

1 Article

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

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

38
3940 **1. INTRODUCTION**41
42
43

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-

44 valent or 9-valent vaccine Gardasil, for its prevention. Both Cervarix [2] and Gardasil [3]
45 use purified recombinant genotype-specific HPV major capsid L1 proteins assembled in the
46 form of virus-like particles (VLPs) as the active ingredient (the antigen). Since the
47 assembled VLPs are purified recombinant proteins, they are poor immunogens and require
48 the assistance of specially designed adjuvants to generate a robust and persistent immune
49 response as other purified, subunit and synthetic antigens usually do in many newly
50 developed vaccines [4]. In Cervarix, the adjuvant is AS04 [2], a compound created by
51 combining a Toll-like receptor (TLR) 4 agonist MPL (3-O-desacyl-4'-monophosphoryl
52 lipid A) and aluminum hydroxide. MPL is a detoxified derivative of the lipopolysaccharide
53 (LPS) isolated from *Salmonella minnesota* R595 strain and LPS is a specific agonist of
54 TLR 4. In chemical structure, a single negatively charged phosphate of the linear MPL is
55 bound to the cationic aluminum through an ionic bond so that the free molecular chains of
56 LPS can react with TLR 4 of the immune cells. The MPL within AS04 enhances the
57 initiation of the immune response through activation of the innate immunity, leading to an
58 enhanced cellular and humoral adaptive immune response [5].
59

60 The adjuvant in Gardasil is amorphous aluminum hydroxyphosphate sulfate (AAHS).
61 Each dose of Gardasil9 contains approximately 500 mcg of AAHS as adjuvant [3]. Both
62 AS04 and AAHS are made from the same starting chemical of aluminum hydroxide [6-9]
63 whose hydroxyl groups have been partially replaced by phosphate-containing molecules,
64 namely by MPL to form AS04 [6] and by an inorganic phosphate to form AAHS [7]
65 through ligand exchange. The crucial difference between AS04 and AAHS is that MPL is a
66 TLR agonist and inorganic phosphate is immunologically inert.
67

68 In animal experiments, anti-HPV L1 VLP responses from mice immunized with
69 AAHS-formulated HPV16 vaccine have been shown to be substantially greater than those
70 produced by mice immunized with the same antigen formulated with aluminum hydroxide
71 or with aluminum phosphate [10]. In human studies, vaccination with Gardasil has been
72 shown to induce significantly higher early innate proinflammatory cytokine/chemokine
73 responses than Cervarix in women [11]. The peripheral blood mononuclear cells (PBMCs)
74 of healthy women vaccinated with Gardasil have been shown to be associated with
75 significant changes in the expression and function of immune innate and regulatory
76 receptors [12]. These results indicate that Gardasil is capable of augmenting innate immune
77 response, at least comparable to Cervarix if not in greater magnitude even though its
78 aluminum adjuvant does not contain MPL. A TLR agonist component equivalent to MPL is
79 neither a part of AAHS, nor mentioned in the description for Gardasil [3]. The mechanism
80 by which AAHS exerts its adjuvant effects in Gardasil vaccination is unclear or has not
81 been published.
82

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

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

109

110 Previous testing of 16 samples from different vaccine lots revealed that Gardasil4
111 contains fragments of HPV L1 gene DNA firmly bound to the insoluble, proteinase-
112 resistant fraction of the vaccine, presumably AAHS nanoparticles [21]. Since free DNA
113 released from dying host cells and bound to aluminum salts at the site of vaccine injection
114 is known to be transfected into the cytoplasm of antigen-bearing dendritic cells in
115 promoting MHC class II presentation and enhancing dendritic cell –T-cell interactions as a
116 mechanism of augmenting the immunogenicity of vaccination [14, 15], the HPV L1 gene
117 DNA fragments bound to AAHS in Gardasil4 must have provided such an instant premade
118 TLR 9 agonist which is needed to enhance the initiation of the immune response through
119 activation of the innate immunity, leading to an enhanced cellular and humoral adaptive
120 immune response in Gardasil vaccination. However, for efficacy and safety of HPV
121 vaccination, the types and quantity of HPV L1 gene DNA as TLR agonist have not been
122 defined and standardized for Gardasil as MPL for Cervarix. This article reports the
123 technical challenges in using a routine diagnostic PCR protocol for detection of the
124 genotype-specific HPV L1 gene DNAs bound to AAHS in the HPV vaccine Gardasil9.

