vaccines

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Manuscript ID	Journal	Section / Special Issue	Title	Status	Submission Date
vaccines-476060	Vaccines		Toll-like receptor 9 agonist in HPV vaccine Gardasil9	Rejected by editor	2019-03-17 18:21:05

Toll-like receptor 9 agonist in HPV vaccine Gardasil9

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Received: date; Accepted: date; Published: date

Abstract: Gardasil9 is a recombinant human papillomavirus (HPV) 9-valent vaccine, containing purified major capsid L1 protein of HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 re-assembled into virus-like particles (VLPs) as the active ingredient. Since the antigens are purified recombinant proteins, Gardasil9 needs a potent adjuvant to enhance the initiation of the immune response through activation of innate immunity of the host to generate high and sustained levels of antibodies for maintaining efficacy of vaccination. Historically, the aluminum salt, amorphous aluminum hydroxyphosphate sulfate or AAHS which is listed as the adjuvant for Gardasil9, was known to require a Toll-like receptor agonist, such as phospholipids, to work in combination to achieve its potent adjuvant effects in the recombinant hepatitis B vaccine, Recombivax HB®. However, there are no phospholipids in the purified HPV L1 proteins or in the Gardasil9 formulation. Since the Food and Drug Administration has informed the public that Gardasil4 does contain recombinant HPV L1-specific DNA fragments, these HPV DNA fragments may serve as Toll-like receptor 9 agonist in Gardasil9 vaccination. The author has tested 5 samples of Gardasil9 from 4 manufacturing lots by PCR amplification with a set of degenerate primers followed by heminested PCR or by another 5 sets of non-degenerate nested PCR primers in an attempt to detect all 9 vaccine-relevant HPV type-specific L1 gene DNAs bound to AAHS in the vaccine. Sanger sequencing of the PCR products confirmed the presence of HPV 18, 11, 16 and 6 L1 gene DNA bound to insoluble AAHS nanoparticles, but unevenly distributed even within one vaccine sample. In addition, these genotype-specific HPV DNA fragments were at least partially in non-B conformations. Since no L1 gene DNA of HPV 31, 33, 45, 52, and 58 was amplified by the commonly used degenerate PCR primers, the results suggest that these latter 5 type-specific HPV DNAs may all be in non-B conformations or have been removed as contaminants by a special purification protocol. Further research is warranted to standardize the HPV DNA fragments in Gardasil which are known to be potent Toll-like receptor 9 agonist.

Keywords: Gardasil9; Gardasil; HPV vaccine; HPV DNA; non-B conformations; topological conformational change; Toll-like receptor 9 agonist; AAHS; amorphous aluminum hydroxyphosphate sulfate; DNA sequencing

1. INTRODUCTION

Human papillomavirus (HPV) is the agent of a common sexually transmitted infection [1]. There are two FDA-approved HPV vaccines, the bivalent vaccine Cervarix and the 4-

valent or 9-valent vaccine Gardasil, for its prevention. Both Cervarix [2] and Gardasil [3] use purified recombinant genotype-specific HPV major capsid L1 proteins assembled in the form of virus-like particles (VLPs) as the active ingredient (the antigen). Since the assembled VLPs are purified recombinant proteins, they are poor immunogens and require the assistance of specially designed adjuvants to generate a robust and persistent immune response as other purified, subunit and synthetic antigens usually do in many newly developed vaccines [4]. In Cervarix, the adjuvant is AS04 [2], a compound created by combining a Toll-like receptor (TLR) 4 agonist MPL (3-O-desacyl-4'-monophosphoryl lipid A) and aluminum hydroxide. MPL is a detoxified derivative of the lipopolysaccharide (LPS) isolated from *Salmonella minnesota* R595 strain and LPS is a specific agonist of TLR 4. In chemical structure, a single negatively charged phosphate of the linear MPL is bound to the cationic aluminum through an ionic bond so that the free molecular chains of LPS can react with TLR 4 of the immune cells. The MPL within AS04 enhances the initiation of the immune response through activation of the innate immunity, leading to an enhanced cellular and humoral adaptive immune response [5].

The adjuvant in Gardasil is amorphous aluminum hydroxyphosphate sulfate (AAHS). Each dose of Gardasil9 contains approximately 500 mcg of AAHS as adjuvant [3]. Both AS04 and AAHS are made from the same starting chemical of aluminum hydroxide [6-9] whose hydroxyl groups have been partially replaced by phosphate-containing molecules, namely by MPL to form AS04 [6] and by an inorganic phosphate to form AAHS [7] through ligand exchange. The crucial difference between AS04 and AAHS is that MPL is a TLR agonist and inorganic phosphate is immunologically inert.

In animal experiments, anti-HPV L1 VLP responses from mice immunized with AAHS-formulated HPV16 vaccine have been shown to be substantially greater than those produced by mice immunized with the same antigen formulated with aluminum hydroxide or with aluminum phosphate [10]. In human studies, vaccination with Gardasil has been shown to induce significantly higher early innate proinflammatory cytokine/chemokine responses than Cervarix in women [11]. The peripheral blood mononuclear cells (PBMCs) of healthy women vaccinated with Gardasil have been shown to be associated with significant changes in the expression and function of immune innate and regulatory receptors [12]. These results indicate that Gardasil is capable of augmenting innate immune response, at least comparable to Cervarix if not in greater magnitude even though its aluminum adjuvant does not contain MPL. A TLR agonist component equivalent to MPL is neither a part of AAHS, nor mentioned in the description for Gardasil [3]. The mechanism by which AAHS exerts its adjuvant effects in Gardasil vaccination is unclear or has not been published.

Aluminum salts, including various forms of aluminum hydroxide and aluminum phosphate, have been used as vaccine adjuvants for over 80 years. However, the mechanisms of their action remain largely unknown and controversial. Recent research progress has led us to believe that pattern recognition receptors (PRRs) of the innate

immune system, particularly TLRs and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), can modulate and control the generation of humoral and cellular immune responses in vaccination [13]. Aluminum salts invariably induce cell damage and local inflammation at the site of injection. It has been suggested that at least as an adjuvant in animal vaccination experiments with protein antigen, the cationic aluminum binds the phosphate backbone of the free DNA released from the dying host cells at the injection site of inflammation and transfect the host nucleic acids into the APCs, exerting its adjuvant effects by activation of STING and IFN regulatory factor 3 (IRF3) [14, 15]. Internalized nucleic acids in the APCs are potent TLR agonists in enhancing the required immune responses in vaccination [16]. Internalization of the aluminum salt particles by immune cells may also lead to phagosomal destabilization resulting in the activation of NLR protein NLRP3 [17], probably by inducing the production of endogenous uric acid, which in turn activates NLRP3 within APCs [18]. All these proposed immunological effects induced by aluminum adjuvants in vaccination follow or are the consequences of generation or release of certain endogenous chemicals as a result of cell damages caused by the aluminum salts at the site of vaccine injection; the real immune mediators are the uric acid and the nucleic acids from the host cells, not the aluminum salt itself. Based on the studies of Cervarix, HPV vaccines need an exogenous, pre-made, ready-to-use, instant potent TLR agonist immediately available at the time of vaccination to enhance the innate immune responses of the host to overcome the poor immunogenicity of the purified HPV L1 proteins re-assembled as VLPs during vaccine manufacturing [19, 20]. Such a TLR agonist has not been listed in Gardasil formulation [3].

