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Melting Profiles May Affect Detection of Residual HPV L1 Gene DNA Fragments in Gardasil®

Sin Hang Lee*

Milford Hospital and [1]Milford Molecular Laboratory, 2044 Bridgeport Avenue, Milford, CT 06460, USA

Abstract: Gardasil® is a quadrivalent human papillomavirus (HPV) protein-based vaccine containing genotype-specific L1 capsid proteins of HPV-16, HPV-18, HPV-6 and HPV-11 in the form of virus-like-particles (VLPs) as the active ingredient. The VLPs are produced by a DNA recombinant technology. It is uncertain if the residual HPV L1 gene DNA fragments in the vaccine products are considered contaminants or excipients of the Gardasil® vaccine. Because naked viral DNA fragments, if present in the vaccine, may bind to the insoluble amorphous aluminum hydroxyphosphate sulfate (AAHS) adjuvant which may help deliver the foreign DNA into macrophages, causing unintended pathophysiologic effects, experiments were undertaken to develop tests for HPV L1 gene DNA fragments in the final products of Gardasil® by polymerase chain reaction (PCR) and direct DNA sequencing. The results showed that while the HPV-11 and HPV-18 L1 gene DNA fragments in Gardasil® were readily amplified by the common GP6/MY11 degenerate consensus primers, the HPV-16 L1 gene DNA may need specially designed non-degenerate PCR primers for amplification at different regions of the L1 gene and different stringency conditions for detection. These variable melting profiles of HPV DNA in the insoluble fraction of the Gardasil® vaccine suggest that the HPV DNA fragments are firmly bound to the aluminum AAHS adjuvant. All methods developed for detecting residual HPV DNA in the vaccine Gardasil® for quality assurance must take into consideration the variable melting profiles of the DNA to avoid false negative results.

Keywords: Gardasil®, human papilloma virus, HPV, vaccine, melting profiles, HPV L1 gene DNA, AAHS nanoparticles, PCR, DNA Sequencing, detection.

INTRODUCTION

Gardasil® (Merck & Co.) is a protein-based vaccine in which the active ingredient is a mixture of L1 capsid proteins of the human papillomavirus (HPV) types -16, -18, -6 and -11. The antigenic proteins are produced by a DNA recombinant technology in which the genotype-specific "viral genes coding for the capsid proteins" [1] are inserted into the plasmid pGAL110 for transformation of the yeast spheroplasts [2]. Then the L1 capsid proteins can self-assemble into virus-like-particles (VLPs). HPV VLPs adsorbed to the amorphous aluminum hydroxyphosphate sulfate (AAHS) nanoparticles are highly effective in eliciting production of genotype-specific antibodies against HPV-16, HPV-18, HPV-6 and HPV-11 in mammals [3].

It is well known to the industry that the U.S. Food and Drug Administration (FDA) specifically requires vaccine developers to show that VLPs do not *encapsidate* "specific" nucleic acid sequences from the expression system, and especially those encoding VLPs components [4]. To comply with this requirement, the viral and plasmid DNA residues in the Gardasil® vaccine are removed as contaminants by a highly effective patented process [5] so that the final vaccine products do not contain viral DNA as stated by the vaccine manufacturer [6-8].

*Address correspondence to this author at the Milford Hospital and [1]Milford Molecular Laboratory, 2044 Bridgeport Avenue, Milford, CT 06460, USA; Tel: 203-878-1438; Fax: 203-387-1431; Email address: shlee01@snet.net

The FDA has recently announced that Gardasil® does contain recombinant HPV L1-specific DNA fragments [9]. However, it is still not clear if all or only some of the Gardasil® lots in use contain these HPV L1 gene DNA fragments as vaccine excipients. Since naked viral and bacterial DNA fragments firmly bound to insoluble aluminum salts can be carried into tissue macrophages through phagocytosis to initiate a series of DNA-related immune reactions [10-13] which may not be the intended effects of a protein-based preventive vaccine [14-21], it is desirable to test for residual HPV L1 gene DNA in the vaccine products as a quality assurance procedure. According to a World Health Organization (WHO) report on vaccinology, the currently available HPV assays differ in their analytical performance with regard to type-specific sensitivity and specificity [22]. None of the available test kits and laboratory protocols has been shown to be capable of detecting residual HPV DNA in vaccine products because the HPV DNA fragments in the Gardasil® vaccine are probably bound to the insoluble AAHS adjuvant [23]. This paper reports necessary modifications of the standard nested PCR/DNA sequencing technology for the detection and genotyping of the HPV L1 gene DNA fragments in the final Gardasil® products.

METHODS

A total of 16 unopened vials or manufacturer-prefilled syringes of Gardasil® were used for this study. Their lot numbers were #1437Z, #1511Z, # 0553AA, #NL35360, #NP23400, #NN33070, #NL01490, #NM25110, #NL39620,

0929-8673/13 $58.00+.00

#NK16180, #NK00140, #NM08120, #NL13560, #NL49190, #NN28160, and #NM29390. These vaccine samples were purchased by licensed health care professionals in various countries and sent to the author's laboratory to be tested for the presence of residual HPV DNA in the vaccine because they suspected that the residual recombinant HPV DNA might be a contributing factor in some of the unexpected symptoms observed in the women vaccinated with Gardasil®.

To test for residual HPV L1 gene DNA fragments in the supernatant of Gardasil®, an aliquot of 100μL of the vaccine suspension was centrifuged at ~16,000*g* for 10 min in a 1.5mL microcentrifuge tube at room temperature. The entire supernatant was transferred to another 1.5mL microcentrifuge tube containing 500μL of 95% ethanol, 12 μL of molecular grade water, and 68μL of 3M sodium acetate. After the precipitates were washed 3 times each with 1mL of 70% ethanol and the final ethanol suspension was centrifuged at ~16,000*g* for 5 min, the pellet was air-dried.