125

126 **2. MATERIALS AND METHODS**

127

128 *2.1. Gardasil9 vaccine samples*

129

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

136

137 2.2. PCR and sequencing primers

138

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

142

143 MY09 forward = 5'-CGTCCMARRGGAWACTGATC-3'

144 MY11 reverse = 5'-GCMCAGGGWCATAAYAATGG-3' (also in heminested PCR)

145 GP6 forward heminested = 5'-GAAAAATAAACTGTAAATCA-3'

146

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

150

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

153

154 2.3. Preparation of Samples for PCR

155

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

168

169 2.4. PCR Amplification of HPV L1 Gene DNAs for Sanger sequencing

170

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

173 DNA polymerase, magnesium ions, denaturing agents, and dNTPs with stabilizing
174 additives (HiFi DNA Tech, LLC, Trumbull, CT, USA), 1 μL of 10 μM MY09 primer, 1
175 μL of 10 μM MY11 primer and 2 μL of molecular grade water were mixed in a final
176 volume of 25 μL in a thin-walled PCR tube for low temperature PCR amplification. The
177 LoTemp® thermocycling steps were set for an initial heating at 85°C for 10 min, followed
178 by 30 cycles, each set at 85°C for 30 sec, 40°C for 30 sec, and 65°C for 1 min. The final
179 extension was 65°C for 10 min. A trace of each of the primary PCR products (about 0.2 μL)
180 was transferred by a micro-glass rod to another 25 μL complete PCR mixture containing 20
181 μL of ready-to-use LoTemp® PCR mix, 1 μL of 10 μM GP6 forward primer, and 1 μL
182 of 10 μM reverse primer and 3 μL of water for heminested PCR or nested PCR. After
183 completion of the primary and the nested PCR, a 5 μL aliquot of the PCR products was
184 pipetted out from each tube and mixed with 2 μL loading fluid for electrophoresis in a 2%
185 agarose gel containing ethidium bromide. The gel was examined under UV light for the
186 PCR product bands in the agarose gel. An HPV 16 plasmid DNA positive control and a no
187 sample negative control (1 μL of water added instead of sample) were included in each
188 primary and heminested or nested PCR run.

189

190 *2.5. Direct Automated DNA Sequencing of the heminested or nested PCR amplicons*

191

192 For DNA sequencing, a trace of the positive nested PCR products (about 0.2 μL) was
193 transferred directly with a micro-glass rod from the heminested or nested PCR tube into a
194 20 μL volume of a cycle sequencing reaction mixture consisting of 14.5 μL water, 3.5 μL
195 of 5 \times buffer, 1 μL of BigDye Terminator 1.1 (Applied Biosystems) and 1 μL of 10 μM olar
196 sequencing primer solution in TE buffer. After thermal cycling according to the
197 manufacturer's recommendation for 20 cycles, the reaction mixture was loaded in an
198 automated ABI 3130 four-capillary Genetic Analyzer or an Applied Biosystems SeqStudio
199 Genetic Analyzer for sequence analysis. Alignment analysis of a 45 - 60 base sequence in
200 the hypervariable region of the L1 gene excised from the computer-generated base calling
201 electropherogram was performed against various standard HPV genotype sequences
202 retrieved from the GenBank, using the on-line BLAST (Basic Local Alignment Search
203 Tool) system to validate the specific HPV genotyping and for visual sequence analyses.
204 Throughout the entire period when this study was carried out, no routine diagnostic HPV
205 tests were performed in the laboratory and the procedures of sample preparation for primary
206 PCR, nested PCR and DNA sequencing were performed in different rooms to avoid cross
207 contamination by HPV DNA from other sources.

208

209 **3. RESULTS**

210

211 *3.1. Short-segment L1 gene DNA sequence analysis for HPV genotyping*

212

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

216 33 (KU550675), HPV 45 (KU049756), HPV 52 (LC373207) and HPV 58 (KY225967), the
 217 9 HPV genotypes included in Gardasil9, can be reliably diagnosed by BLAST analysis of a
 218 45-base sequence immediately downstream of the 20-base degenerate MY11 primer site.
 219 The size of the amplicon defined by the GP6 and MY11 primers of these HPV genotypes
 220 varies from 181 bp to 187 bp [22], as shown in Figure 1.

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

Figure 1

HPV	Ending 65-base L1 gene sequences of PCR amplicon defined by GP6 and MY11 primers (3'-5')	Size of amplicon
6	GTGGTATCTACCACAGTAACAACAGTTGATTACCCCAACAAATACCATTGTTATGTCCCTGGGC	181bp
11	GTGGTATCTACCACAGTAACAACAGATGATTACCCCAACAAATACCATTGTTATGTCCCTGGGC	181
16	GTAAGTATCAACAACAGTAACAATAAGTTGGTTACCCCAACAAATGCCATTATTGTGCCCTGTGC	184
18	GTGGTATCTACCACAGTAACAATAAATTGATTATGCCAGCAGATAACCATTGTTATGACCCCTGTGC	187
31	GTGGTATCTACCACAGTAACAATAACTGATTGCCCAACAAATACCATTATTGTGTCCCTGAGC	184
33	GTGGTATCTACCACAGTAACAATACTCTGATTGCCCAACAAATACCATTATTATGACCTTGTGC	181
45	GTAAGTGTCCACTACAGTAACAACAACACTGATTATGCCCAACAAATACCATTGTTATGGCCCTGGGC	187
52	GTGGTATCCACAACGTGACAAACAACAGTATGCCCAACATATGCCATTATTGTGGCCCTGCGC	181
58	GTGGTATCAACCACGGTAACAATAAAGTATGCCCAACAAATGCCATTGTTATGACCTTGTGC	181