Previous testing of 16 samples from different vaccine lots revealed that Gardasil4 contains fragments of HPV L1 gene DNA firmly bound to the insoluble, proteinase-resistant fraction of the vaccine, presumably AAHS nanoparticles [21]. Since free DNA released from dying host cells and bound to aluminum salts at the site of vaccine injection is known to be transfected 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 [14, 15], the HPV L1 gene DNA fragments bound to AAHS in Gardasil4 must have provided such an instant premade TLR 9 agonist which is needed to enhance the initiation of the immune response through activation of the innate immunity, leading to an enhanced cellular and humoral adaptive immune response in Gardasil vaccination. However, for efficacy and safety of HPV vaccination, the types and quantity of HPV L1 gene DNA as TLR agonist have not been defined and standardized for Gardasil as MPL for Cervarix. This article reports the technical challenges in using a routine diagnostic PCR protocol for detection of the genotype-specific HPV L1 gene DNAs bound to AAHS in the HPV vaccine Gardasil9.

2. MATERIALS AND METHODS

2.1. Gardasil9 vaccine samples

A total of 5 Gardasil9 vials or manufacturer-prefilled vaccine syringes with intact original packages were submitted to the author's laboratory by health care professionals to be tested for the presence of HPV L1 gene DNA fragments at the request of their patients or the guardians of their patients. The lot numbers printed on the labels of these vaccine samples were N020139, K001502(x 2, registered as A and B for testing), R000303 and M045743.

2.2. PCR and sequencing primers

The sequences of the well characterized MY09 and MY11 degenerate primers and the GP6 primer for PCR amplification of a conserved segment of the HPV L1 gene in routine Sanger-sequencing-based diagnostics [22] were:

MY09 forward = 5'-CGTCCMARRGGAWACTGATC-3'
MY11 reverse = 5'-GCMCAGGGWCATAAYAATGG-3' (also in heminested PCR)
GP6 forward heminested = 5'-GAAAAATAAACTGTAAATCA-3'

The sequences of additional non-degenerate nested PCR reverse primers, referred to as primer R16, R31, R45, R52 and R58, were listed under the appropriate Results section in this article.

All primers were diluted in TE buffer pH 7.4 (Sigma Chemical Co., St. Louis, MO) to a 10 μmolar working solution.

2.3. Preparation of Samples for PCR

After the contents of the vaccine samples were mixed well, an aliquot of 100 μL of the vaccine suspension was centrifuged at ~16,000 × g for 10 min in a 1.5 mL microcentrifuge tube. The pellet was re-suspended and washed twice with 1 mL of 70% ethanol each and the final ethanol suspension was centrifuged at ~16,000 × g for 5 min. The washed pellet was air-dried. The dried pellet was re-suspended in 100 μL of 0.1 mg/mL proteinase K (Sigma Chemical Co., St. Louis, MO) in a buffer consisting of 50 mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.1. The mixture was digested at 45˚C - 55˚C overnight and was exhaustively washed with the same Tween 20 buffer pH 8.1, 4 times, 1 mL each time. After heating at 95˚C for 10 min to inactivate any residual proteinase K, a 1 μL aliquot of the washed and heated particle suspension was used to initiate each primary PCR with a pair of MY09/MY11 degenerate primers followed by a GP6/MY11 heminested PCR or a set of nested PCRs.

2.4. PCR Amplification of HPV L1 Gene DNAs for Sanger sequencing

For the primary PCR, 1 μL aliquot of the washed and heated vaccine particle suspension, 20 μL of LoTemp® master mix containing manufacturer-optimized HiFi®

DNA polymerase, magnesium ions, denaturing agents, and dNTPs with stabilizing additives (HiFi DNA Tech, LLC, Trumbull, CT, USA), 1 µL of 10 µmolar MY09 primer, 1 µL of 10 µmolar MY11 primer and 2 µL of molecular grade water were mixed in a final volume of 25 µL in a thin-walled PCR tube for low temperature PCR amplification. The LoTemp® thermocycling steps were set for an initial heating at 85˚C for 10 min, followed by 30 cycles, each set 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. A trace of each of the primary PCR products (about 0.2 µL) was transferred by a micro-glass rod to another 25 µL complete PCR mixture containing 20 µL of ready-to-use LoTemp® PCR mix, 1 µL of 10 µmolar GP6 forward primer, and 1 µL of 10 µmolar reverse primer and 3 µL of water for heminested PCR or nested PCR. After completion of the primary and the nested PCR, a 5 µL aliquot of the PCR products was pipetted out from each tube and mixed with 2 µL loading fluid for electrophoresis in a 2% agarose gel containing ethidium bromide. The gel was examined under UV light for the PCR product bands in the agarose gel. An HPV 16 plasmid DNA positive control and a no sample negative control (1 µL of water added instead of sample) were included in each primary and heminested or nested PCR run.

2.5. Direct Automated DNA Sequencing of the heminested or nested PCR amplicons

For DNA sequencing, a trace of the positive nested PCR products (about 0.2 µL) was transferred directly with a micro-glass rod from the heminested or nested PCR tube into a 20 µL volume of a cycle sequencing reaction mixture consisting of 14.5 µL water, 3.5 µL of 5 × buffer, 1 µL of BigDye Terminator 1.1 (Applied Biosystems) and 1 µL of 10 µMolar sequencing primer solution in TE buffer. 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 or an Applied Biosystems SeqStudio Genetic Analyzer for sequence analysis. Alignment analysis of a 45 - 60 base sequence in the hypervariable region of the L1 gene excised from the computer-generated base calling electropherogram was performed against various standard HPV genotype sequences retrieved from the GenBank, using the on-line BLAST (Basic Local Alignment Search Tool) system to validate the specific HPV genotyping and for visual sequence analyses. Throughout the entire period when this study was carried out, no routine diagnostic HPV tests were performed in the laboratory and the procedures of sample preparation for primary PCR, nested PCR and DNA sequencing were performed in different rooms to avoid cross contamination by HPV DNA from other sources.

3. RESULTS

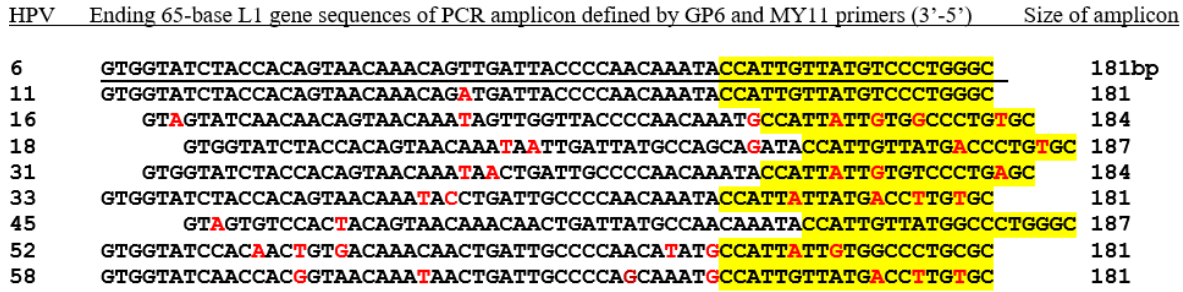
3.1. Short-segment L1 gene DNA sequence analysis for HPV genotyping

Based on alignment of a highly conserved sequence with hypervariable regions of the HPV L1 gene retrieved from the GenBank database, the L1 gene of HPV 6 (KX514429), HPV 11 (U55993), HPV 16 (AF125673), HPV 18 (EF202155), HPV 31 (KX638481), HPV

33 (KU550675), HPV 45 (KU049756), HPV 52 (LC373207) and HPV 58 (KY225967), the 9 HPV genotypes included in Gardasil9, can be reliably diagnosed by BLAST analysis of a 45-base sequence immediately downstream of the 20-base degenerate MY11 primer site. The size of the amplicon defined by the GP6 and MY11 primers of these HPV genotypes varies from 181 bp to 187 bp [22], as shown in Figure 1.