The dried pellet was re-suspended in100μL of 0.1 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) in a buffer consisting of 50mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1. The mixture was digested at 45-55°C overnight. After heat inactivation of the proteinase K digestate in a metal block at 95°C for 10 min, 1μL of the unspun digestate was used for each primary PCR followed by nested (hemi-nested, or same-nested) PCR with various nested PCR primer pairs (Fig. **1**).

To test HPV DNA in the insoluble part of the Gardasil® vaccine, the pellet of the centrifuged 100μL vaccine suspension described above was washed twice with 1mL of 70% ethanol each and the final ethanol suspension was centrifuged at ~16,000g for 5 min. The washed pellet was air-dried. The dried pellet was re-suspended in 100μL of proteinase K solution as described above. The suspension was digested at 45-55°C overnight and centrifuged at ~16,000g for 5 min the next day. The supernatant was transferred to a 1.5mL centrifuge tube and was heated at 95°C for 10 min to inactivate the proteinase K. One μL of the unspun digestate was used for each primary PCR followed by nested PCR.

The pellet of the proteinase K digestate of the insoluble part of Gardasil® was re-suspended in100μL of buffer. After heating at 95°C for 10 min, 1μL of the washed and heated insoluble particle suspension was used for each primary PCR followed by nested PCR.

For routine HPV L1 gene DNA detection, the well-characterized degenerate MY09/MY11 primer pair was used to initiate a primary PCR. All primary PCR products were blindly transferred by micro-glass rod to a heminested PCR mixture containing GP6 and MY11, or GP5 and MY09 as the primer pair. A LoTemp® PCR protocol in which 1μL of sample, 1μL of 10μM forward primer, 1μL of 10μM reverse primer, 2μL of molecular grade water and 20μL of LoTemp® ready-to-use PCR mix containing HiFi® DNA polymerase in a total volume of 25μL was used. The thermocycling steps were set at a temperature program not to exceed 85°C for all PCRs. Thermocycling for a low stringency PCR was programmed for an initial heating at 85°C for 2 min, followed by 30 cycles at 85°C for 30 sec, 40°C for 30 sec, and 65°C for 1 min. The final extension was 65°C for 10 min. For high stringency PCR, the annealing temperature was raised to 50°C for 30 sec from 40°C for 30 sec; and all other temperature steps were unchanged. All primary and heminested PCR products were subjected to agarose gel electrophoresis with ethidium bromide staining and visually examined under ultraviolet light for a specific amplicon band to be further validated by DNA sequencing [23-29].

Short target sequence HPV genotyping was performed by direct automated cycle DNA sequencing [23-29]. Briefly, a trace of the positive nested PCR product was transferred directly with a micro-glass rod from the positive nested PCR tube into a 20μL volume of a cycle sequencing reaction mixture consisting of 14.5 μL water, 3.5 μL of 5 X buffer, 1μL of BigDye Terminator 1.1 (Applied Biosystems) and 1 μL of 10 μM sequencing primer. After thermal cycling according to the manufacturer's recommendation for 20 cycles, the reaction mixture was loaded in an automated ABI 3130 four-capillary Genetic Analyzer for sequence analysis. Alignment analysis of a 45-60 bp sequence in the hypervariable region of the L1 gene excised from the computer-generated electropherogram was performed against standard HPV genotype sequences stored in the GenBank, using the on-line BLAST system to validate the specific HPV genotyping.

Transferring of all primary PCR products to the nested PCR mixture and nested PCR products to the sequencing reaction mixture was accomplished with micro-glass rods to avoid micropipetting aerosol and performed by experienced molecular technologists [29]. Negative water and primer controls were included in each PCR run of no more than 4 samples in one run. All PCR primers, including the MY09, MY11, GP5 and GP6 oligonucleotides, were tested, as previously described [24], against standard plasmid DNA of HPV types -16, -18, -11 or -6B purchased from ATCC to ensure that 1-10 copies of plasmid DNA from each genotype could be detected by the nested PCR protocol designed for this project.

After testing all 16 samples of Gardasil® in which only HPV-11 and/or HPV-18 L1 gene DNA fragments were detected by the GP6/MY11 degenerate consensus PCR primer pair [23], experiments were designed to investigate if HPV-16 L1 gene DNA fragments were also present in the vaccine. A "same-nested PCR" protocol with non-degenerate primers for repeated selective amplification was used to target a segment of the HPV-16 L1 gene DNA for detection. To perform the same-nested PCR for HPV-16 DNA detection, both the primary and nested PCRs in tandem were conducted with the same primers selected from one of the two oligonucleotide pairs referred to as HPV16MY11+/HPV16GP6+ and HPV16F/HPV16R, respectively (Fig. **1**). The latter pair of PCR primers was reported to be effective in selective amplification of HPV-16 L1 gene DNA in human samples infected by more than one HPV genotype [30]. The annealing site for the HPV16F primer is positioned between the GP6 and MY11 binding sites, and the HPV16R annealing site between the GP6 and MY09 binding sites (Fig. **1**). The annealing sites for HPV16MY11+ and HPV16GP6+ are identical to those for the MY11 and GP6 primers, respectively.

RESULTS

No PCR products were visualized at agarose gel electrophoresis when the aqueous supernatant of the whole vaccine