226

227

228 3.2. Selective amplification of HPV 18 and HPV 11 DNA

229

230 Since most invasive cervical cancers are associated with or preceded by persistent
 231 infection by one of numerous genotypes of HPV [23, 24], laboratory tests for HPV in
 232 specimens obtained from patients have been developed to amplify all clinically relevant
 233 HPV genotype L1 gene DNAs by MY09/MY11 degenerate primer PCR followed by
 234 GP6/MY11 heminested PCR for initial detection. DNA sequencing is performed on a PCR
 235 amplicon for accurate genotyping in follow-up of the patients with persistent HPV infection
 236 [22, 23]. Theoretically, Gardasil9 may contain 9 genotype-specific HPV L1 gene DNAs,
 237 and all 9 genotypes of HPV L1 gene DNA were expected to be co-amplified by the degenerate
 238 MY09/MY11 primary PCR primers and the GP6/MY11 heminested PCR primers if these
 239 DNAs were in B conformation.

240

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

247

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

252

253 Left panel: Lanes 1-4 = Lot #N020139 (labeled M18-39) showing 4 invisible MY09/MY11
 254 primary PCR products (upper) and 4 GP6/MY11 heminested PCR bands (lower).

255 Right panel: GP6/MY11 heminested PCR products only. Lanes 1-4 = Lot #K001502(A);

256 Lanes 5-8 = Lot #K001502(B); Lanes 9-12 = Lot #R000303; Lanes 13-16 = Lot

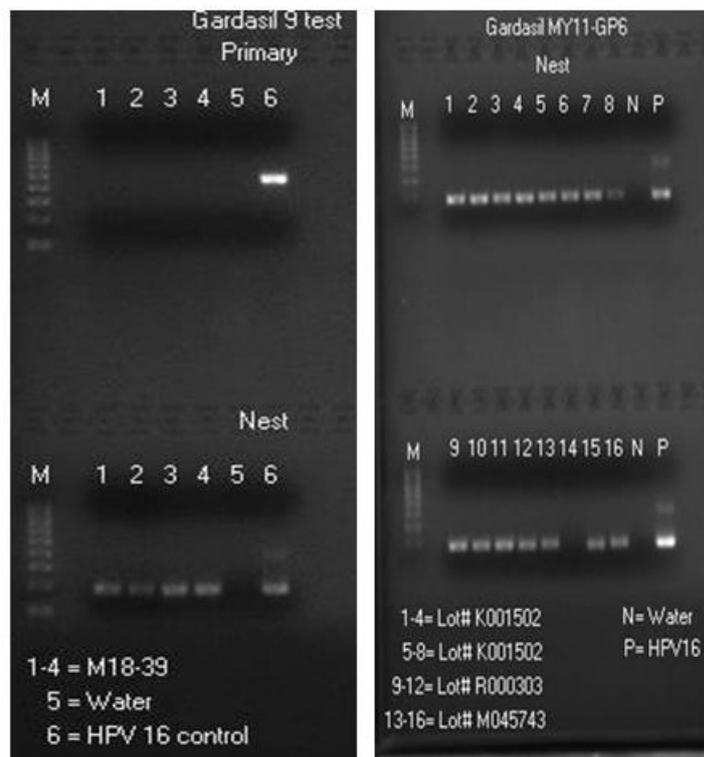
257 #M045743.

258 N=negative, no sample control. P= HPV 16 positive control.

259

260

261



262

263

264

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

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

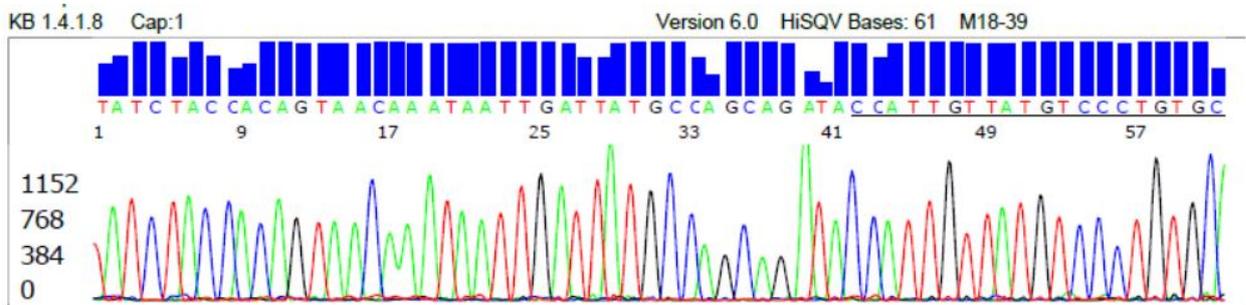
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275