Figure 1 Alignment of the ending 65-base sequences of the 181-187 bp amplicons of the Gardasil9 HPV L1 genes defined by GP6/MY11 heminested PCR primers. The MY11 degenerate primer binding sites are yellow-highlighted. The letters in red color represent single nucleotide polymorphisms which can be used to distinguish the sequences of other HPV genotypes from that of HPV 6 and from one another.

Figure 1



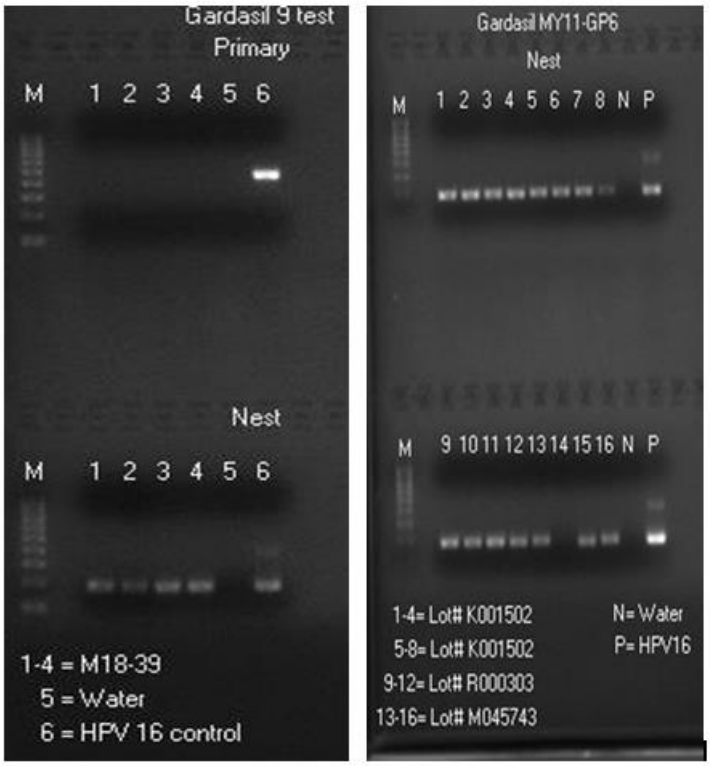
3.2. Selective amplification of HPV 18 and HPV 11 DNA

Since most invasive cervical cancers are associated with or preceded by persistent infection by one of numerous genotypes of HPV [23, 24], laboratory tests for HPV in specimens obtained from patients have been developed to amplify all clinically relevant HPV genotype L1 gene DNAs by MY09/MY11 degenerate primer PCR followed by GP6/MY11 heminested PCR for initial detection. DNA sequencing is performed on a PCR amplicon for accurate genotyping in follow-up of the patients with persistent HPV infection [22, 23]. Theoretically, Gardasil9 may contain 9 genotype-specific HPV L1 gene DNAs, and all 9 genotypes of HPV L1 gene DNA were expected be co-amplified by the degenerate MY09/MY11 primary PCR primers and the GP6/MY11 heminested PCR primers if these DNAs were in B conformation.

As demonstrated in Figure 2, using 1 μL of washed and heated insoluble nanoparticle suspension as the template to initiate each MY09/MY11 primary PCR followed by GP6/MY11 heminested PCR invariably generated a 181-187 bp HPV L1 gene DNA amplicon, indicating that the HPV L1 gene DNA fragments in Gardasil9 were firmly bound to AAHS nanoparticles, the only water-insoluble and proteinase K-resistant ingredient in the vaccine formulation [3].

Figure 2 Image of agarose gel electrophoresis showing products of HPV DNA primary and heminested PCR products in the left panel and heminested PCR products only on the right panel. There were four duplicate PCR sets on each of the 5 Gardasil9 digestates.

Left panel: Lanes 1-4 = Lot #N020139 (labeled M18-39) showing 4 invisible MY09/MY11 primary PCR products (upper) and 4 GP6/MY11 heminested PCR bands (lower).
Right panel: GP6/MY11 heminested PCR products only. Lanes 1-4 = Lot #K001502(A); Lanes 5-8 = Lot #K001502(B); Lanes 9-12 = Lot #R000303; Lanes 13-16 = Lot #M045743.
N=negative, no sample control. P= HPV 16 positive control.



Sanger sequencing with GP6 primer carried out on all these 20 GP6/MY11 heminested PCR products showed a segment of HPV 18 L1 gene sequence (Figure 3) in 1 of the 4 heminested PCR tubes of Lot #N020139, in 1 of the 8 heminested PCR tubes of Lot #K001502, in 1 of the 4 heminested PCR tubes of Lot #R000303, and in 2 of the 4 heminested PCR tubes of Lot #M045743. A sequence of synthetic HPV 11 L1 gene DNA (Figure 4) was generated with the heminested PCR products in 1 of the 4 tubes of Lot #M045743. In other words, Sanger sequencing of 20 heminested PCR products generated only 6 readable DNA sequences. Five of the 6 sequences (5/6) were those of HPV 18 and

one (1/6) was that of HPV 11(synthetic).

Figure 3 Electropherogram of a segent of HPV 18 L1 gene (Seq ID: EF202155)-MY11 primer underlined.

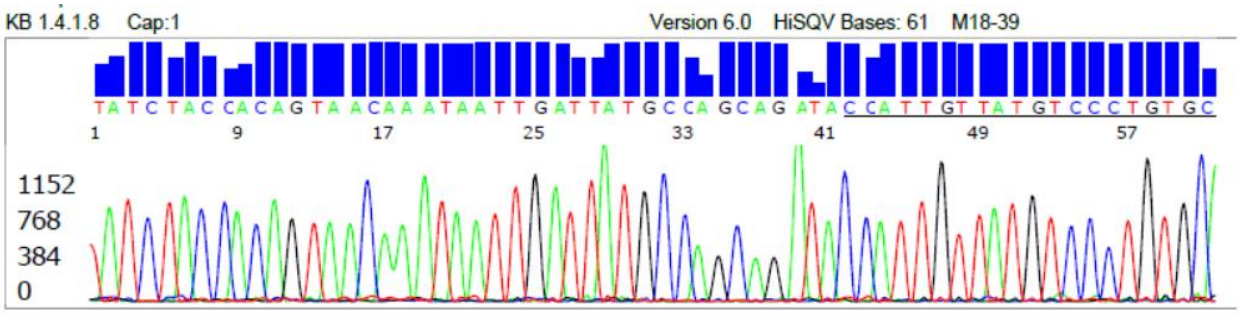
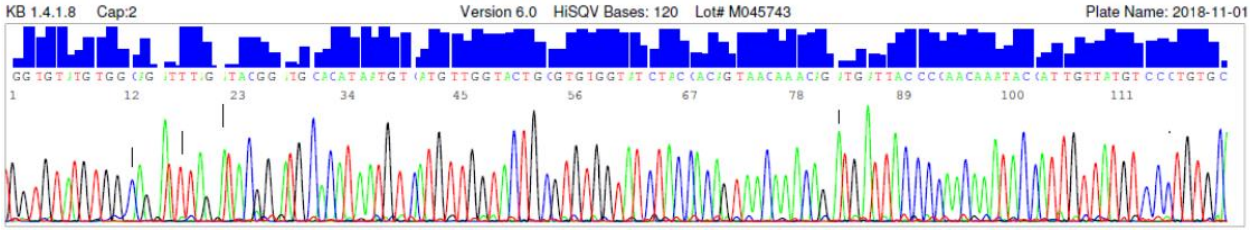


Figure 4 HPV 11 – a segment of DNA modified from HPV 6 L1 gene DNA (Seq ID:KX14429) with one A>C, one G>T and two T>A mutations at positions indicated by 4 arrows in creating a synthetic HPV 11 L1 gene (Seq ID: U55993) for vaccine manufacturing.



