Toll-like Receptor 9 Agonists in HPV Vaccine Gardasil9

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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 ingredients. Since the antigens are purified recombinant proteins, in theory Gardasil9 needs a potent adjuvant to generate high and sustained levels of antibodies. Historically, amorphous aluminum hydroxyphosphate sulfate (AAHS), listed as the adjuvant for Gardasil9, was known to require one or more Toll-like receptor agonists, such as the phospholipids in the recombinant hepatitis B vaccine, Recombivax HB®. However, there are no phospholipids in the purified HPV L1 proteins or in Gardasil9. But the Food and Drug Administration (FDA) reports that Gardasil4 does contain recombinant HPV L1-specific DNA fragments, and they may serve as Toll-like receptor 9 agonists in Gardasil9. 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 confirmed the presence of HPV 18, 11, 16 and 6 L1 gene DNA bound to insoluble AAHS nanoparticles, but they were unevenly distributed even within the same vaccine sample. Also, these 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 may all be in non-B conformations or may have been removed as contaminants by a purification protocol. Further research is warranted to standardize the HPV DNA fragments in Gardasil which are known to be potent Toll-like receptor 9 agonists.

Introduction

Human papillomavirus (HPV) is the agent of a common sexually transmitted infection according to the Centers for Disease Control and Prevention (CDC, 2019). 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 (GlaxoSmithKline, 2019) and Gardasil (Merck & Co., Inc., 2019) use purified recombinant genotype-specific HPV major capsid L1 proteins re-assembled in the form of virus-like particles (VLPs) as their active ingredients (their antigens).

Because the assembled VLPs are purified recombinant proteins, by themselves they are relatively weak 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, as pointed out by the National Institutes of Health (NIH, 2019). In Cervarix, the adjuvant is AS04 (GlaxoSmithKline, 2019), 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 according to standard theory to an enhanced cellular and humoral adaptive immune response (Tagliabue & Rappuoli, 2008).

The adjuvant in Gardasil is amorphous aluminum hydroxyphosphate sulfate (AAHS). Each dose of Gardasil9 contains approximately 500 mcg of AAHS as its adjuvant (Merck & Co., Inc., 2019). Both AS04 and AAHS are made from the same starting chemical of aluminum hydroxide (Iyer, HogenEsch & Hem, 2003; EMEA, 2006; Didierlaurent, 2009; Egan, Belfast, Giménez, Sitrin, & Mancinelli, 2009), the hydroxyl groups of which have been partially replaced by phosphate-containing molecules, namely, by MPL, to form AS04 (Tagliabue & Rappuoli, 2008) and by an inorganic phosphate to form AAHS through ligand exchange (Egan, Belfast, Giménez, Sitrin, & Mancinelli, 2009). 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 vaccinated 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 (Caulfield et al, 2007). In human studies, vaccination with Gardasil has been shown to induce significantly higher early innate proinflammatory cytokine/chemokine responses than Cervarix in women (Herrin et al., 2014). 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 (Colmenares et al., 2012). These results indicate that Gardasil can augment the innate immune response, at a level comparable to Cervarix, if not greater, 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 Gardasil9 (Merck & Co., Inc., 2019). The mechanism by which AAHS exerts its adjuvant effects in either Gardasil4 or Gardasil9 has not been fully explained or published. Although AAHS and other aluminum salts, including various other forms of aluminum hydroxide and also of aluminum phosphate, have been used as vaccine adjuvants for over 80 years, how they work remains largely unknown, or at best the theories that have been proposed are controversial. Recent research progress has led the author, and certain others, 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 to vaccination (Maisonneuve, Bertholet, Philpott & De Gregorio, 2014).

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) (Marichal et al., 2011; McKee et al., 2013). Internalized nucleic acids in the APCs are potent TLR agonists in enhancing the desired immune responses (Mohsen, Zha, Cabral-Miranda, & Bachmann, 2017). Internalization of the aluminum salt particles by immune cells may also lead to phagosomal destabilization resulting in the activation of NLR protein NLRP3 (Hornung et al., 2008), probably by inducing the production of endogenous uric acid, which in turn activates NLRP3 within APCs (Kool et al., 2008). 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 damage 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 (Kool et al., 2008). 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 relatively weak immunogenicity of the purified HPV L1 proteins re-assembled as VLPs during vaccine manufacturing (Mach et al., 2006; Frazer, 2018). Such a TLR agonist has not been listed in the Gardasil9 formulation (Merck & Co., Inc., 2019).

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 that vaccine, presumably AAHS nanoparticles (Lee, 2012). 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 to T-cell interactions as a mechanism of augmenting the immunogenicity of vaccination (Marichal et al., 2011; McKee et al., 2013), the HPV L1 gene DNA fragments bound to AAHS in Gardasil4 are expected to provide such an instant premade TLR 9 agonist 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 Gardasil9 vaccination. However, with respect to the efficacy and safety of HPV vaccination, the type and quantity of HPV L1 gene DNA as a TLR agonist have not been defined and standardized for Gardasil vaccines as MPL was 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.

Materials and Methods

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, respectively.

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 (Lee, 2012a) 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, to be paired with GP6 forward primer, referred to as primer R16, R31, R45, R52 and R58, were as follows:

R16: 5'-AATGGCATTTGTTGGGGTAAC for binding site 3'-GTTACCCCAACAAATGCCATT

R31: 5'-GCTCAGGGACACAATAATGGT   3'-ACCATTATTGTGTCCCTGAGC

R45: 5'-ATAACAATGGTATTTGTTGGC   3'-GCCAACAAATACCATTGTTAT

R52: 5'-GCGCAGGGCCACAATAATGGC   3'-GCCATTATTGTGGCCCTGCGC

R58: 5'-GGTCATAACAATGGCATTTGC   3'-GCAAATGCCATTGTTATGACC

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

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 and resuspended in 100μL of buffer. 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.