276

277 Figure 3 Electropherogram of a segent of HPV 18 L1 gene (Seq ID: EF202155)-

278 MY11 primer underlined.



279

280

281

282

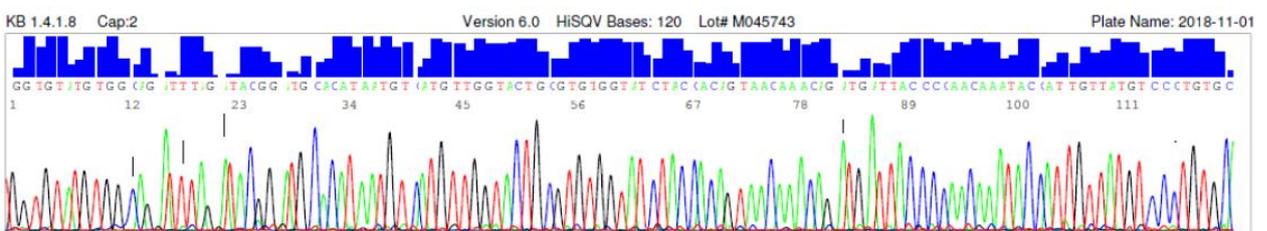
283

284 Figure 4 HPV 11 – a segment of DNA modified from HPV 6 L1 gene DNA

285 (Seq ID:KX14429) with one A>C, one G>T and two T>A mutations at positions indicated

286 by 4 arrows in creating a synthetic HPV 11 L1 gene (Seq ID: U55993) for vaccine

287 manufacturing.



288

289 *3. 3. Multiple HPV DNA sequences generated by MY09/MY11 degenerate primers*

290

291 Sequencing with GP6 primer of the 14 GP6/MY11 primer heminested PCR products

292 other than those 6 mentioned above yielded 13 mixed HPV L1 gene DNA sequences. The

293 heminested PCR products shown in Lane 14 (Figure 2) did not generate a sequence (1 of 4

294 aliquots from Lot #M045743).

295 The 13 mixed DNA sequences could be separated into two patterns, each consisting

296 of at least two mixed amplicons, one being 6 bases longer than the other(s), as shown in

297 Figure 5, and one being 3 bases longer than the other(s) as shown in Figure 6.

298

299

300

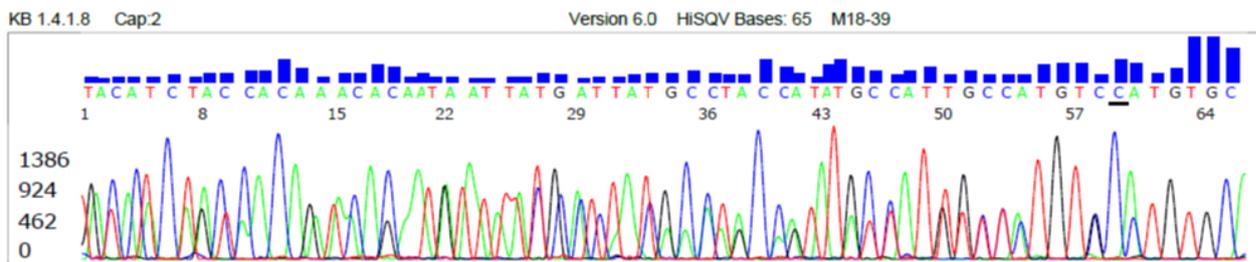
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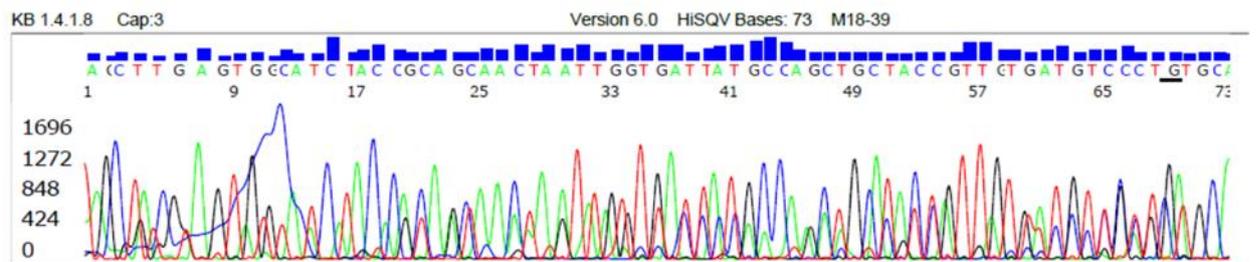
304