3. 3. Multiple HPV DNA sequences generated by MY09/MY11 degenerate primers

Sequencing with GP6 primer of the 14 GP6/MY11 primer heminested PCR products other than those 6 mentioned above yielded 13 mixed HPV L1 gene DNA sequences. The heminested PCR products shown in Lane 14 (Figure 2) did not generate a sequence (1 of 4 aliquots from Lot #M045743).

The 13 mixed DNA sequences could be separated into two patterns, each consisting of at least two mixed amplicons, one being 6 bases longer than the other(s), as shown in Figure 5, and one being 3 bases longer than the other(s) as shown in Figure 6.

Figure 5 Mixed HPV DNA sequences. One was 6 bases longer than the other(s). The MY11 primer of the shorter sequence started from the underlined base "C" at position 59, reading right to left.

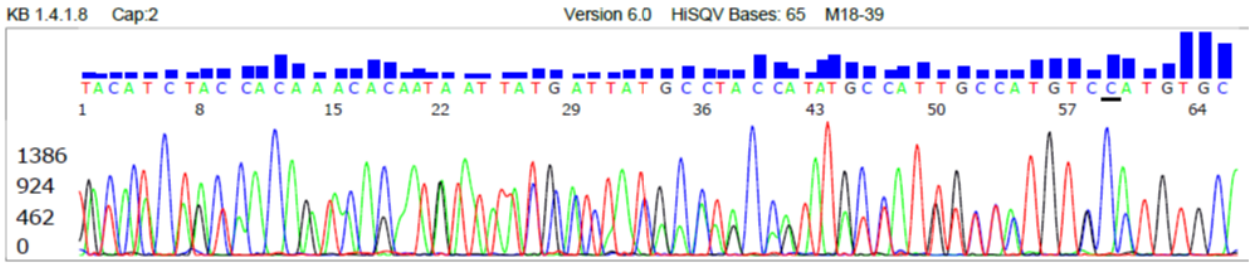
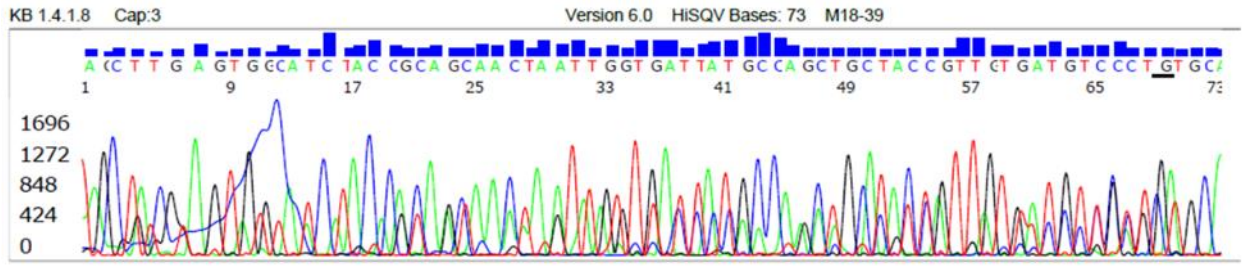


Figure 6 Mixed HPV DNA sequences. One was 3 bases longer than the other(s); the first base "C" of the MY11 primer of the shorter amplicon was overshadowed by a big "G" (underlined) of the longer amplicon 3 bases downstream of its first base "C" at position 72.



According to the sequence alignment in Figure 1, the unreadable superimposed sequences illustrated in Figure 5 must represent the sequence of an HPV 18 PCR amplicon plus one or more of the 5 HPV genotypes with a 181 bp-long PCR amplicon, all defined by the GP6 and MY11 primer binding sites. By the same token, the electropherogram of Figure 6 indicates that there were at least two amplicons in the PCR products; at least one was 3 bases longer than the other(s). Based on analysis the terminal sequences of the electropherograms of Figures 5 and 6, there were at least 3 genotype-specific HPV L1 gene DNA amplicons in the MY09/MY11 primary PCR and the GP6/MY11 heminested PCR products illustrated in Figure 2. One of the 3 was HPV 18, and at least one was an HPV L1 gene DNA with 3 bases shorter and another with 6 bases shorter than HPV 18 in their PCR amplicon sizes defined by the GP6 and MY11 primers.

3. 4. No amplification of HPV 31, 33, 45, 52 and 58 L1 gene DNA by MY09/MY11 degenerate PCR primers

In order to test if there were any L1 gene DNA amplicons of the HPV 31, 33, 45, 52 and 58 genotypes in the MY09/MY11 primary PCR products, each of the 14 primary

PCR products generated (see Section ***3.3.)*** which did not yield a single heminested PCR amplicon for successful Sanger sequencing was re-amplified in 5 sets of nested PCRs, using the combination of a GP6 forward primer and one of the following non-degenerate reverse primers for each nested PCR set.

R16: 5'-AATGGCATTTGTTGGGGTAAC for the binding site **3′-GTTACCCCAACAAATGCCATT**

R31: 5′-GCTCAGGGACACAATAATGGT 3′-ACCATTATTGTGTCCCTGAGC

R45: 5′-ATAACAATGGTATTTGTTGGC 3′-GCCAACAAATACCATTGTTAT

R52: 5′-GCGCAGGGCCACAATAATGGC 3′-GCCATTATTGTGGCCCTGCGC

R58: 5′-GGTCATAACAATGGCATTTGC 3′-GCAAATGCCATTGTTATGACC

These 5 non-degenerate reverse PCR primers were located internal of the MY11 primer binding site of each HPV L1 gene and were designed to match a segment of the targeted type-specific HPV DNA (Figure 1). Since the last 9 nucleotides at the 3'end sequence of primer R31 designed for HPV 31 DNA amplification are identical to the sequence of HPV 33 in the corresponding position, no separate reverse primer for HPV 33 amplification was considered necessary.

After completion of all 70 (14x5) nested PCRs, each of the 13 primary PCR products which led to a visualized heminested PCR product band consisting of multiple sequences (refer to Section 3.2. above) yielded 5 HPV nested PCR product bands at gel electrophoresis, as expected. The primary PCR products as shown in Lane 14, Figure 2 which yielded no visible heminested PCR band also generated no visible nested PCR products. All 70 nested PCR products, regardless of yielding a visible band on gel electrophoresis or not, were subjected to Sanger sequencing with GP6 primer. Visual and BLAST analyses of these Sanger sequencing results did not reveal any PCR amplicons of L1 gene DNA of HPV 31, 33, 45, 52 or 58 in the MY09/MY11 primary PCR products which could be selectively amplified by a pair of non-degenerate nested PCR primers for a successful DNA sequencing. However, these non-degenerate nested PCR primers did selectively re-amplify some of the L1 gene DNA amplicons of HPV 6, 11, 16 or 18 to be used as templates for Sanger sequencing from the MY09/MY11 primary PCR products containing mixed genotype DNAs.