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 molecular grade 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.

Figure 1. Alignment of the ending 65-base sequences of the 181-187 bp amplicons of the Gardasil9 HPV L1 genes defined by the 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.

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.
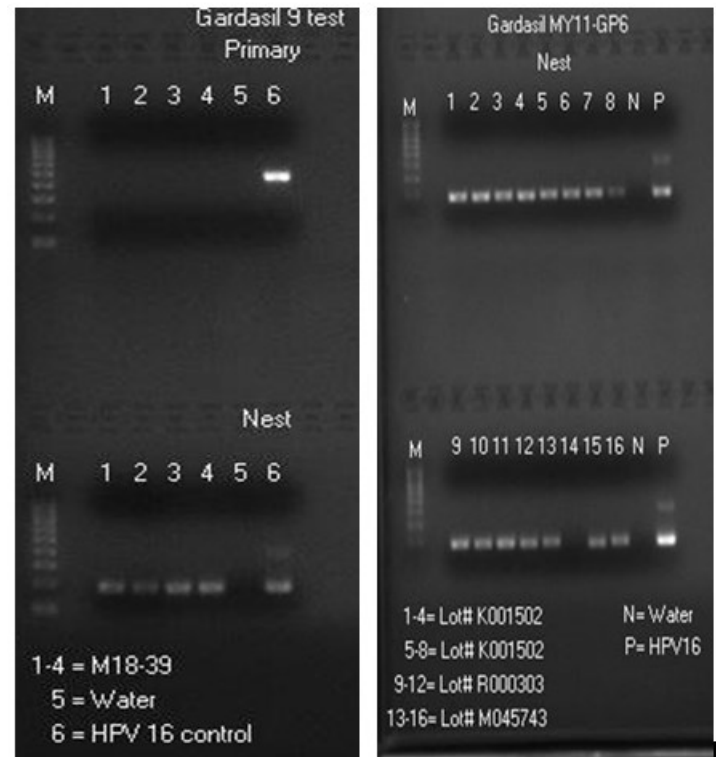

Figure 2. Image of agarose gel electrophoresis showing products of HPV DNA primary and heminested PCR products in the left panel and heminested PCR products only on the right panel. There were four duplicate PCR sets on each of the 5 Gardasil9 digestates.

Left panel: Lanes 1-4 = Lot #N020139 (labeled M18-39) showing 4 invisible MY09/MY11 primary PCR products (upper) and 4 visible 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. M=molecular ruler.

RESULTS

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

Based on alignment of the highly conserved sequence with hypervariable regions of the HPV 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 (Lee, 2012a), as shown in Figure 1.

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 (Wallin et al., 1999; Ciotti et al., 2006), 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 (Lee, 2012a; Wallin et al., 1999). 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 (Merck & Co., Inc., 2019).

Figure 3. Electropherogram of a segment of HPV 18 L1 gene (Seq ID: EF202155)- MY11 primer underlined.
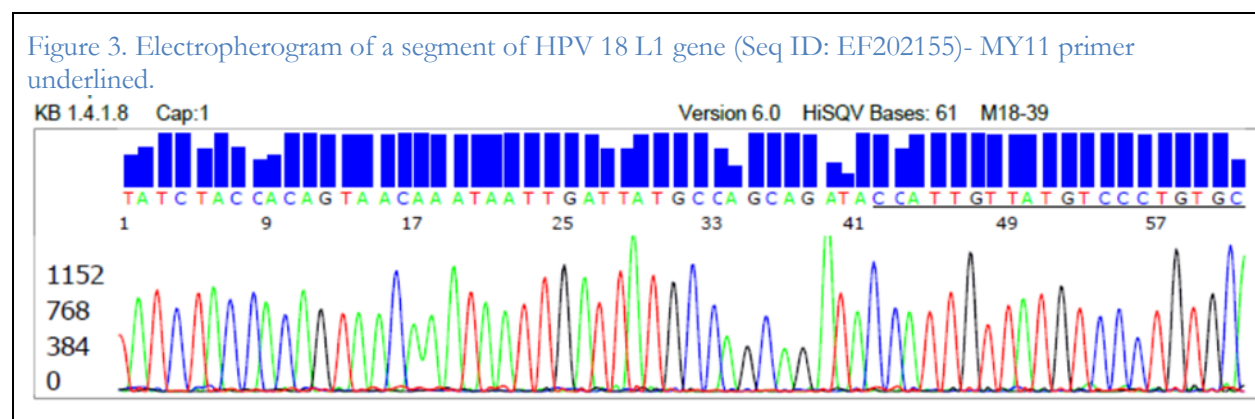
Sanger sequencing with GP6 primer carried out on all these 20 GP6/MY11 heminested PCR products showed a segment of HPV 18 L1 gene sequence (Figure 3) in 1 of the 4 heminested PCR tubes of Lot #N020139, in 1 of the 8 heminested PCR tubes of Lot #K001502, in 1 of the 4 heminested PCR tubes of Lot #R000303, 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