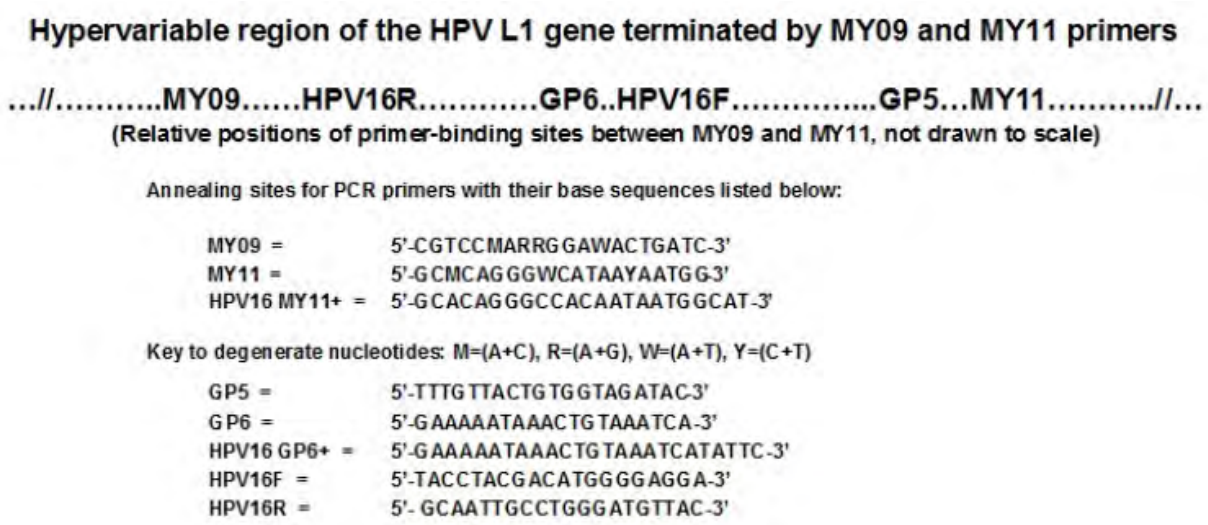

Fig. (1). Region commonly targeted for HPV L1 gene DNA amplification by PCR.

or the supernatant of the proteinase K-digestate of the pellets was tested. These negative results indicated that the residual HPV DNA fragments were not in the aqueous solution, nor were they encapsulated in the VLPs.

A GP6/MY11 nested PCR amplicon, ~190 bp in size, was obtained in all 16 vaccine samples tested when the suspension of the proteinase K-digested pellet was used as the primary PCR template. Direct automated DNA sequencing showed all ~190 bp amplicons generated by the GP6/MY11 nested PCR to be a synthetic HPV-11 L1 gene DNA (GenBank Locus #SCU55993) or an HPV-18 L1 gene DNA (GenBank Locus #JF728188), or a mixture of both, as previously reported [23]. However, the MY09/MY11 primary PCR and the GP5/MY09 nested PCR did not generate any visible amplicons at agarose gel electrophoresis even when the digested pellet was used as the template, indicating that the MY09 binding site of the HPV L1 gene in the Gardasil® samples tested was not available for effective PCR amplification.

When the degenerate consensus GP6/MY11 primer pair was used, a specific HPV L1 gene nested PCR amplicon was readily obtained at both low stringency thermocycling and high stringency thermocycling although more intense bands of PCR products were obtained under low stringency PCR condition with an annealing temperature set at 40°C (Fig. **2**). This observation was consistent with the findings with human cervicovaginal specimens infected with HPV that low stringency PCR was preferred when a not fully matched consensus primer pair was used for the detection of various genotypes of HPV DNA [29].

The GP6/MY11 nested PCR could not effectively amplify any HPV-16 L1 gene DNA in the Gardasil® vaccine samples tested. To amplify the HPV-16 DNA fragments, a non-degenerate HPV16MY11+/HPV16GP6+ primer pair was needed and two same-nested PCRs were required to generate a specific HPV-16 amplicon for DNA sequencing. For illustration, in the first HPV16MY11+/HPV16GP6+ nested PCR, there was no amplicon band visualized at gel electrophoresis when either 40ºC or 50ºC was used as the annealing temperature in thermocycling (Fig. **2**). The ~190 bp amplicon of HPV-16 L1 gene DNA only appeared after the second same-nested PCR with the HPV16MY11+/HPV16 GP6+ primer pair amplification under low stringency PCR condition with a 40ºC annealing temperature (Fig. **3**).

The position of the DNA fragment of the HPV-16 L1 gene in the Gardasil® vaccine detectable by target nested PCR was not constant. For example, in at least one sample tested (lot #NL39620) a segment of the HPV-16 L1 gene DNA was amplified by a pair of HPV-16F/HPV-R PCR primers (Fig. **2**, lower right, lane 1a), but not by a pair of HPV16MY11+/HPV16GP6+ primers in a parallel experiment (Fig. **2**, lower left lane 1b and lower right lane 1b). This result indicated that the MY11-binding site of the HPV-16 L1 gene DNA in this vaccine lot was not available for PCR amplification. Notably, the same-nested PCR amplification with the HPV-16F/HPV16R primer pair was only successful under high stringency PCR with an annealing temperature set at 50ºC. For other vaccine lots tested positive for HPV-16 L1 gene DNA fragments, the GP6/MY11 and the HPV16MY1+/HPV16GP6+ same-nested PCRs were successful with both 40°C and 50°C annealing temperatures although the specific amplicon bands were always more intense with the lower stringency amplification with a 40ºC annealing temperature.

Co-existence of an HPV-11 L1 gene DNA and an HPV-18 L1 gene DNA in the same vaccine sample caused sequencing failure because the DNA fragments of both HPV genotypes were co-amplified by the GP6/MY11 consensus primers, as previously reported [23]. However, the presence of HPV-16 L1 gene DNA in the same vaccine sample did not interfere with generation by a GP6/MY11 nested PCR of a useful amplicon for sequencing validation of an HPV-18 DNA, or of a useful amplicon for sequencing validation of an HPV-11 DNA. For illustration, when aliquots from a single pellet of Gardasil® lot #NL01490 were used to start an MY09/MY11 primary PCR followed by a GP6/MY11 nested PCR and used to initiate a series of HPV16MY11+/HPV16GP6+ same-nested PCRs, and then the two final nested PCR products were used for direct DNA