3.4.1. In the absence of HPV 16 DNA, primer R16 amplified HPV 6 and HPV 11 L1 DNA

Figure 7 When HPV 16 DNA was present in the mixed genotype MY09/MY11 primary PCR products, the non-degenerate R16 primer was able to selectively amplify the HPV 16 DNA for Sanger sequencing. The R16 primer is 15 bases internal to the MY 11

primer-binding site (see Figure 1) and fully matches the natural HPV 16 binding site sequence (underlined in the electropherogram below).

Natural HPV 16 sequence at primer binding site: GTTACCCCAACAAATGCCATT

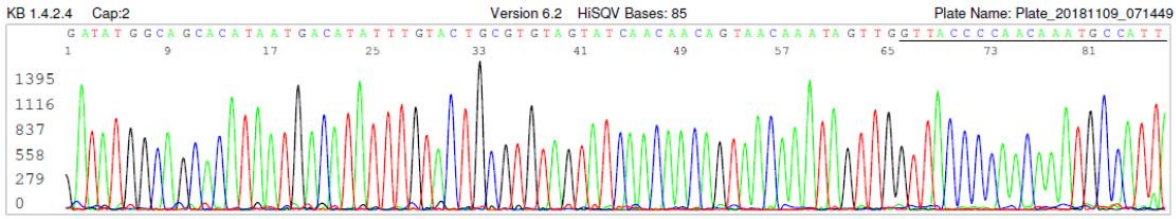


Figure 8 When HPV 16 DNA was absent in the mixed genotype primary PCR products, the non-degenerate R16 primer might anneal to a segment of HPV 6 L1 gene DNA to generate a template for Sanger sequencing even though there were two mismatched nucleotides between primer R16 and the template primer blinding site with one mismatch being at the 3' terminus (primer R16 underlined in electropherogram). The HPV 6 natural primer binding site sequence is placed over the R16 primer with mismatched nucleotides in red color as follows.

Natural sequence of HPV 6 at primer binding site: ATTACCCCAACAAATACCATT

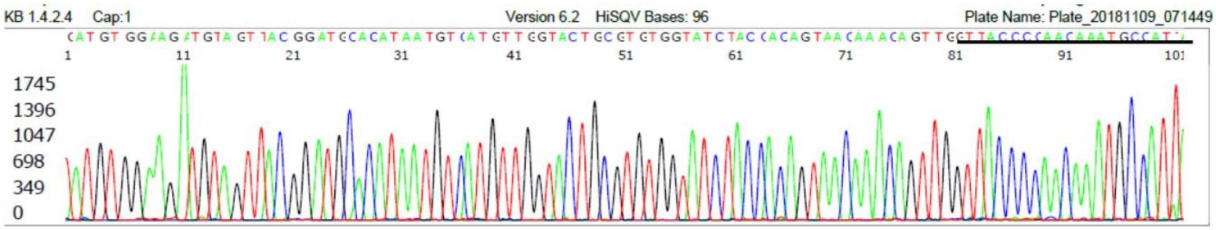
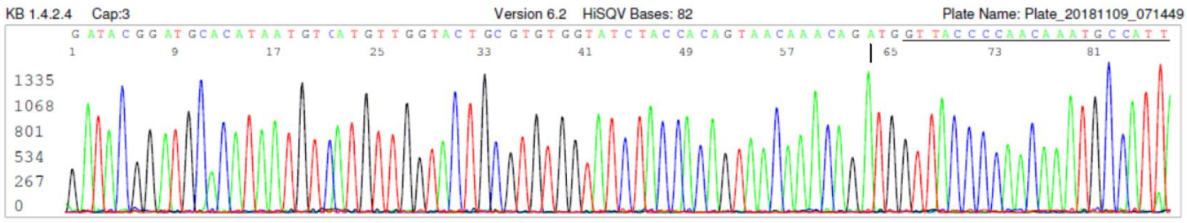


Figure 9 When HPV 16 DNA was absent in the mixed genotype primary PCR products, the non-degenerate R16 primer might anneal to a segment of HPV 11 L1 gene DNA to generate a template for Sanger sequencing even though there were two mismatched nucleotides between primer R16 and the template primer blinding site with one mismatch being at the 3' terminus. Note: The sequence of the synthetic HPV 11 L1 gene and the natural HPV 6 L1 gene have the same DNA sequence in this segment except for a T>A mutation indicated by an arrow in the electropherogram illustrated in Figure 9 below.

Natural sequence HPV 11 at primer binding site: ATTACCCCAACAAATACCATT

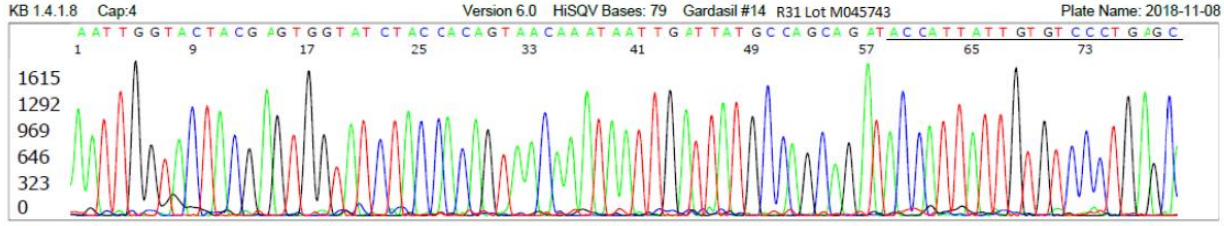


3.4.2. Topological conformational change at the primer binding site led to PCR failure

As for all other Gardasil9 samples, four 1µL aliquots were pipetted from one 100µL AAHS suspension derived from a sample of Lot #M045743, to initiate 4 individual MY09/MY11 primary PCRs, followed by 4 corresponding GP6/MY11 heminested PCRs. The results were shown by gel electrophoresis in Lanes 13-16, Figure 2. The MY09/MY11 primary PCR products which generated no visible GP6/MY11 heminested PCR product band in Lane 14 (Figure 2) were re-amplified by a set of 5 pairs of non-degenerate nested PCR primers, and the nested PCR products were re-sequenced with GP6 primer as described above even though the nested PCR products were not visible at gel electrophoresis. Three (3) DNA sequences ending with non-degenerate primer R31, R45 and R58 were generated from the 5 nested PCR amplicons derived from the Lane 14 primary PCR products. These 3 sequences are illustrated in Figures 10, 11 and 12 as follows.

Figure 10 DNA sequencing electropherogram of a GP6/R31 nested PCR amplicon generated from Lane 14 MY09/MY11 primary PCR products, showing a sequence of HPV 18 L1 gene DNA amplified by primer R31. The R31 sequence was underlined and had one extra nucleotide "A" at the 3' end compared to the degenerate MY11 primer for HPV 18 shown in Figure 3. The natural sequence of HPV 18 has 4 mismatched bases (in red color) against the R31 primer.