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.
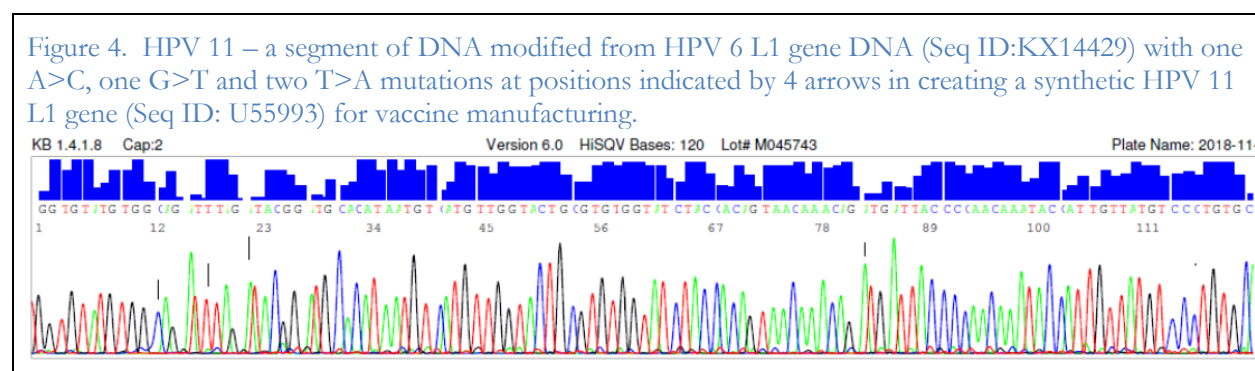
heminested PCR products in 1 of the 4 tubes of Lot #M045743. In other words, Sanger sequencing of 20 heminested PCR products generated only 6 readable DNA sequences. Five of the 6 sequences

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. Sequencing of the invisible heminested PCR products shown in Lane 14 (Figure 2) with GP6 primer did not generate a sequence (1 of 4 aliquots from Lot #M045743).

Figure 5. Mixed HPV DNA sequences. At least one PCR amplicon was 6 bases longer than the other(s)-(position 60-65, reading CTGTGC with 2 A-overhangs). The MY11 primer of the shorter sequence started from the underlined base "C" at position 59, reading right to left.
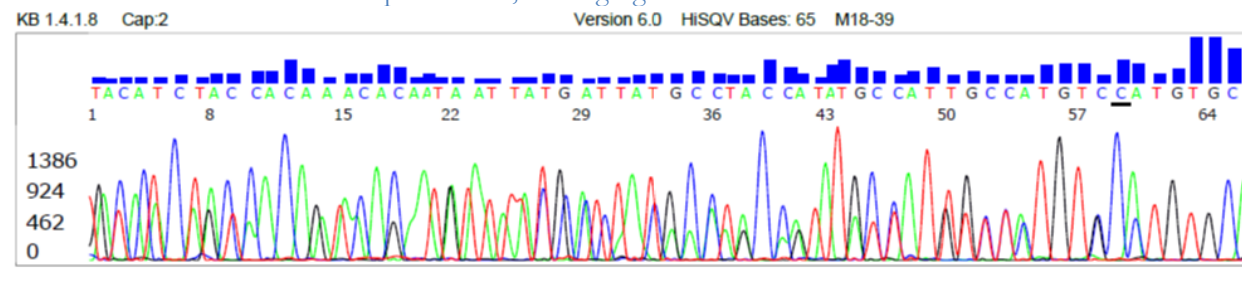
The 13 mixed DNA sequences could be separated into two patterns, each consisting of at least two mixed amplicons, one being 6 bases longer than the other(s), as shown in Figure 5, and one being 3 bases longer than the other(s) as shown in Figure 6. According to the sequence alignment in Figure 1, the 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, because the HPV 18 PCR

Figure 6. Mixed HPV DNA sequences. At least one PCR amplicon was 3 bases longer than the other(s)-(position 70-72, reading TGC with 2 A-overhangs); the first base "C" of the MY11 primer of the shorter amplicon (reading from right to left) was overshadowed by a big "G" (underlined) of the longer amplicon 3 bases downstream of its first base "C" at position 72.
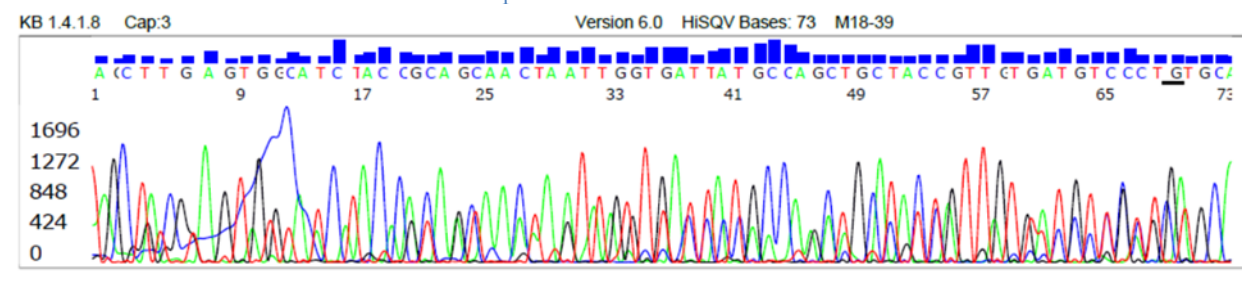
amplicon defined by the GP6 and MY11 primers is the longest with a clear CTGTGC ending in any mixed sequence combinations. By the same token, the electropherogram of Figure 6 indicates that there were at least two amplicons in the PCR products; at least one was 3 bases longer than the other(s). Based 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.

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)* which did not yield a single heminested PCR amplicon for successful Sanger sequencing was re-amplified in 5 sets of nested PCRs, each using the combination of a GP6 and one of the R16, R31, R45, R52 and R58 as forward and reverse primers.

The 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.