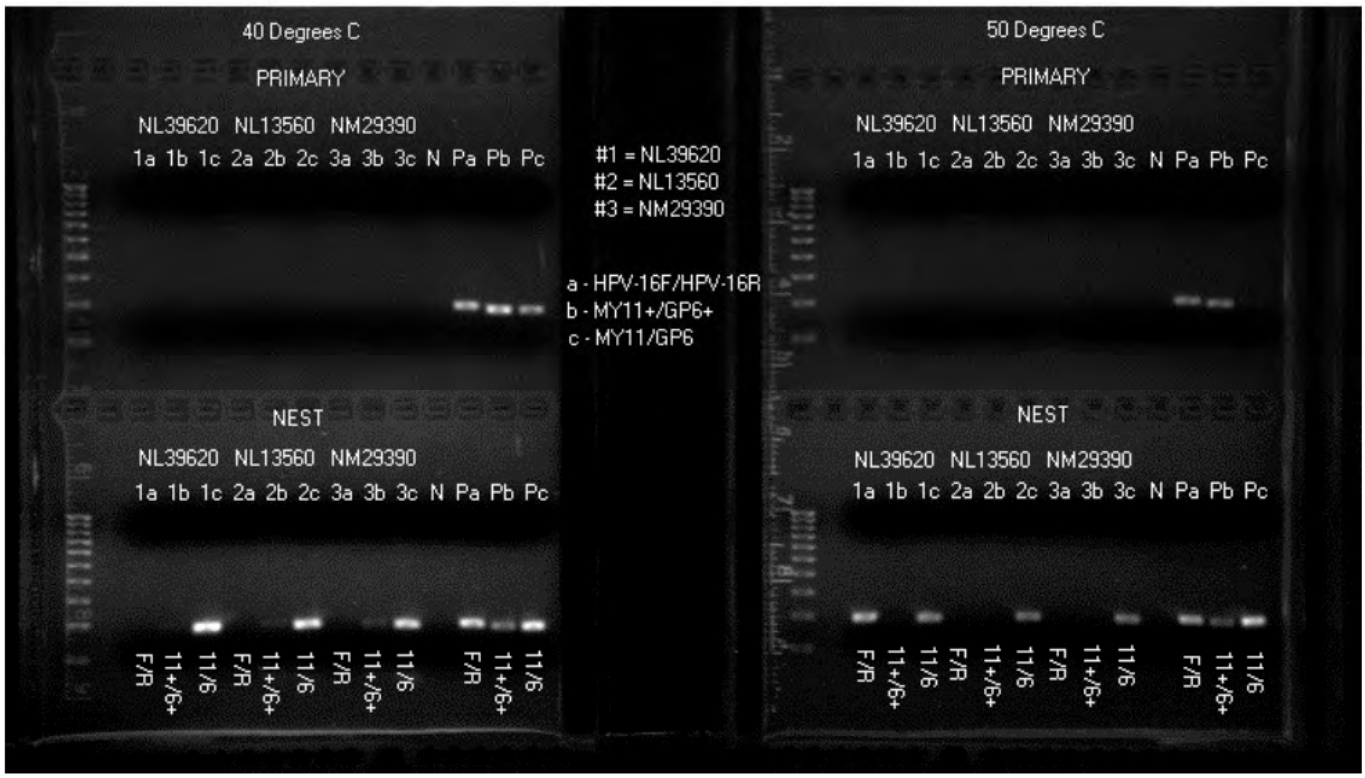

Fig. (2). Parallel same-nested PCR amplifications of 3 Gardasil® lot samples (#1, #2, #3) each with 3 primer pairs (a, b, c) under low (left) and high (right) stringency conditions. The left panel of gel electrophoresis shows PCR products after 30 cycles of LoTemp® thermocycling with annealing temperature set at 40°C. Each of the 3 HPV L1 gene PCR primer pairs, a, b and c, generated a ~190 bp PCR product at the primary PCR and the same-nested PCR (Pa, Pb, Pc) when HPV-16 plasmid DNA was used as the positive control to start the primary PCR. However, when the pellets of 3 Gardasil® samples were tested, only the GP6/MY11 same-nested PCR products (1c, 2c, 3c) were visible and useful as the template for direct DNA sequencing. DNA sequencing confirmed the genotypes of the nested PCR amplicons to be: 1c=HPV-11; 2c=a mixture of HPV-11 and HPV-18; 3c=HPV-18. The right panel shows PCR products after 30 cycles of LoTemp® thermocycling with the annealing temperature set at 50ºC. All other experimental conditions were identical to those described for the left panel. The results were also identical to those shown on the left panel except that lighter PCR product bands were obtained in lanes 1c, 2c, 3c, Pa, Pb and Pc. In addition, a same-nested PCR product band appeared in lane 1a, an HPV-16 L1 gene amplicon generated by the HPV16F/HPV16R primer pair at high stringency condition only (See Figures 6 and 7 for DNA sequencing confirmation of co-existence of HPV-16 and synthetic HPV-11 L1 gene DNA fragments in lot #NL39620).
40 Degree C = annealing temperature was set at 40°C
50 Degree C = annealing temperature was set at 50°C
Primary = primary PCR
Nest = same-nested PCR using identical primers as those for primary PCR
F/R = HPV16F/HPV16R primer pair (see Figure 1 for sequences)
11+/6+ = MY11+/GP6+ = HPV16MY11+/HPV16GP6+ primer pair (see Figure 1 for sequences)
11/6 = degenerate consensus GP6/MY11 primer pair (see Figure 1 for sequences)
N = Negative water control
P = HPV-16 plasmid DNA positive control

sequencing, using GP6 and HPV16GP6+ as the sequencing primer, respectively, the electropherogram showed no interference of the base-calling for an HPV-18 genotyping (Fig. **4**) by the coexistent HPV-16 DNA in the PCR system. The sequence of the non-degenerate HPV16MY11+ primer with fully matched bases for HPV-16 DNA was shown at the MY11-binding site (Fig. **5**). The melting profile of the HPV-16 L1 gene DNA in the vaccine apparently prevented it from participation in the PCR amplification process by a degenerate MY11 primer which functioned effectively when an HPV-18 L1 gene fragment in the same sample was amplified. The control HPV-16 plasmid DNA was amplified by all 3 primer pairs (Fig. **2**, Pa, Pb, Pc).