Natural HPV 18 sequence at MY11 primer binding site: CCATTGTTATGACCCTGTGC



This sequence (Figure 10) indicates that the HPV 18 DNA in 1 of the 4 aliquots from Lot #M045743 was not exponentially amplified by the MY11 degenerate primer **as** the HPV 18 DNA in other aliquots from the same vaccine sample. An R31 primer with a 3'-ACCATT end instead of the MY11 primer with a 3'-CCATT end was needed to yield an HPV 18 PCR amplicon in this aliquot to be used as the template for DNA sequencing. It was previously reported that non-degenerate HPV 16 MY11 primer with 3'-end extension was required to amplify some of the HPV 16 L1 DNA fragments bound to AAHS in Gardasil4 to generate a visible PCR amplicon for Sanger sequencing because binding of the HPV dsDNA to aluminum salts may cause topological conformational changes at the MY11 primer binding site, turning a segment of the dsDNA into a non-B conformation [25, 26].

It was also found that in the same primary PCR products described above there were DNAs other than those of HPV 18 whose sequence was shown in Figure 10. As illustrated in Figures 11 and 12 below, the R45 and R58 primers, both shifted internally from the MY11 primer binding site, when pairing with the GP6 primer, were able to re-amplify more than one HPV type-specific DNAs which had been prematurely terminated during MY09/MY11 primary PCR due to topological conformational changes at the 3' end of the MY11 primer site.

Figure 11 Nested PCR with R45 primer which was shifted 10 nucleotides inward compared to the primer used for Figure 10 yielded more than one type of HPV L1 gene DNAs. Note: The underlined R45 primer in the electropherogram had two mismatches (in red color) against the natural HPV 18 DNA primer binding site in this location.

Natural HPV 18 sequence at primer binding site: GCCAGCAGATACCATTGTTAT

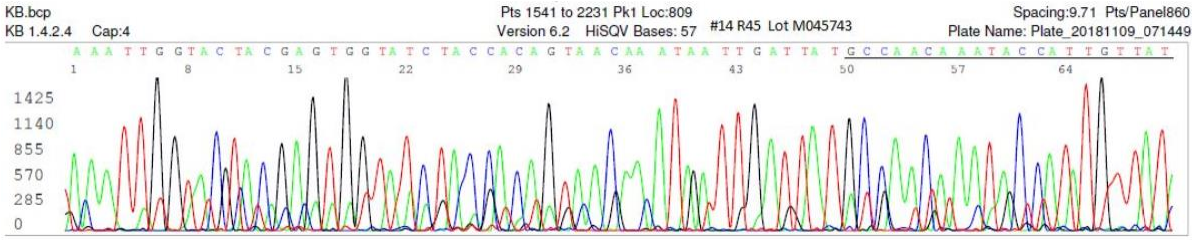
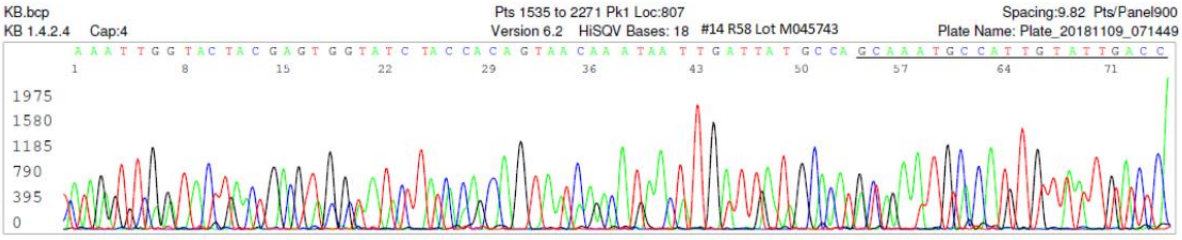


Figure 12 Nested PCR with R58 primer which was shifted 6 nucleotides inward compared to the primer used for Figure 10 yielded also more than one type of HPV L1 gene DNAs. Note: The underlined R58 primer in the electropherogram had two mismatches (in red) with the natural HPV 18 DNA primer binding site in this location.

Natural HPV 18 sequence at primer binding site: GCAGATACCATTGTTATGACC



To summarize briefly: Most HPV 18 L1 gene DNA fragments bound to AAHS in Gardasil were in B conformation and readily amplified by MY09/MY11 degenerate primary PCR primers and by the subsequent GP6/MY11 heminested PCR primers to produce one dominant HPV 18 PCR amplicon as shown in Figure 3, or as one of multiple PCR amplicons as shown in mixed sequences (Figure 5). However, in 1 of 4 tested aliquots from Gardasil9 Lot M045743, the HPV 18 DNA could not be exponentially amplified by the degenerate MY11 primer. The sequencing data presented above showed that replacing the MY11 primer with a non-degenerate primer to re-amplify the primary PCR products yielded templates for GP6 primer sequencing with 5 different results as follows.

```
AACAAATAATTGATTATGCCAGCAGATACCATTGTTATGACCCTGTGC-3' HPV18 (ID#EF202155)
                              CCATTGTTATGTCCCTGTGC  MY11 No template generated
              GTTACCCCAACAAATGCCATT                 R16  No template generated
                            ACCATTATTGTGTCCCTGAGC  R31  Figure 10
                  GCCAACAAATACCATTGTTAT             R45  Figure 11
                            GCCATTATTGTGGCCCTGCGC  R52  No template generated
                     GCAAATGCCATTGTTATGACC          R58  Figure 12
```

In the sequence alignment presented above, nucleotide mismatches in each primer against the natural HPV 18 sequence in this region were typed in red color. The highly conserved sequence CCATT for all HPV L1 genes were highlighted yellow. PCR primer needs at least 6 matched nucleotides with the template to initiate a chain reaction [27]. Mismatch at the 3' terminus is usually not tolerated for PCR amplification. The fact that the MY09/MY11 primary PCR products of the HPV 18 L1 DNA segment in this particular sample aliquot was re-amplifiable by primer 31, primer 45 and primer 58, but not by primer MY11 to generate a template for Sanger sequencing indicates that there was a topological conformational change at the MY11 primer binding site of the AAHS-bound HPV 18 L1 gene DNA, rendering a portion of its DNA with the sequence GTTATGACCCTGTGC (underlined) unavailable for template-directed enzymatic DNA synthesis. Moving the PCR primer inward was necessary to provide a stable primer/template duplex to initiate a template-directed enzymatic primer extension.

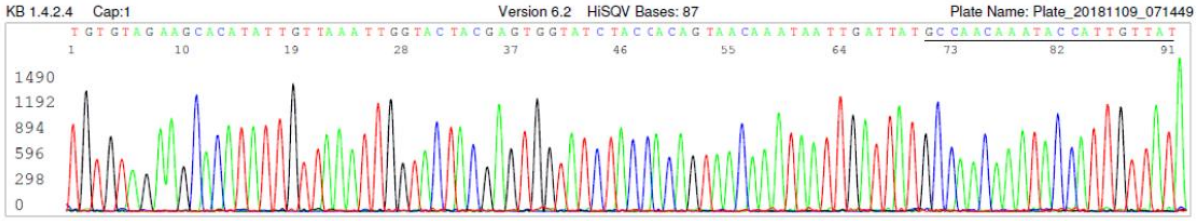
3.4.3. In the absence of HPV 45 DNA, primer R45 amplified HPV 18 DNA

When HPV 45 DNA was absent in the mixed genotype MY09/MY11 primary PCR products, the non-degenerate R45 primer might anneal to a segment of HPV 18 L1

gene DNA to generate a template for Sanger sequencing. There are only two mismatched nucleotides between the R45 sequence and the natural HPV 18 L1 gene sequence at the primer binding site (Figure 13).