Figure 7. Natural HPV 16 sequence at primer binding site: GTTACCCCAACAAATGCCATT
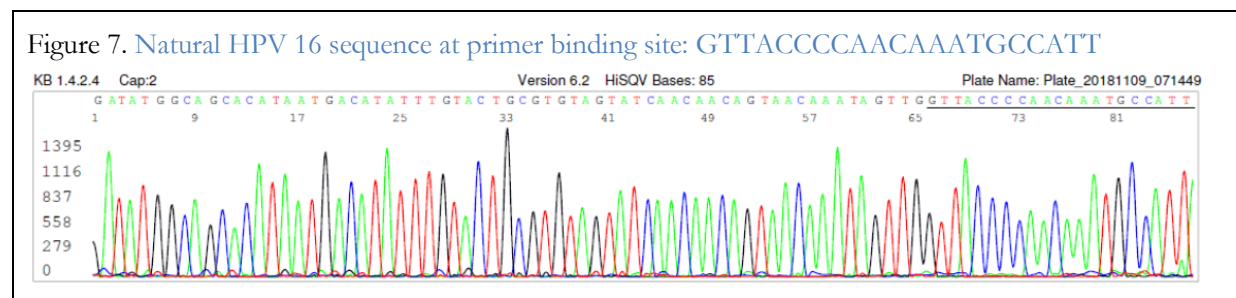
After completion of all 70 (14x5) nested PCRs, each of the 13 primary PCR products which led to a visualized heminested PCR product band consisting of multiple sequences yielded 5 HPV nested PCR product bands at gel electrophoresis, as expected. The primary PCR products as shown in Lane 14, Figure 2 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 data 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, as illustrated below.

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

When HPV 16 DNA was present in the mixed genotype MY09/MY11 primary PCR products, the non-degenerate GP6/R16 primer pair selectively amplified 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 of Figure 7).

When HPV 16 DNA was absent in the mixed genotype primary PCR products, the non-degenerate R16 primer pair was able to 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 primer blinding site of the template 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 2 mismatched nucleotides in red color as in Figure 8.
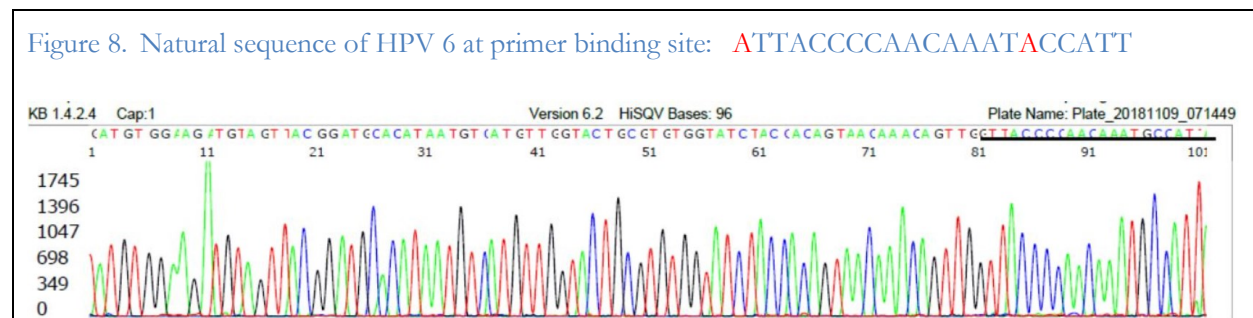
Figure 8. Natural sequence of HPV 6 at primer binding site: ATTACCCCAACAAATACCATT

When HPV 16 DNA was absent in the mixed genotype primary PCR products, the non-degenerate R16 primer was able to 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

Figure 9. Natural sequence HPV 11 at primer binding site: ATTACCCCAACAAATACCATT
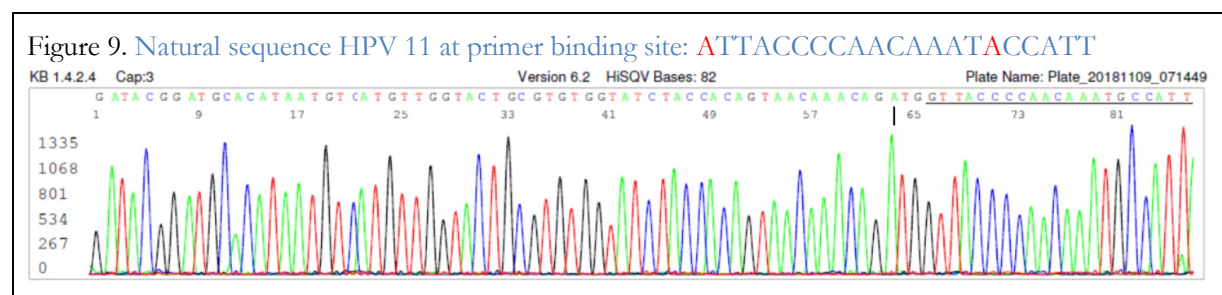
the synthetic HPV 11 L1 gene and the natural HPV 6 L1 gene have the same DNA sequence in this segment except for a T>A mutation indicated by a dark vertical line at 63 in the electropherogram illustrated in Figure 9.

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

As for all other Gardasil9 samples tested, 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 heminested PCR products

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 although the nested PCR amplicons were not visible as bands on agarose gel electrophoresis. These 3 sequences are illustrated in Figures 10, 11 and 12 as follows.

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 is underlined; it has one extra nucleotide "A" at the 3' end compared to the degenerate MY11 primer sequence for HPV 18 shown in Figure 3. The natural sequence of HPV 18 with 4 mismatched bases (in red color) is placed over the underlined R31 primer in the electropherogram of Figure 10. This sequence found there 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 needed 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 (Lee, 2013; Lee, 2014).