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



308

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



312

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

324 *3. 4. No amplification of HPV 31, 33, 45, 52 and 58 L1 gene DNA by MY09/MY11*
 325 *degenerate PCR primers*

326

327 In order to test if there were any L1 gene DNA amplicons of the HPV 31, 33, 45,
 328 52 and 58 genotypes in the MY09/MY11 primary PCR products, each of the 14 primary

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

333 **R16:** 5' -AATGGCATTGTGTTGGGGTAAAC for the binding site 3' -GTTACCCCAACAAATGCCATT

334 **R31:** 5' -GCTCAGGGACACAATAATGGT 3' -ACCATTATTGTGTCCCTGAGC

335 **R45:** 5' -ATAACAATGGTATTTGTTGGC 3' -GCCAACAAATACCATTGTTAT

336 **R52:** 5' -GCGCAGGGCCACAATAATGGC 3' -GCCATTATTGTGGCCCTGCGC

337 **R58:** 5' -GGTCATAACAATGGCATTTC 3' -GCAAATGCCATTGTTATGACC

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

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

358 3.4.1. In the absence of HPV 16 DNA, primer R16 amplified HPV 6 and HPV 11 L1 DNA

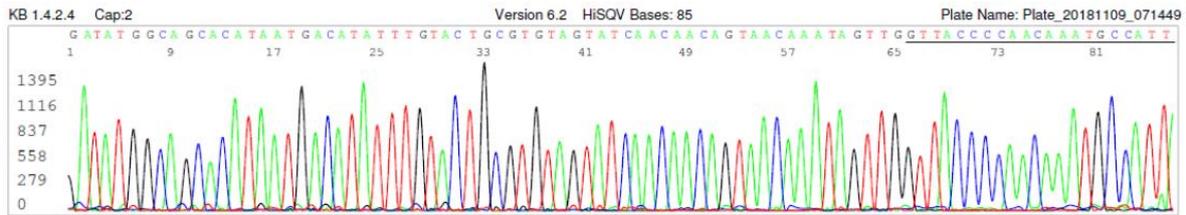
359 Figure 7 When HPV 16 DNA was present in the mixed genotype MY09/MY11
 360 primary PCR products, the non-degenerate R16 primer was able to selectively amplify the
 361 HPV 16 DNA for Sanger sequencing. The R16 primer is 15 bases internal to the MY 11

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

364

365

366 **Natural HPV 16 sequence at primer binding site: GTTACCCCAACAAATGCCATT**

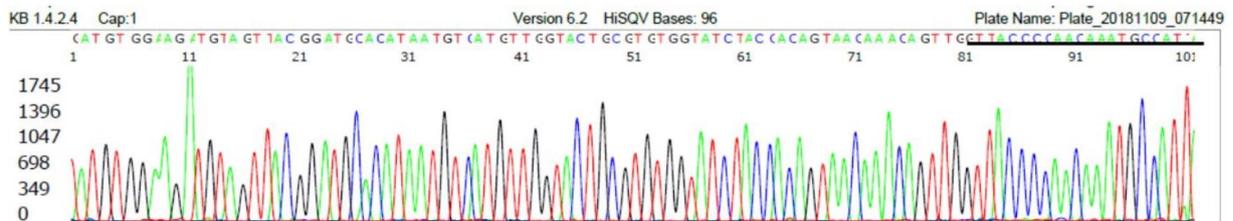


367

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

375

376 **Natural sequence of HPV 6 at primer binding site: ATTACCCCAACAAATACCATT**



377

378

379

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

387

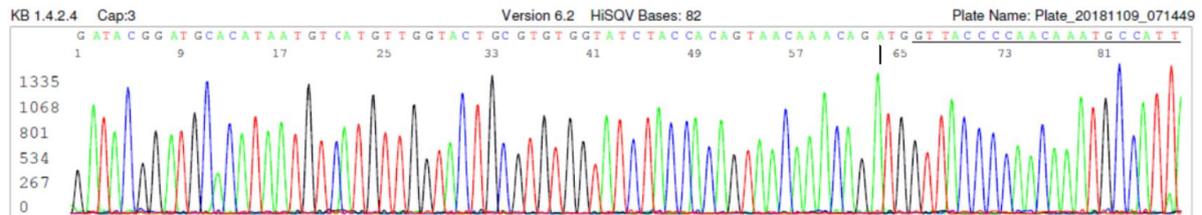
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391

Natural sequence HPV 11 at primer binding site: **ATTACCCCAACAAATACCAT**T



392

393 *3.4.2. Topological conformational change at the primer binding site led to PCR failure*

394

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.