Figure 13 The HPV 18 DNA in a mixed genotype MY09/MY11 primary PCR products was amplified by a non-degenerate primer R45 (underlined).

Natural HPV 18 sequence at primer binding site: GCCAGCAGATACCATTGTTAT

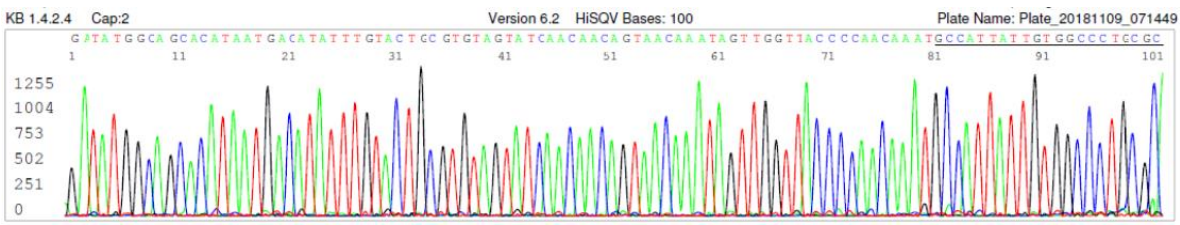


3.4.4. In the absence of HPV 52 DNA, primer R52 amplified HPV 16 DNA

When HPV 52 DNA was absent in the mixed genotype MY09/MY11 primary PCR products, the non-degenerate R52 primer might anneal to a segment of HPV 16 L1 gene DNA to generate a template for Sanger sequencing. There is only one mismatched nucleotide (typed in red) between the R52 sequence and the natural HPV 16 L1 gene sequence at the primer binding site (Figure 14).

Figure 14 The HPV 16 DNA in a mixed genotype MY09/MY11 primary PCR products was amplified by a non-degenerate primer R52 (underlined).

Natural HPV 16 sequence at primer binding site: GCCATTATTGTGGCCCTGTGC

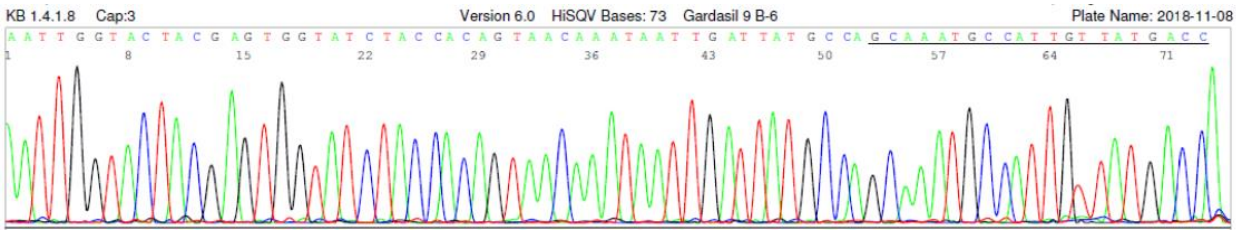


3.4.5. In the absence of HPV 58 DNA, primer R58 amplified HPV 18 DNA

When HPV 58 DNA was absent in the mixed genotype MY09/MY11 primary PCR products, the non-degenerate R58 primer might anneal to a segment of HPV 18 L1 gene DNA to generate a template for Sanger sequencing. There are only two mismatched nucleotides (typed in red) between the R58 sequence and the natural HPV 18 L1 gene sequence at the primer binding site (Figure 15).

Figure 15 The HPV 18 DNA in a mixed genotype MY09/MY11 primary PCR products was amplified by a non-degenerate primer R58 (underlined).

Natural HPV 18 sequence at primer binding site: GCAGATACCATTGTTATGACC



4. Discussion

4.1. HPV L1 gene DNA bound to AAHS in Gardasil9

As advised by the FDA, Gardasil contains recombinant HPV L1-specific DNA fragments. These HPV DNA fragments are not contaminants [28].The current study based on testing 5 Gardasil9 samples and a previous report based on testing 16 Gardasil4 samples [21] confirm that both Gardasil4 and Gradasil9 contain type-specific HPV L1 gene DNA fragments. Since these DNA fragments were found to be in the water-insoluble AAHS particles which were proteinase K-resistant and the DNA remained bound to the proteinase-digested particles after exhaustive washings in TE buffer with detergent Tween 20, the HPV DNA detected must be bound to AAHS via ligand exchange as the phospholipids bound to AAHS in creation of a potent adjuvant for the recombinant hepatitis B vaccine, Recombivax HB® [7]. Among the officially listed ingredients of Gardasil9 which include VLPs, AAHS, sodium chloride, L-histidine, polysorbate 80, sodium borate, yeast protein and water for injection [3], AAHS is the only water-insoluable, proteinase-resistant component.

4.2. Most HPV L1 gene DNA fragments bound to AAHS are in non-B conformations

Multi-valent Gardasil vaccines are produced by separate fermentation and the VLPs of each HPV type are adsorbed on AAHS before the monovalent bulk adsorbed products are combined [3, 8]. As recombinant HPV L1 gene DNA fragments are not contaminants,

they are not targets for removal as for other contaminants during vaccine manufacturing. Therefore, 9 type-specific HPV L1 gene DNAs are expected to be present in the 9-valent vaccine Gardasil9. However, as demonstrated in the current study routine MY09/MY11 degenerate primer PCR amplification was able to generate only amplicons of HPV 18, 11, 16 and 6 for sequencing validation after testing 5 samples of Gardasil9. As those in Gardasil4 [21], HPV 18 and HPV 11 L1 gene DNAs in Gardasil9 are most commonly detected, suggesting that these two types of HPV DNA are more likely in B conformation when bound to the AAHS particles. However, as illustrated in Figures 10-12, even HPV 18 DNA can undergo topological conformational change which may interfere with template-directed enzmatic DNA synthesis during PCR amplfication. Successful generation of one single HPV DNA amplicon by PCR as the template for Sanger sequencing does not exclude the possibility that there are other genotype-specific HPV DNAs also in the tested sample. Previous studies on Gardasil4 samples showed that the AAHS-bound HPV 16 and HPV 6 genotype-specific L1 gene DNAs were not amplifiable by MY09/MY11 degenerate PCR primers [21, 25, 26]. The current study on Gardasil9 samples shows that using non-degenerate primer nested PCRs and shifting the primer binding sites inwards could amplify some of the AAHS-bound HPV 16 and HPV 6 type-specific L1 gene DNAs in Gardasil9 which had been replicated by the MY09 degenerate primer as linear PCR amplification products. The failure to detect any type-specific L1 gene DNA of HPV 31, 33, 45, 52 and 58 suggests that all these 5 type-specific DNAs were in non-B conformations. Alternatively, all the L1 gene DNA fragments of the these 5 HPV genotypes in the 4 tested lots of Gardasil9 have been removed as "contaminants" during the manufacturing process.