Figure 10. Natural HPV 18 sequence at MY11 primer binding site: CCATTGTTATGACCCTGTGC
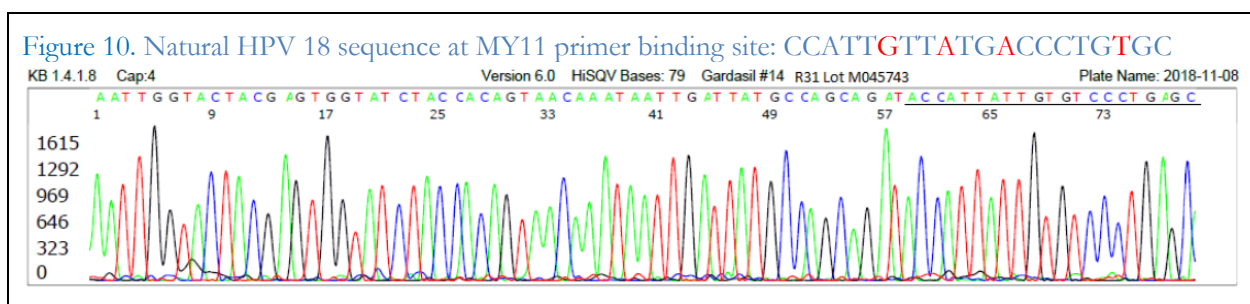
It was also found that in the same primary PCR products described above there were DNAs other than those of HPV 18 the sequence of which was shown in Figure 10. As illustrated in Figures 11 and 12 below, the R45 and R58 primers, both shifted internally from the MY11 primer binding site, when pairing with the GP6 primer, re-amplified 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. Natural HPV 18 sequence at primer binding site: GCCAGCAGATACCATTGTTAT
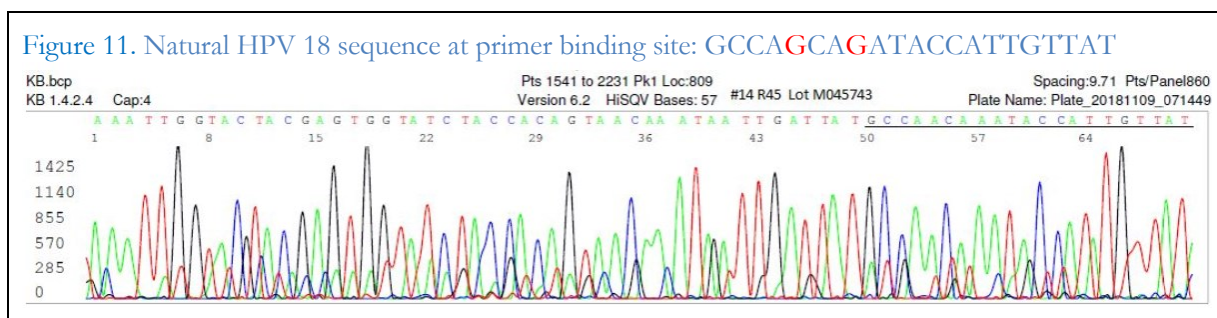
As seen in Figure 11, nested PCR with R45 primer, shifted 10 nucleotides inward compared to the primer used for Figure 10, yielded more than one type of HPV L1 gene DNAs. The computer-generated sequence downstream of the underlined primer in Figure 11 is that of HPV 18 L1 gene. The underlined R45 primer in the electropherogram had two mismatches (in red) compared against the natural HPV 18 DNA primer binding site in this location.

Figure 12. Natural HPV 18 sequence at primer binding site: GCAGATACCATTGTTATGACC
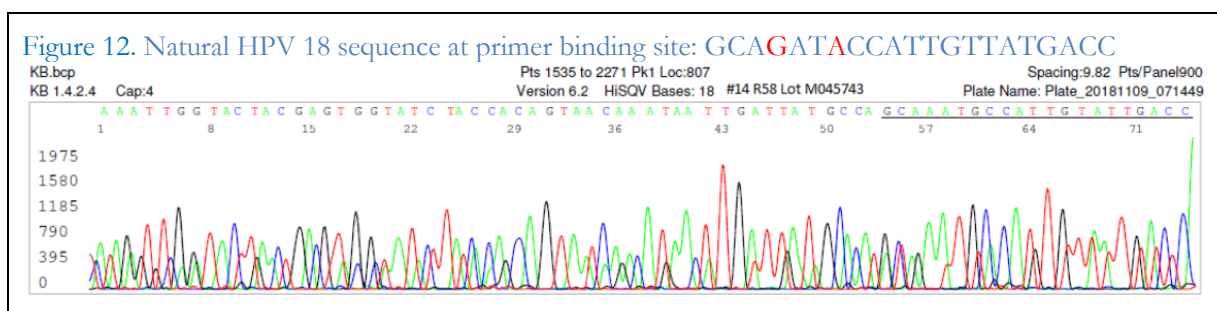
In Figure 12, nested PCR with R58 primer, shifted 6 nucleotides inward compared to the primer used for Figure 10, also yielded more than one type of HPV L1 gene DNAs. The computer-generated sequence downstream of the underlined primer is that of HPV 18 L1 gene. The underlined R58 primer in the electropherogram in this instance also had two mismatches (in red) from the natural HPV 18 DNA primer binding site in this location.

Summarizing, most HPV 18 L1 gene DNA fragments bound to AAHS in Gardasil were in B conformation and readily amplified by the 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 (the mismatched bases in primers typed in red).

