1% agarose gels, and DNA fragments with the expected size were cloned using the TA (thymine, adenine) vector (Invitrogen which is routinely used in our laboratory) and sequenced. Sequence analysis was facilitated by using several computer programs, including Genetic Computer Group (GCG) and DNAStar.

RESULTS AND DISCUSSION

A total of 100-500 µg genomic DNAs were extracted from 28 tumor and non-tumor tissue samples collected from nine tumored and two newly hatched green turtles. Prior to PCR amplification, these DNAs were analyzed by electrophoresis using 1% agarose gel and by spectrophotometer to ascertain the quality of these prepared DNAs. To detect papillomaviral sequence, these DNAs were subjected to PCR amplification using three pairs of primers. Following PCR amplification, amplified products were analyzed by electrophoresis on 2% NuSieve gels.

No PCR products were obtained for all the DNA samples tested when they were exposed to the oligonucleotide primers of HPV-F/R and L1C1/L1C2. Further amplifications by lowering the annealing temperature (42-46°C) while increasing $MgCl_2$ concentration (2.5-4.0 mM) did not result in positive signals when these two sets of primers were used.

In contrast, a 230-bp PCR product was observed when the primer pair of MY11/MY09 was used (Fig. 1). Although this product migrated faster than the control DNA containing papillomaviral genome, it was very distinctive and detected in most of samples. Under the current amplification conditions, no other PCR fragments were detected. We detected this fragment in all 23 DNAs from tumors and 5 of 6 tissues from tumored green turtles in this study. The PCR amplification also showed 3 out of 5 DNAs prepared from cell cultures established from tumored turtles to be positive, including green turtle lung cells which generated tumor-like aggregates in vitro.

Analysis of brain and lung tissues harvested from newly hatched green turtles for the papillomaviral sequence revealed the former was negative while the latter was infected (Table 1). These data suggest a wide distribution of the viral sequence among the tumored green turtles. Amplification of this fragment from lung tissue of newly hatched green turtles was not expected. However, this finding may hint at the possibility of vertical transmission of agent causing FP. It should be pointed out that little is known regarding the mode of transmission of the disease and the most susceptible age of green turtles for the infection.

To further analyze this amplified product, the PCR fragments from both tumor and tissue samples were cloned in a TA vector according to manufacturer's instructions. Four clones, two from each sample, were then sequenced in both directions using the Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA) on an automated sequencer (Applied Biosystems Inc. Model 373A).

Comparison of nucleotide sequences of all the four clones revealed that they were identical to each other, as shown in Figure 2. This result indicates a consistent amplification from a DNA portion common in all preparations. Comparative analysis of this piece of nucleotide sequence, using GCG and DNAStar Computer Programs indicated, a 37-44% nucleotide similarity to eight known L-1 sequences of animal papillomaviruses and 36-41% of four human papillomaviruses (Table 2).

Recent reports from several investigators have indicated the presence of herpesviral and retroviral genomes in FP diseased green turtles. However, the role of these viruses in causing FP is not known and the causative agent or agents of FP have not been identified. Our attempts to examine papillomavirus, an agent commonly seen to induce tumors in animals and human, in

association with FP will facilitate the discovery of the causes of FP. We have successfully amplified a PCR fragment from the tumor tissues and cell cultures derived from tumored green turtles using an oligonucleotide primer pair derived from the L1 region of papillomavirus.

Despite a low sequence homology to other papillomaviruses examined, the detected fragment could be a truly papillomaviral sequence since papillomaviruses are known to be highly diverse. Our data represent, to the best of our knowledge, the first report of detection of papillomavirus in tumor tissues of green turtles. In addition, we have recently identified papilloma-like virus particles associated with in vitro formation of tumor-like aggregates in cell cultures established from green turtles with fibropapillomas. These preliminary data may suggest a possible association between papillomavirus and FP.

Our future work will be focused on the isolation and characterization of this viral agent and the determination of its role in causing fibropapillomatosis in Hawaiian green turtles.

Table 1.-- PCR Detection of papillomaviral sequence in tumors, tissues from tumored and non-tumored green turtles, and cell cultures derived from tumored animals.

Sample	No. tested	No. positive	No. negative
Tumors	21	21	0
Tissues-A[a]	6	5	1
Tissues-B[b]	2	1	1
Cell[c]	5	3	2

[a] = tissues from tumored animals: lung, kidney, heart, skin and soft tissue around eye (+); spleen (-)

[b] = tissues from newly hatched green turtles: lung (+) and brain (-). The hatchlings were obtained from Sea Life Park Hawaii where they were hatched in captivity as the result of a long-term captive breeding program. The two hatchlings had no hope of survival due to severe natural deformities.

[c] = cell cultures derived from tumored turtles: tumor, testis, and lung (+); skin and spleen (-)

Table 2.-- Percentage nucleotide sequence homology between PCR-amplified product from green sea turtles with fibropapilloma and known mammalian papillomaviruses.

Virus*	Homology (%)
BPV1	44
BPV2	43
BPV4	43
COPV	41
DPV	37
EEPV	38
MRPV	42
PCPV	39
HPV6a	41
HPV41	36
HPV44	36
HPV67	37

* BPV = bovine papillomavirus
COPV = canine oral papillomavirus
DPV = deer papillomavirus
EEPV = European elk papillomavirus
MRPV = multimammate rat papillomavirus
PCPV = pygmy Chimpanzee papillomavirus
HPV = human papillomavirus

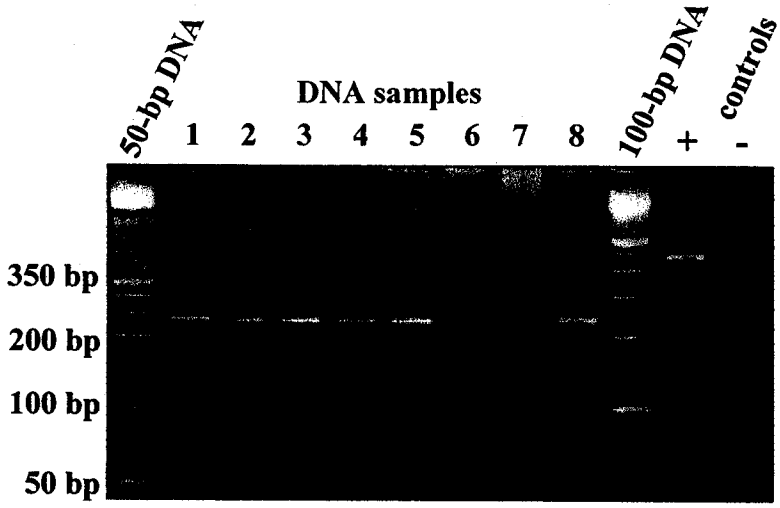

Figure 1.-- PCR detection of papilloma-like viral sequence in selected tissues of green sea turtles with fibropapilloma.

1 50
GGGGTACGCC CAGGGACATA ACAATGG TGGAGTGTTCCAC ACTATGTCAT

51 100
CGGGGATCCG ATGTTCACGC AGAAACACAA CCTGACTATA TTTGGCTGTC

101 150
CCTGGGCTAC TTCCTAGTCT CCCACTGTAA TGTACGTGTG GTGTGGGGTC

151 200
ATCCCCTGTC TCTTCAGGGA ACAGGTTGAA TGGCAGTCCT GGTAGCTCTT

201 235
CTCCCTGGAT CAGTTTCCCC TGGGACGTCT AGACC

Figure 2.--Nucleotide sequence of PCR amplified product from green sea turtles with fibro-papilloma using a pair of primers derived from human papillomavirus 16.