4.3. Topological conformational change of HPV DNA bound to AAHS is genotype-dependent

In all tested aliquots of 5 Gardasil9 samples from 4 vaccine lots, HPV 18 and/or HPV 11 L1 gene DNA fragments can be amplified by the MY09/MY11 degenerate PCR primers, as reported previously on Gardasil4 [21]. Only rarely, as shown in Figures 10-12, HPV 18 L1 gene DNA in a fraction of the Gardasil9 shows a topological conformational change. In contrast, the HPV 16 L1 gene DNA fragments were not exponentially amplifiable by the MY09/MY11 degenerate primers, and require non-degenerate primers with a 3'end extension or primers targeting another segment of L1 gene for PCR amplification as reported previously on Gardasil4 [25, 26]. In the current study, a non-degenerate primer shifted 15 nucleotides inward (R16) at the MY11 binding site generated an HPV 16 nested PCR amplicon for Sanger sequencing validation (Figure 7). An HPV 16 amplicon was also generated when an extra "G" nucleotide was added to the 3' end of the MY 11 primer (R52), as shown in Figure 14. These results suggest that topological conformational change occurred in the HPV 16 MY11 primer binding site 5 nucleotides upstream of the 3' terminus because at least a 6-base matched sequence at 3'end of the primer is needed for template-directed primer extension in enzymatic DNA synthesis [27]. Apparently, when the phosphate backbone of the HPV DNA binds the AAHS, the HPV 16 L1 gene DNA in Gardasil is more prone to topolotical conformational change than the HPV 18 L1 gene DNA at this location.

4.4. PCR amplification of HPV DNA by primer with a mismatch at 3' terminus

In the absence of a fully matched complementary target, the primer designed to amplify a segment of HPV 16 L1 gene DNA (R16) can initiate a PCR to amplify a segment of HPV 6 DNA (Figure 8) or a segment of HPV 11 DNA (Figure 9) even though there is a single base mismatch at the 3'terminus of a 21-nucleotide primer. A highly processive DNA polymerase can "by-pass" one single terminal nucleotide mismatch in template-

590 directed enzymatic DNA synthesis, a phenomenon which was previously observed and
591 reported when a non-degenerate GP6 primer was used to amplify a segment of HPV 52
592 DNA [29].

593 *4.5. HPV L1 gene DNA as TLR 9 agonist in Gardasil vaccination*

594 Based on animal and *in vitro* studies of the HPV vaccine Cervarix, aluminum
595 hydroxide makes little contribution to the early innate response stimulated by AS04 and
596 there is no evidence that aluminum hydroxide acts synergistically with MPL to enhance the
597 magnitude of cytokine production or to enhance the infiltration of APCs in the draining
598 lymph nodes 24 hours after injection. Neither does aluminum hydroxide alter substantially
599 the type of cytokines and recruited cells induced by MPL. Both AS04 and MPL, but not
600 aluminum salt alone, can induce TNF-α secretion in monocytes. It is MPL which plays the
601 crucial role in AS04 as a TLR 4 agonist for the stimulation of an innate immune response in
602 Cervarix vaccination [6].
603
604 AAHS, also a derivative of aluminum hydroxide, was first used officially as an
605 adjuvant in RECOMBIVAX HB® Hepatitis B Vaccine (Recombinant), initially approved
606 in 1983 [30]. The effect of the adjuvant in the latter vaccine depends on replacing some of
607 the hydroxyl groups of its parent chemical, aluminum hydroxide, with inorganic phosphates
608 by ligand exchange [7] so that the phospholipid moiety of the viral surface antigen [31] can
609 bind to the cationic aluminum loosely to serve as a TLR 4 agonist in vaccination [32],
610 similar to MPL bound to aluminum hydroxide in AS04, in boosting antibody production.
611 For optimum immune response, AAHS needs a pre-made TLR 4 agonist which happens to
612 be the phospholipid part of the viral surface antigen [31] to fulfill its extraordinary adjuvant
613 effects in RECOMBIVAX HB® vaccination. In other words, AAHS needs a pre-made,
614 ready-to-use TLR agonist to carry out its expected potent adjuvant function in a vaccine.
615 However, the re-assembled HPV L1 protein VLPs do not provide a phospholipid. The
616 PCR/sequencing results presented above and the data previously reported [21] indicate that
617 the HPV L1 gene DNA fragments are the only known TLR 9 agonist in Gardasil
618 vaccination as MPL being a TLR 4 agonist in Cervarix vaccination. The sequencing data
619 presented in this report suggest that most of the HPV DNAs bound to AAHS in Gardasil
620 are in non-B conformations which can function as a long-acting TLR 9 agonist in
621 vaccination because DNA bound to minerals and colloidal particles in non-B conformations
622 are known to resist DNase degradation [33].
623
624 TLR 9 is one of the intracellular TLRs situated in the membrane of the endolysosomal
625 compartments of APCs. It samples the content of these compartments for the presence of
626 dsDNA agonists. Humans develop intracellular TLRs during the long history of vertebrate
627 evolution, principally specialized in viral recognition [34]. Now, TLR 9 has evolved as
628 innate immune sensor for viral, bacterial, fungal and protozoan DNA and is also activated
629 by synthetic oligodeoxyribonucleotide (ODN) with a phosphorothioate backbone and an
630 unmethylated CpG motif [35]. Natural TLR 9 agonists are the various kinds of dsDNA with
631 a phosphodiester and 2’ deoxyribose backbone, like those found in bacterial and viral
632 genomes or in self-DNA when the latter is delivered to the endolysosomal compartments of

633 the host's dendritic cells [35], for example as aluminum salt/DNA complexes [14, 15].
634 Until recently the prevailing paradigm was that TLR 9 recognized unmethylated CpG
635 motifs, which are abundant in bacterial DNA but relatively scarce in mammalian DNA
636 [36]. However, it is known now that the dependence on CpG motifs for TLR 9 activation
637 is restricted to synthetic phosphorothioate oligodeoxynucleotides (PS-ODNs), and that
638 natural phosphodiester oligodeoxynucleotides (PD-ODNs) bind and activate TLR 9 via the
639 2' deoxyribose backbone in a sequence-independent manner [37].
640
641 The resulting immune responses to TLR 9 activation include induction of pro-
642 inflammatory and Th1 cytokines (for example, IL-6, IL-1, TNFα, IFNγ and IL-12). In
643 particular, IL-12 and Type I IFNs induced by pDCs via TLR 9 induce strong Th1 type
644 immunity and CTL cytotoxicity. Stimulating endosomal TLRs is particularly effective at
645 promoting the generation of CTL responses capable of eliminating viral pathogens and
646 cancer [38]. A recent human case report demonstrated that complete regression of a
647 widespread cutaneous malignant tumor was achieved after combined systemic and direct
648 intratumoral injection of Gardasil9 [39], suggesting that this vaccine may have therapeutic
649 utility for squamous cell carcinomas which cannot be surgically excised. The only plausible
650 immunological mechanism by which Gardasil9 exerts its therapeutic activity against
651 widespread cancer is through its TLR 9 agonist.
652

653 **5. Conclusions**

654
655 HPV DNA fragments bound to AAHS are part of the essential ingredients of
656 Gardasil4 and Gardasil9, and are mostly in non-B conformations. These HPV DNA
657 fragments may function as potent long-acting TLR 9 agonist in augmenting the induction of
658 pro-inflammatory and Th1 cytokines to enhance the immune responses to HPV vaccination.
659 Since the immunological effects of the AAHS-bound HPV DNA have not been studied by
660 the vaccine industry and the HPV vaccine Gardasil9 with its TLR 9 agonist may have
661 immunotherapeutic effects on cancers, further research on the immunological roles of the
662 HPV DNA fragments bound to AAHS as an active ingredient in Gardasil is warranted.
663

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