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AACAAATAATTGATTATGCCAGCAGATACCATTGTTATGACCCTGTGC    HPV18 sequence (ID#EF202155)
                        CCATTGTTATGACCCTGTGC    MY11 No template generated
            GTTACCCCAACAAATGCCATT               R16  No template generated
AACAAA··················ACCATTATTGTGTCCCTGAGC   R31 Template generated Figure 10
AACAAA···········GCCAACAAATACCATTGTTAT          R45 Template generated Figure 11
            GCCATTATTGTGGCCCTGCGC   R52  No template generated
AACAAA···············GCAAATGCCATTGTTATGACC           R58 Template generated Figure 12
```

In the sequence alignment presented above, the highly conserved sequence CCATT for all HPV L1 genes are highlighted yellow in its reference position which is also the ending of the degenerate MY11 primer. PCR primer requires at least 6 nucleotides that match those in the template to initiate a polymerase chain reaction (Ryu, Choi, & Lee, 2000). A 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 (which is underlined in the sequence spelled out above) 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.

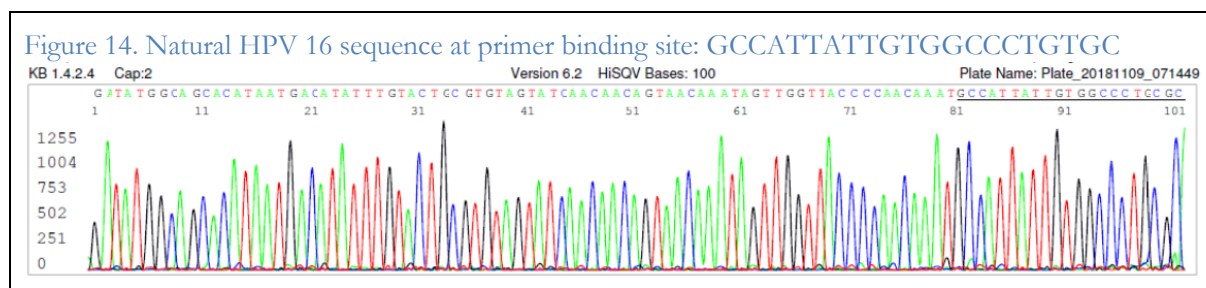*4.3. In the absence of HPV 45 DNA, primer R45 amplified HPV 18 DNA*

When HPV 45 DNA was absent in the mixed genotype MY09/MY11 primary PCR products, the non-degenerate R45 primer could 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

Figure 13. Natural HPV 18 sequence at primer binding site: GCCAGCAGATACCATTGTTAT
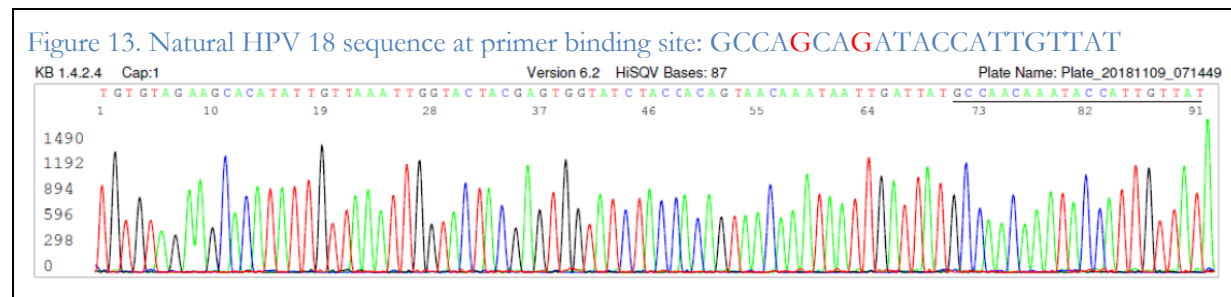
sequence and the natural HPV 18 L1 gene sequence at the primer binding site as shown in Figure 13. There the HPV 18 DNA in a mixed genotype MY09/MY11 with primary PCR products was amplified by a non-degenerate primer R45 (underlined in the electropherogram of Figure 13).

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 could 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 as seen in Figure 14. There, the HPV 16 DNA in a mixed genotype MY09/MY11 the primary PCR products were amplified by a non-degenerate primer R52 (as underlined in Figure 14).

Figure 14. Natural HPV 16 sequence at primer binding site: GCCATTATTGTGGCCCTGTGC

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

Figure 15. Natural HPV 18 sequence at primer binding site: GCAGATACCATTGTTATGACC
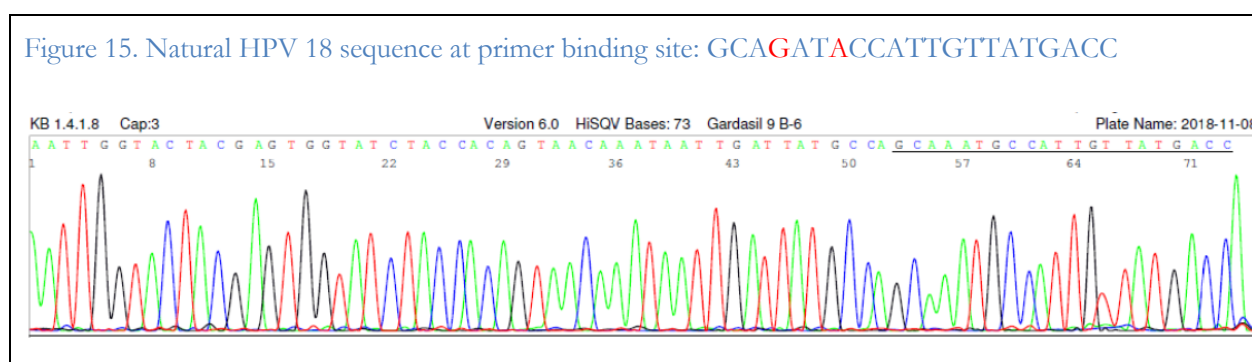
Discussion

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 (FDA, 2011). The current study based on testing 5 Gardasil9 samples and a previous report based on testing 16 Gardasil4 samples (Lee, 2012) 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. If so, it can work as a potent adjuvant in Gardasil9 as the phospholipids bound to AAHS in creation of a potent adjuvant for the recombinant hepatitis B vaccine, Recombivax HB® (Egan, Belfast, Giménez, Sitrin, & Mancinelli, 2009). Among the officially listed ingredients of

Gardasil9, including the VLPs, AAHS, sodium chloride, L-histidine, polysorbate 80, sodium borate, yeast protein and water for injection (Merck & Co., Inc., 2019), AAHS is the only water-insoluable, proteinase-resistant component.

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

Multi-valent Gardasil vaccines are produced by separate fermentation. The purified and reassembled VLPs of each HPV type are adsorbed on AAHS before the monovalent bulk adsorbed products are combined (EMEA, 2006; Merck & Co., Inc., 2019). As recombinant HPV L1 gene DNA fragments are not contaminants, they are not targets for removal as are 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 only generated amplicons of HPV 18, 11, 16 and 6 for sequencing validation in tests of 5 samples of Gardasil9. As in Gardasil4 (Lee, 2012), 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 may be other genotype-specific HPV DNAs also present in any given sample. Previous studies on Gardasil4 samples showed that the AAHS-bound HPV 16 and HPV 6 genotype-specific L1 gene DNAs could not be amplified by MY09/MY11 degenerate PCR primers (Lee, 2012; Lee, 2013; Lee, 2014). 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 5 of these specific DNAs may be in non-B conformations. Alternatively, all the L1 gene DNA fragments of the these 5 HPV genotypes in the 4 tested lots of Gardasil9 may have been removed as "contaminants" during the manufacturing process.

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 (Lee, 2012). 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 (Lee, 2013; Lee, 2014). In the current study, a non-degenerate primer shifted 15 nucleotides inward (R16) from 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 (Ryu, Choi, & Lee, 2000). 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. 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-directed enzymatic DNA synthesis, a phenomenon which was previously observed and reported when a non-degenerate GP6 primer was used to amplify a segment of HPV 52 DNA (Hong, Lee, Ge & Zhou, 2013).