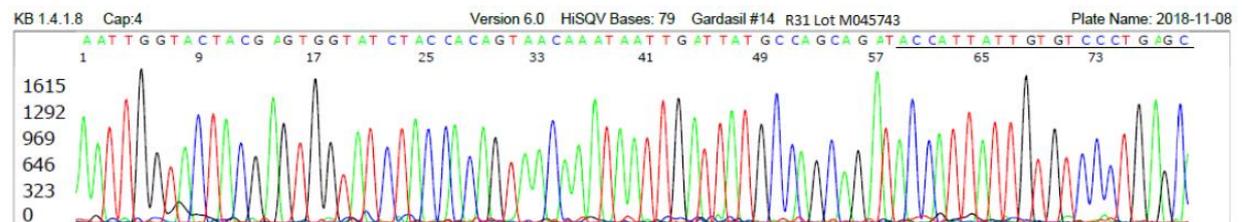
406

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.

412

413

Natural HPV 18 sequence at MY11 primer binding site: **CCATTGTTATGACCCTGTGC**



414

415 This sequence (Figure 10) indicates that the HPV 18 DNA in 1 of the 4 aliquots
 416 from Lot #M045743 was not exponentially amplified by the MY11 degenerate primer as
 417 the HPV 18 DNA in other aliquots from the same vaccine sample. An R31 primer with a
 418 3'-ACCATT end instead of the MY11 primer with a 3'-CCATT end was needed to yield an
 419 HPV 18 PCR amplicon in this aliquot to be used as the template for DNA sequencing. It
 420 was previously reported that non-degenerate HPV 16 MY11 primer with 3'-end extension
 421 was required to amplify some of the HPV 16 L1 DNA fragments bound to AAHS in
 422 Gardasil4 to generate a visible PCR amplicon for Sanger sequencing because binding of the
 423 HPV dsDNA to aluminum salts may cause topological conformational changes at the
 424 MY11 primer binding site, turning a segment of the dsDNA into a non-B conformation [25,
 425 26].