For another illustrative example, aliquots of a single pellet of Gardasil® lot #NL39620 were used as the material to start in parallel a GP6/MY11 same-nested PCR, an HPV16MY11+/HPV16GP6+ same-nested PCR, and an HPV16F/HPV16R same-nested PCR. Only the HPV16F/HPV16R and GP6/MY11 primer pairs generated an HPV DNA band (Fig. **2**, lower right lanes 1a and 1c). When the latter two nested PCR products were used as the templates for direct DNA sequencing, using HPV16F and GP6 as the sequencing primer, respectively, the electropherogram showed no interference of the HPV-11 base-calling (Fig. **6**) by the coexistence of an HPV-16 DNA (Fig. **7**) in the PCR system. There was no evidence of PCR co-amplification of

the DNA of two HPV genotypes by the consensus degenerate GP6/MY11 primer pair.

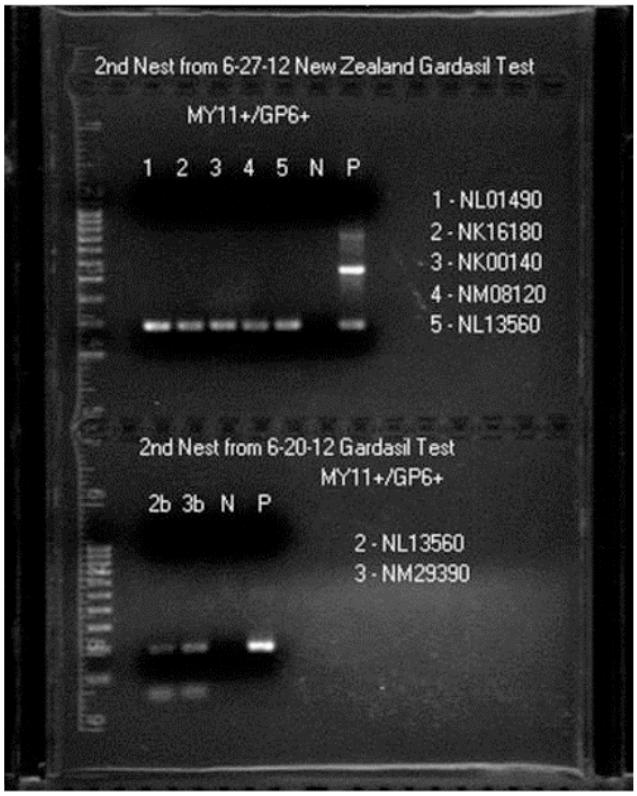

Fig. (3). Second same-nested PCR for detection of HPV-16 L1 gene DNA in Gardasil® samples. To selectively amplify the HPV-16 L1 gene DNA fragments terminated by the GP6 and MY11 primer endings, an elongated non-degenerate primer pair, HPV16MY11+/HPV16GP6+, was used to perform a second same-nested PCR under low stringency condition. As shown in the upper half of this gel electrophoresis photograph, an HPV DNA amplicon of ~190 bp was generated and visualized in lanes 1-5, all confirmed to be a sequence of HPV-16 L1 gene (See Figures 4 and 5 for DNA sequencing confirmation of co-existence of HPV-16 and HPV-18 in lot #NL01490). The lower half of the gel shows two lighter amplicon bands (lanes 2b and 3b) of a second same-nested PCR of the HPV-16 L1 gene DNA from an earlier experiment (lanes 2b and 3b in Figure 2), indicating that the efficiency of PCR amplification of the HPV-16 fragments in the vaccine is subjected to technical variations, such as the uneven amounts of the suspended pellets pipetted to initiate a primary PCR.
MY11+/GP6+ = HPV16MY11+/HPV16GP6+ primer pair (see Figure 1 for sequences)
N = Negative water control.
P = HPV-16 plasmid DNA positive control.

DISCUSSION

Vaccine manufacturers have traditionally considered residual DNA of the viral gene and the plasmid DNA left over from production to be contaminants and have made great efforts to remove these DNA residues from protein-based preventive vaccines [4, 14]. According to a publication of the Centers for Disease Control and Prevention (CDC) the only vaccines listed with DNA as expected excipients are the rotavirus and varicella vaccines [31]. Listed as excipients for Gardasil® are "yeast protein, vitamins, amino acids, mineral salts, carbohydrates, amorphous aluminum hydroxyphosphate sulfate, L-histidine, polysorbate 80 and sodium borate"[31]. The viral and plasmid DNA residues in the Gardasil® vaccine have been removed as contaminants by a highly effective patented process [5]. Since naked HPV DNA, if present in Gardasil®, may bind to the AAHS nanoparticles to form a stable DNA/AAHS complex [23] which can be delivered into antigen-presenting cells and macrophages after intramuscular injection [11-13] with potential unintended pathophysiologic consequences [14-21], a reliable method is needed to determine if there is any residual HPV DNA bound to the AAHS adjuvant in the final vaccine products as a measure of quality assurance.

To test for a specific DNA in small quantities usually depends on amplification of a target DNA segment with a pair of PCR primers followed by determining the DNA sequence of the PCR amplicon. However, aluminum-DNA complexes differ from other metal-DNA complexes. More than one form of DNA with different melting profiles can exist at any time in the presence of aluminum. The different DNA-aluminum complexes lead to a variety of reactions with DNA [32]. The experimental data presented in this report confirm that the melting profiles of the HPV L1 gene DNA bound to AAHS particles differ from those of free HPV DNA in solution.

First, the binding site for the degenerate MY09 primer of the HPV L1 gene DNA in the 16 vaccine lots tested is not available for effective PCR amplification. The second unexpected observation is that the coexistent HPV-16 L1 gene DNA cannot be amplified by a pair of GP6/MY11 degenerate consensus primers although the GP6/MY11 primers consistently generate a PCR amplicon from the HPV-11 L1 gene DNA and the HPV-18 gene DNA as a single genotype template or a mixture of the two templates present in one vaccine sample. It is well known that under identical PCR conditions the HPV-16 L1 gene DNA is almost invariably co-amplified along with other HPV DNA in clinical mixed HPV infection specimens [30]. Co-amplification of the L1 gene DNA of HPV-16 and that of HPV-18 by the GP6/MY11 primer pair leading to sequencing failure is a common phenomenon in clinical laboratory practice [29].