5. HPV L1 gene DNA as a TLR 9 agonist in Gardasil vaccination

Based on animal and *in vitro* studies of the HPV vaccine Cervarix, aluminum hydroxide makes little contribution to the early innate response stimulated by AS04 and there is no evidence that aluminum hydroxide acts synergistically with MPL to enhance the magnitude of cytokine production or to enhance the infiltration of APCs in the draining lymph nodes 24 hours after injection. Neither does aluminum hydroxide alter substantially the type of cytokines and recruited cells induced by MPL. Both AS04 and MPL, but not aluminum salt alone, can induce TNF-α secretion in monocytes. It is MPL which plays the crucial role in AS04 as a TLR 4 agonist for the stimulation of an innate immune response in Cervarix vaccination (Didierlaurent et al., 2009).

AAHS, also a derivative of aluminum hydroxide, was first used officially as an adjuvant in RECOMBIVAX HB® Hepatitis B Vaccine (Recombinant) in the 1980s (Merck & Co., Inc., 2018). The effect of the adjuvant in the latter vaccine depends on replacing some of the hydroxyl groups of its parent chemical, aluminum hydroxide, with inorganic phosphates by ligand exchange (Egan, Belfast, Giménez, Sitrin, & Mancinelli, 2009) so that the phospholipid moiety of the viral surface antigen (Gavilanes, Gonzalez-Ros & Peterson, 1982) can bind to the cationic aluminum loosely to serve as a TLR 4 agonist in vaccination (Wong-Baeza et al., 2015), similar to MPL bound to aluminum hydroxide in AS04, in boosting antibody production. For optimum immune response, AAHS needs a pre-made TLR 4 agonist which happens to be the phospholipid part of the viral surface antigen (Gavilanes, Gonzalez-Ros & Peterson, 1982) to fulfill its extraordinary adjuvant effects in RECOMBIVAX HB® vaccination. In other words, AAHS needs a pre-made, ready-to-use TLR agonist to perform its expected potent adjuvant function in a vaccine. However, the re-assembled HPV L1 protein VLPs do not provide a phospholipid. The PCR/sequencing results presented above and the data previously reported (Lee, 2012) indicate that the HPV L1 gene DNA fragments are the only known TLR 9 agonist in Gardasil vaccination as MPL is in Cervarix vaccination. The sequencing data presented in this report suggest that most of the HPV DNAs bound to AAHS in Gardasil are in non-B conformations which can function as long-acting TLR 9

agonists in vaccination because DNA bound to minerals and colloidal particles in non-B conformations are known to resist DNase degradation (Cai, Huang & Zhang, 2006).

TLR 9 is one of the intracellular TLRs situated in the membrane of the endolysosomal compartments of APCs. It samples the content of these compartments for the presence of dsDNA agonists. It is hypothesized that humans developed intracellular TLRs during a long history of vertebrate evolution, principally specialized in viral recognition (Barreiro et al., 2009). Now, TLR 9 serves as an innate immune sensor for viral, bacterial, fungal and protozoan DNA and is also activated by synthetic oligodeoxyribonucleotide (ODN) with a phosphorothioate backbone and an unmethylated CpG motif (Brencicova & Diebold, 2013). Natural TLR 9 agonists are the various kinds of dsDNA with a phosphodiester and 2' deoxyribose backbone, like those found in bacterial and viral genomes or in self-DNA when the latter is delivered to the endolysosomal compartments of the host's dendritic cells (Brencicova & Diebold, 2013), for example as aluminum salt/DNA complexes (Marichal et al., 2011; McKee et al., 2013). Until recently the prevailing paradigm was that TLR 9 recognized unmethylated CpG motifs, which are abundant in bacterial DNA but relatively scarce in mammalian DNA (Krieg et al., 1995). However, it is known now that the dependence on CpG motifs for TLR 9 activation is restricted to synthetic phosphorothioate oligodeoxynucleotides (PS-ODNs), and that natural phosphodiester oligodeoxynucleotides (PD-ODNs) bind and activate TLR 9 via the 2' deoxyribose backbone in a sequence-independent manner (Li, Berke & Modis, 2012).