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

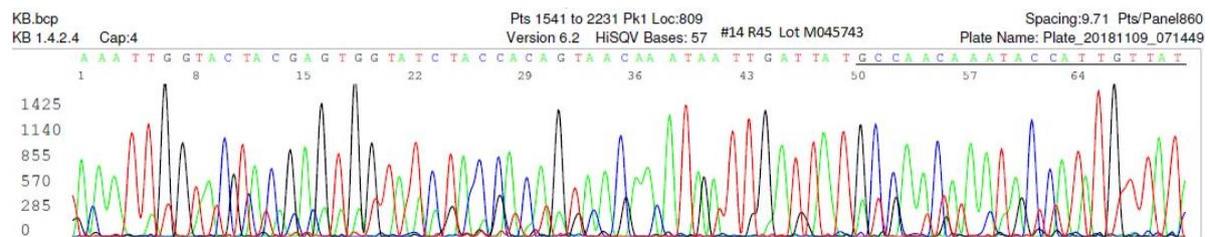
433

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

438

439

Natural HPV 18 sequence at primer binding site: GCCAGCAGATACCATTGTTAT



440

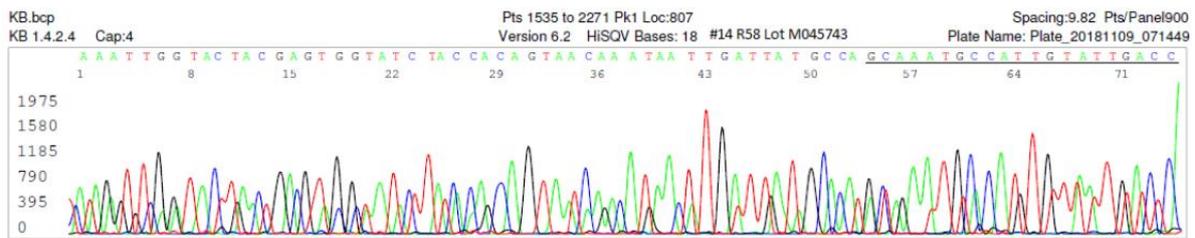
441

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

446

447

Natural HPV 18 sequence at primer binding site: GCAGATACCATTGTTATGACC



448

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

458

459 AACAAATAATTGATTATGCCAGCAGATACCATTGTTATGACCCTGTGC-3' HPV18 (ID#EF202155)

460 CCATTGTTATGTCCTGTGC MY11 No template generated

461 GTTACCCCAACAATGCCATT R16 No template generated

462 ACCATTATTGTGTCCTGAGC R31 Figure 10

463 GCCAACAAATACCATTGTTAT R45 Figure 11

464 GCCATTATTGTGCCCTGCGC R52 No template generated

465 GCAAATGCCATTGTTATGACC R58 Figure 12

466

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

480

481 3.4.3. In the absence of HPV 45 DNA, primer R45 amplified HPV 18 DNA

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

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

487

488

489

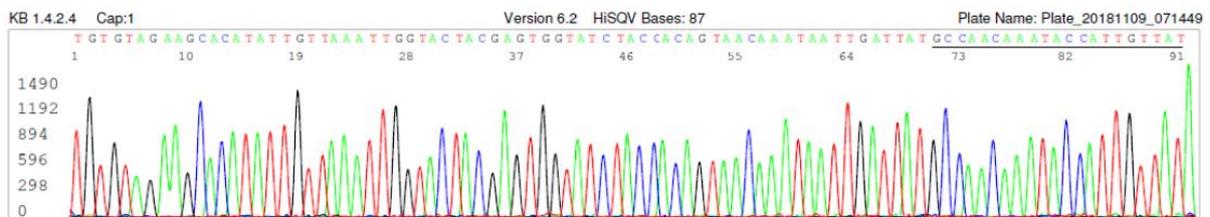
490

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

493

494

Natural HPV 18 sequence at primer binding site: GCCAGCAGATACCATTGTTAT



495

496

497 3.4.4. In the absence of HPV 52 DNA, primer R52 amplified HPV 16 DNA

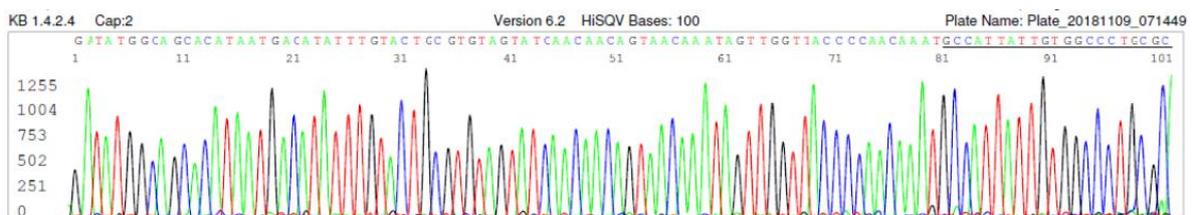
498 When HPV 52 DNA was absent in the mixed genotype MY09/MY11 primary PCR
 499 products, the non-degenerate R52 primer might anneal to a segment of HPV 16 L1 gene
 500 DNA to generate a template for Sanger sequencing. There is only one mismatched
 501 nucleotide (typed in red) between the R52 sequence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

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

505

506

Natural HPV 16 sequence at primer binding site: GCCATTATTGTTGGCCCTGTGC



507

508 3.4.5. In the absence of HPV 58 DNA, primer R58 amplified HPV 18 DNA

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

514

515

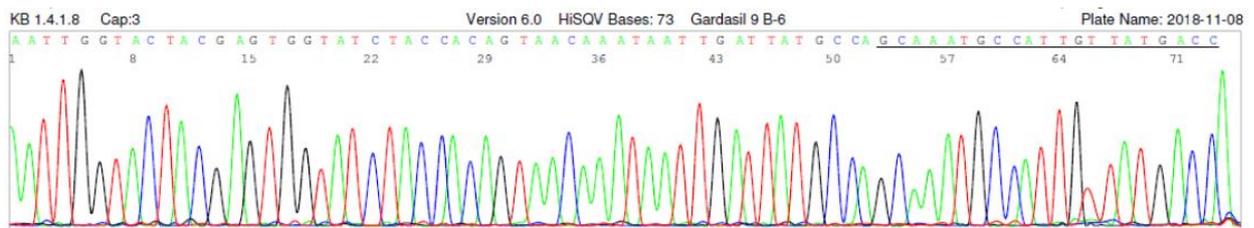
516

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

519

520

Natural HPV 18 sequence at primer binding site: GCAGATACCATTGTTATGACC



521

522

523 4. Discussion

524 4.1. HPV L1 gene DNA bound to AAHS in Gardasil9

525 As advised by the FDA, Gardasil contains recombinant HPV L1-specific DNA
 526 fragments. These HPV DNA fragments are not contaminants [28]. The current study based
 527 on testing 5 Gardasil9 samples and a previous report based on testing 16 Gardasil4 samples
 528 [21] confirm that both Gardasil4 and Gardasil9 contain type-specific HPV L1 gene DNA
 529 fragments. Since these DNA fragments were found to be in the water-insoluble AAHS
 530 particles which were proteinase K-resistant and the DNA remained bound to the proteinase-
 531 digested particles after exhaustive washings in TE buffer with detergent Tween 20, the
 532 HPV DNA detected must be bound to AAHS via ligand exchange as the phospholipids
 533 bound to AAHS in creation of a potent adjuvant for the recombinant hepatitis B vaccine,
 534 Recombivax HB® [7]. Among the officially listed ingredients of Gardasil9 which include
 535 VLPs, AAHS, sodium chloride, L-histidine, polysorbate 80, sodium borate, yeast protein
 536 and water for injection [3], AAHS is the only water-insoluble, proteinase-resistant
 537 component.