The HPV-16 L1 gene DNA in the Gardasil® vaccine requires specially designed non-degenerate PCR primers for detection. A pair of modified non-degenerate GP6/MY11 primers with a 3 to 4-base downstream extension referred to as HPV16MY11+ and HPV16GP6+, respectively (Figure 1), is capable of generating an amplicon of HPV-16 DNA by a same-nested PCR for DNA sequencing validation. However, in one Gardasil® sample (Fig. **2**, #NL39620), even the MY11-binding site of the HPV-16 DNA is not available for amplification. In the latter case, a specially designed HPV16F/HPV16R primer pair (Fig. **1**) has to be used for a same-nested PCR to obtain a useful amplicon for DNA sequencing validation (Fig. **2**, right lower, lane 1a and Fig. **7**). The amplicon terminated by the HPV16F/HPV16R primer pair is positioned between the MY09-binding site and MY11-binding site without the MY09 and MY11 termini (Fig. **1**).

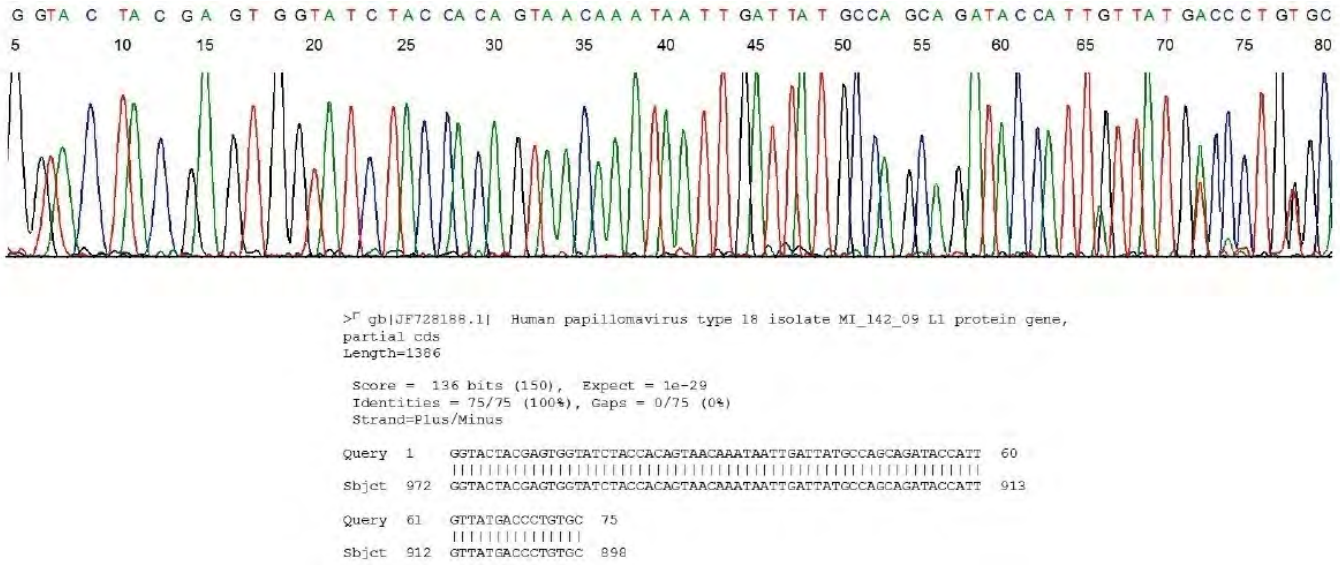

Fig. (4). Base-calling electropherogram of an HPV-18 L1 gene DNA segment amplified by GP6/MY11 PCR primers. An aliquot of the pellet of Gardasil® lot # NL01490 was first amplified by the degenerate MY09/MY11 primers, and then amplified in a nested PCR by a pair of GP6/MY11 degenerate consensus primers. The nested PCR amplicon was sequenced with a GP6 primer. BLAST alignment analysis showed an HPV-18 L1 gene DNA. Note the MY11 primer sequence with its 3 underlined degenerate bases, CCATTGTTATGACCCTGTGC, on the right end of the electropherogram. This MY11 degenerate primer was able to amplify the HPV-18 L1 gene DNA in the Gardasil® vaccine, but not the co-existent HPV-16 L1 gene DNA. As a result, a single PCR amplicon of HPV-18 was generated to be used as the template for this direct DNA sequencing.