The resulting immune responses to TLR 9 activation include induction of pro-inflammatory and Th1 cytokines (for example, IL-6, IL-1, TNFα, IFNγ and IL-12). In particular, IL-12 and Type I IFNs induced by pDCs via TLR 9 induce strong Th1 type immunity and CTL cytotoxicity. Stimulating endosomal TLRs is particularly effective at promoting the generation of CTL responses capable of eliminating viral pathogens and cancer (Dowling & Mansell, 2016). A recent human case report demonstrated that complete regression of a widespread cutaneous malignant tumor was achieved after combined systemic and direct intratumoral injection of Gardasil9 (Nichols et al., 2018), suggesting that this vaccine may have therapeutic utility for squamous cell carcinomas which cannot be surgically excised. The only plausible immunological mechanism by which Gardasil9 exerts its therapeutic activity against widespread cancer is through its TLR 9 agonists.

6. Any TLR 9 agonist is a double-edged sword

Foreign nucleic acids have been known to be *in vivo* active molecules for more than 50 years (Isaacs, & Rotem, 1963). In the past 10 years, experimental research has been directed towards using synthetic CpG rich oligonucleotides with phosphorothioate backbone as a TLR 9 agonist to stimulate the immune system for possible cancer treatment (Vollmer, & Krieg, 2009) and triplex oligonucleotides, a form of non-B DNA, have been used for targeted mutagenesis (Chin, & Glazer, 2009). It is technically challenging to introduce foreign DNA into the target cells in animal experiments because free natural DNAs after being injected into the animal are quickly degraded by various nucleases in the tissue fluids and are excreted through the kidneys. In contrast, synthetic CpG rich oligonucleotides with a phosphorothioate backbone are highly resistant to degradation by nucleases (Stein, Subasinghe, Shinozuka, & Cohen, 1988). In addition, phosphorothioate oligonucleotides are significantly more hydrophobic than their natural phosphodiester, oxygen-

containing counterparts and as a result pass the cell membranes more readily to their intracellular sites of action, *i.e.* the endolysosomal compartments (Juliano, Ming, & Nakagawa, 2012). To introduce natural foreign DNA as a TLR 9 agonist without a phosphorothioate backbone into the target cells of a mammalian host, nanoparticles are usually needed as the DNA carriers, for example in the formulation of DNA vaccines (Poecheim et al., 2015). In Gardasil vaccination, the nanoparticles of AAHS serve as the DNA carriers to bring the HPV L1 gene DNA fragments as TLR 9 agonists into the immune cells. Gardasil has been shown to contain metal nanoparticles in the range of 3-60 μm in size. The metallic elemental compositions of these nanoparticles are CaAlSi, AlSi, SiMgFe, Al,Fe, AlCuFe, FeSiAl, BiBaS, Ti, and TiAlSi as demonstrated by Field Emission Gun Environmental Electron Scanning Microscope equipped with the X-ray microprobe of an Energy Dispersive Spectroscope (Gatti, & Montanari, 2016). All these metal elements, most of which co-exist with aluminum in the AAHS adjuvant, can be in cationic form and bind the phosphate backbone of HPV DNA fragments in the vaccine products, turning the DNA molecules into non-B conformations which may then serve as non-biodegradable long-acting TLR 9 agonists. The aluminum-laden inflammatory cells with activated TLR 9 can enter the lymphatic system, travel throughout the body, cross the blood-brain barrier and merge into the microglial cell population in the brain (Mold, Umar, King, & Exley, 2018). Disorders due to adjuvant-activated TLRs in the form of autoimmune inflammatory reactions in various organs following vaccination have been referred to as 'the adjuvant diseases' (Israeli, Agmon-Levin, Blank, & Shoenfeld, 2009).

The fate of the non-B HPV L1 gene DNA fragments bound to AAHS nanoparticles in the immune cells is totally unknown. Intracellular foreign DNA may have unpredictable and unknown ways to alter the sequences and conformations of the genomic DNA of the host cells (Milot et al., 1992; Doerfler et al., 1997; Würtele, Little, & Chartrand, 2003; Lechardeur, Verkman, & Lukacs, 2005; Bergen, Park, Horner, & Pun, 2008). In addition to being a highly effective long-acting adjuvant in maintaining a sustained high level of anti-HPV L1 protein antibodies and causing autoimmune adjuvant diseases in certain genetically and physically predisposed vaccinees, these intracellular HPV L1 gene DNA in non-B conformations may also induce a mutagenic and genomic instability effect with far-reaching consequences (Bacolla, & Wells, 2009; Zhao, Bacolla, Wang, & Vasquez, 2010).

Conclusions

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

Potential Conflicts of Interest

S. H. Lee is the director of Milford Molecular Diagnostics Laboratory, a CLIA-certified commercial laboratory specialized in developing DNA sequencing-based diagnostic tests implementable in community hospital laboratories.

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