538 4.2. Most HPV L1 gene DNA fragments bound to AAHS are in non-B conformations

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

542 they are not targets for removal as for other contaminants during vaccine manufacturing.
543 Therefore, 9 type-specific HPV L1 gene DNAs are expected to be present in the 9-valent
544 vaccine Gardasil9. However, as demonstrated in the current study routine MY09/MY11
545 degenerate primer PCR amplification was able to generate only amplicons of HPV 18, 11,
546 16 and 6 for sequencing validation after testing 5 samples of Gardasil9. As those in
547 Gardasil4 [21], HPV 18 and HPV 11 L1 gene DNAs in Gardasil9 are most commonly
548 detected, suggesting that these two types of HPV DNA are more likely in B conformation
549 when bound to the AAHS particles. However, as illustrated in Figures 10-12, even HPV 18
550 DNA can undergo topological conformational change which may interfere with template-
551 directed enzymatic DNA synthesis during PCR amplification. Successful generation of one
552 single HPV DNA amplicon by PCR as the template for Sanger sequencing does not exclude
553 the possibility that there are other genotype-specific HPV DNAs also in the tested sample.
554 Previous studies on Gardasil4 samples showed that the AAHS-bound HPV 16 and HPV 6
555 genotype-specific L1 gene DNAs were not amplifiable by MY09/MY11 degenerate PCR
556 primers [21, 25, 26]. The current study on Gardasil9 samples shows that using non-
557 degenerate primer nested PCRs and shifting the primer binding sites inwards could amplify
558 some of the AAHS-bound HPV 16 and HPV 6 type-specific L1 gene DNAs in Gardasil9
559 which had been replicated by the MY09 degenerate primer as linear PCR amplification
560 products. The failure to detect any type-specific L1 gene DNA of HPV 31, 33, 45, 52 and
561 58 suggests that all these 5 type-specific DNAs were in non-B conformations.
562 Alternatively, all the L1 gene DNA fragments of these 5 HPV genotypes in the 4 tested
563 lots of Gardasil9 have been removed as “contaminants” during the manufacturing process.

564 *4.3. Topological conformational change of HPV DNA bound to AAHS is genotype-* 565 *dependent*

566 In all tested aliquots of 5 Gardasil9 samples from 4 vaccine lots, HPV 18 and/or HPV
567 11 L1 gene DNA fragments can be amplified by the MY09/MY11 degenerate PCR primers,
568 as reported previously on Gardasil4 [21]. Only rarely, as shown in Figures 10-12, HPV 18
569 L1 gene DNA in a fraction of the Gardasil9 shows a topological conformational change. In
570 contrast, the HPV 16 L1 gene DNA fragments were not exponentially amplifiable by the
571 MY09/MY11 degenerate primers, and require non-degenerate primers with a 3' end
572 extension or primers targeting another segment of L1 gene for PCR amplification as
573 reported previously on Gardasil4 [25, 26]. In the current study, a non-degenerate primer
574 shifted 15 nucleotides inward (R16) at the MY11 binding site generated an HPV 16 nested
575 PCR amplicon for Sanger sequencing validation (Figure 7). An HPV 16 amplicon was also
576 generated when an extra “G” nucleotide was added to the 3' end of the MY 11 primer
577 (R52), as shown in Figure 14. These results suggest that topological conformational change
578 occurred in the HPV 16 MY11 primer binding site 5 nucleotides upstream of the 3'
579 terminus because at least a 6-base matched sequence at 3' end of the primer is needed for
580 template-directed primer extension in enzymatic DNA synthesis [27]. Apparently, when the
581 phosphate backbone of the HPV DNA binds the AAHS, the HPV 16 L1 gene DNA in
582 Gardasil is more prone to topological conformational change than the HPV 18 L1 gene
583 DNA at this location.

584 *4.4. PCR amplification of HPV DNA by primer with a mismatch at 3' terminus*

585 In the absence of a fully matched complementary target, the primer designed to
586 amplify a segment of HPV 16 L1 gene DNA (R16) can initiate a PCR to amplify a segment
587 of HPV 6 DNA (Figure 8) or a segment of HPV 11 DNA (Figure 9) even though there is a
588 single base mismatch at the 3' terminus of a 21-nucleotide primer. A highly processive
589 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, TNF α , IFN γ 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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665

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