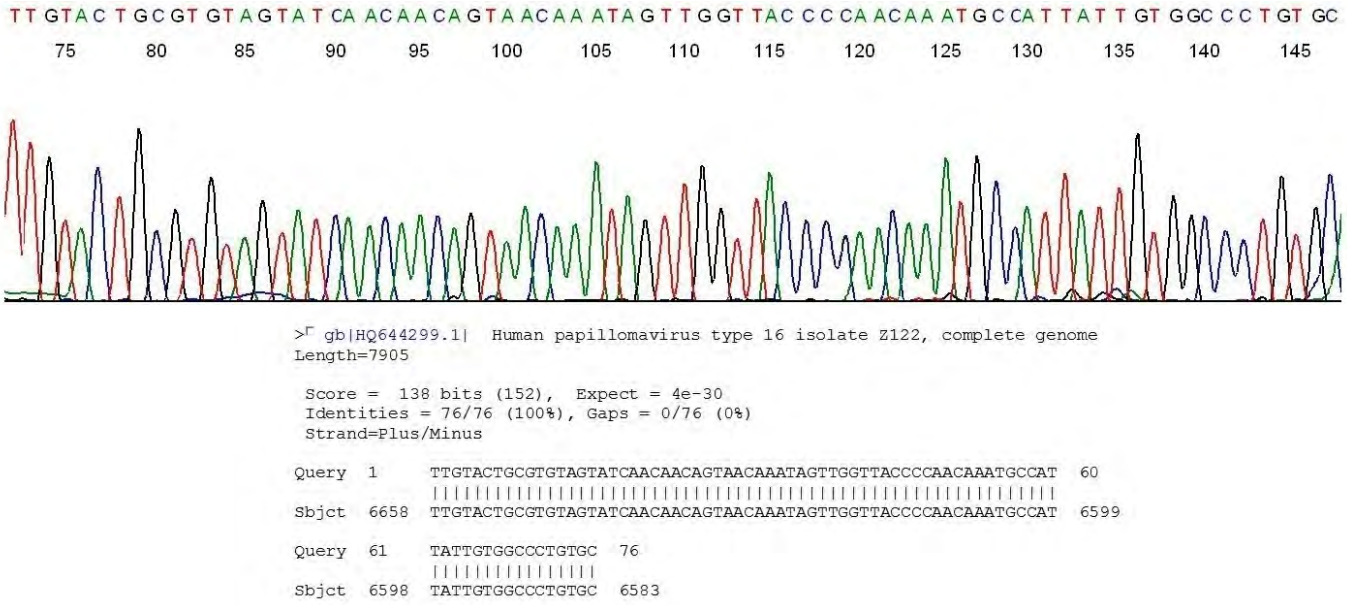

Fig. (5). Base-calling electropherogram of an HPV-16 L1 gene DNA segment amplified by a same-nested PCR with a non-degenerate primer pair. An aliquot of the pellet of Gardasil® lot #NL01490 was amplified by a same-nested PCR with the HPV16MY11+/HPV16GP6+ primer pair (Figure 3, upper half, lane 1). The nested PCR amplicon was sequenced, using HPV16GP6+ as the sequencing primer. BLAST alignment analysis showed an HPV-16 L1 gene DNA. Note the non-degenerate bases of the HPV16MY11+ complementary sequence, ATGCCAT-TATTGTGGCCCTGTGC, on the right end of the electropherogram. Figures 4 and 5 show that the HPV-16 L1 gene DNA in Gardasil® with its special melting profile was not amplified by the commonly used degenerate MY11 primer to interfere with the sequencing base-calling for HPV-18 genotyping (Figure 4). A non-degenerate primer with fully matched bases at the MY11-binding site was needed for the HPV-16 DNA amplification, as demonstrated in this electropherogram.

The procedure developed and reported here uses a LoTemp® ready-to-use PCR mixture containing a highly processive DNA polymerase associated with a proof-reading activity. In this PCR system, the degree of stringency is adjusted by the annealing temperature of the thermocycling steps. A low stringency PCR is conducted with an annealing temperature at 40°C and a high stringency PCR an annealing temperature at 50ºC. While it is preferable to conduct the

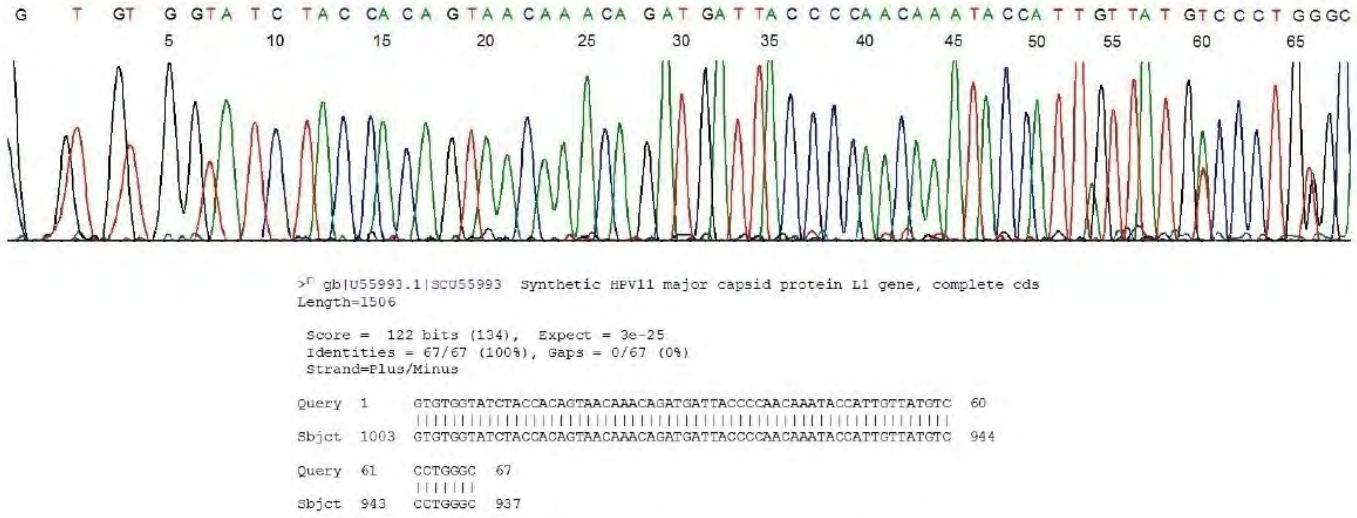

Fig. (6). Base-calling electropherogram of a synthetic HPV-11 L1 gene DNA segment amplified by the GP6/MY11 PCR primer pair. An aliquot of the pellet of Gardasil® lot #NL39620 was first amplified by the degenerate MY09/MY11 primers, and then amplified in a nested PCR by a pair of GP6/MY11 degenerate consensus primers. The nested PCR amplicon was sequenced, using GP6 as the sequencing primer. BLAST alignment analysis showed a synthetic HPV-11 L1 gene DNA. Note the MY11 primer sequence, CCATTGTTATGTCCCTGGGC, with its 3 underlined degenerate bases on the right end of this electropherogram. The MY11 degenerate primer was able to amplify the synthetic HPV-11 L1 gene DNA in the Gardasil® vaccine, but not the co-existent HPV-16 L1 gene DNA. As a result, a single PCR amplicon of a synthetic HPV-11 gene segment was generated to be used as the template for this direct DNA sequencing.

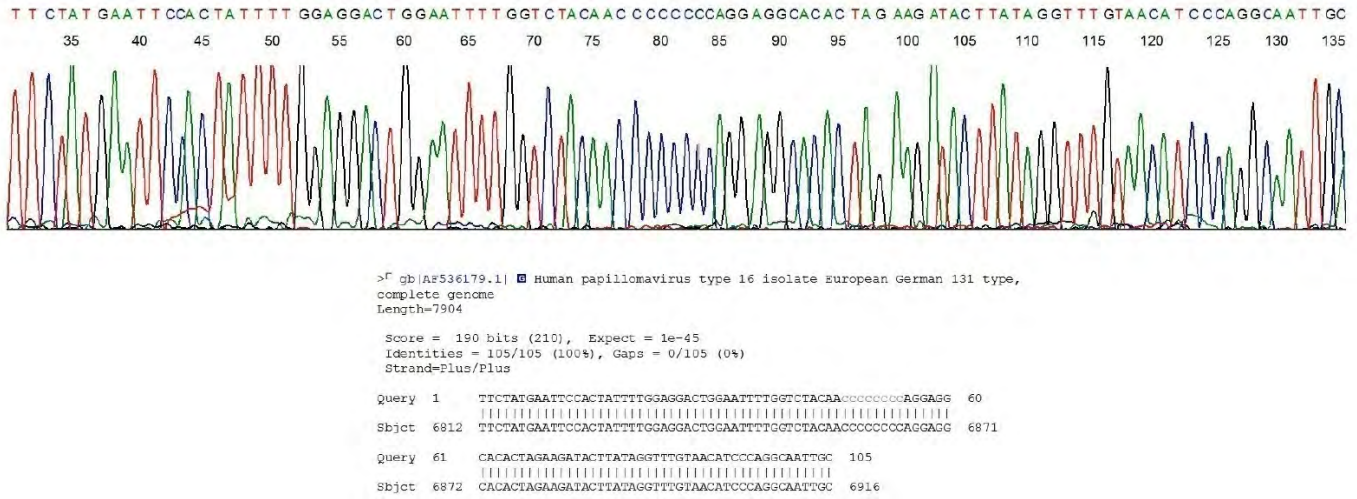

Fig. (7). Base-calling electropherogram of an HPV-16 L1 gene segment amplified by the HPV16F/HPV16R primer pair. Description: An aliquot of the pellet of Gardasil® lot #NL39620 was amplified by a same-nested PCR with a pair of non-degenerate HPV16F/HPV16R primers. The nested PCR amplicon (Figure 2, right panel, lane 1a) was sequenced, using HPV16F as the sequencing primer. BLAST alignment analysis showed an HPV-16 L1 gene DNA. Note the HPV16R primer complementary sequence, GTAACATCCCAGGCAATTGC, on the right end of the electropherogram. The HPV-16 L1 gene DNA in lot #NL39620 with its special melting profile was not amplified by the commonly used degenerate MY11 primer.

GP6/MY11 nested PCR under a low stringency condition for the detection of various genotypes of HPV, the HPV16F/HPV16R primer pair seems to require a high stringency PCR to generate a specific amplicon (Fig. **2**, lanes 1a).

This report represents the first attempt to develop a quality assurance test for residual HPV L1 gene DNA fragments which are in the insoluble fraction of the Gardasil® vaccine [23]. It shows that the GP6/MY11 general consensus PCR primers are capable of amplifying the residues of HPV-11 and HPV-18 L1 gene DNA fragments in the vaccine. But special non-degenerate primers must be used for the HPV-16 DNA amplification. Coexistent HPV-11, HPV-18 and HPV-16 DNA fragments may have different melting profiles when bound to the insoluble AAHS adjuvant, and may require different PCR conditions and special primers for detection. The lack of co-amplification of the HPV-16 gene DNA fragments with a pair of GP6/MY11 degenerate consensus PCR primers along with the HPV-11 or HPV-18 L1 gene DNA fragments co-existent in a vaccine sample indicates that the HPV-16 L1 gene DNA fragments bound to the AAHS particles have assumed a special non-B topological

conformation [33]. AAHS is an adjuvant specially designed for binding phospholipid-containing antigens by ligand exchange of a phosphate group for a hydroxyl group on the adjuvant surface [34]. The phosphate groups on the DNA backbones may bind to the Al^{3+} of the AAHS nanoparticles through the same mechanism [35], forming various non-B topological conformational structures in the dsDNA [36].

After testing 16 samples of Gardasil®, it can be concluded that all samples tested contain residues of synthetic HPV-11 L1 gene DNA and/or HPV-18 gene DNA fragments, including a region defined by the GP6 and MY11 primer termini on both ends. At least 7 of the 16 samples tested also contain HPV-16 L1 gene DNA fragments which can be amplified by a pair of modified non-degenerate primers which anneal at the GP6 and the MY11 binding sites of the HPV-16 template, or a pair of non-degenerate primers positioned between the MY09- and the MY11-binding sites without the MY09 and MY11 termini. Experimental results on testing the remaining samples for the detection of HPV-16 L1 gene DNA are inconclusive due to the limited materials available for method development. It is possible that additional primers may need to be designed to amplify various HPV-16 DNA fragments with different melting profiles in the Gardasil® vaccine. No attempts have been made to amplify the residual HPV-6 L1 gene DNA. If present, the HPV-6 L1 gene DNA in the vaccine is not co-amplified by a pair of GP6/MY11degenerate consensus primers.

Aluminum salts injected as adjuvant can rapidly bind free DNA released from dying host cells [10] and carry the DNA into the cytoplasm of antigen-bearing dendritic cells in promoting MHC class II presentation and enhancing dendritic cell –T-cell interactions as a mechanism of augmenting the immunogenicity of vaccination [37]. On the other hand, residual viral DNA fragments already bound to aluminum adjuvants in the vaccine products, such as those in Gardasil® [23], have received little attention. Routine testing for the presence of residual viral and microbial DNA bound to aluminum adjuvant in vaccines may be warranted for studies of vaccination efficacy and safety because these naked DNA fragments bound to aluminum salts may have topological non-B conformations [33] with different profiles of enzymatic degradation.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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