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Vaccinegate:

Gardasil 9 metagenomic analysis report

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Brief presentation of the results

With these analyses we have finished the first level screening of the Gardasil 9 vaccine.

Results

Presence of adventitious genetic material in residual quantities. The following essential points can be summarized:

Presence of adventitious genetic material as DNA:

- **Bacteria:** The prcentage is significant: 54% of the total DNA, ths contamination can derive mainly from yeast culture, but also from contaminants in the laboratory; more blanks have been made to minimize the error due to environmental contamination, but we will have more accurate data when we make replicates with other laboratories. The bacterial DNA could interact with the adjuvant aluminum and cause allergies, inflammation and autoimmunity. Data to be confirmed.
- **Human and Mouse DNA:** their origin is not known! It may be that human DNA could instead be a cross-contamination from other cell lnes used for the production of vaccines (it is a hypothesis). These DNA could interact with the adjuvant alumin and cause inflammatory and autoimmune reactions.
- **Adventitous viruses: f**ragment L1 of the HPV virus double strain DNA – comes from the antigen manufacturing process; it is a contaminant because it poses security problems as it is not degraded and remains in the macrophages linked to the adjuvant aluminum for a long time; its biological effect is not fully known but it can probably be integrated into the host DNA, stimulate inflammation through the production of proinflammatory cytokines and autoimmune reactions (see research by Prof. Lee).
- **Phages: t**hey derive from the manufacturing process, they are adventitious contaminants of unknown hazards. Can antibodies against phages interact with bacteria in the intestinal bacterial flora? Can they integrate into the bacterial flora?
- **Molluscum contagiosum virus:** it belongs to the family Poxviridae, subfamily Chordopoxvirinae, genus Molluscipoxvirus. The term pox contained in the name of these viruses comes from the vesicles (poxes) produced be the smallpox virus.
- **Retrovirus:** potentially integrated into DNA; they can cause neoplastic transformation and mutations of the host genome; they derive from the contamination of human and mouse DNA, such as possible cross-contamination with other cell lines.
- **Mouse leukemia virus.**
- **Human endogenous retroirus K.**

Adventitious genetic material present as RNA:

- **Bacteria:** the transcripts indicate that the DNA is working.
- **Synthetic constructs (artificial sequences):** they may derive from the antigen production process by genetic recombination with the plasmid; they are potentially able to recombine with human DNA; the link with the adjuvant aluminum can extend and enhance the biological effect (inflammation and autoimmunity).
- **Yeast and its viruses (L-BC virus and narnavirus):** yest RNA can give rise to allergenic proteins (which can bind to adjuvant aluminum), while viruses are not known for effects on human cells and microbiota.
- **Infectious equine anemia virus and mouse leukemia virus:** (the latter) is present both as DNA and RNA and therefore it's a working virus):These viruses derive from the contamination of raw materials and must not be present.

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Metagenomic analysis report on Gardasil 9

Preface

As is known, vaccines are biological drugs used for the prevention of some infectious diseases and are made up of several components: antigens (viruses, inactivated or attenuated bacteria, inactivated toxins, proteins or complex molecules derived from viruses and bacteria, able to stimulate the immune response), adjuvants (substances that increase the ability of the vaccine antigens to induce the antibody immune response), the excipients (substances necessary to formulate the vaccine, or to preserve it from bacterial contamination) and contaminations (trace substances from raw materials, e.g. cell lines for bacteria and virus growth, or from the manufacturing process, e.g. formaldehyde, antibiotics.
During the registration stage of a biological drug, the vaccine is subjected to the controls provided by the EMA guidelines and agreed with the regulatory body according to the specific type of vaccine. These checks are then carried out on a representative number of samples of each batch before marketing.

The producer and the regulatory body in charge of the control
are responsible for the compliance of the product sold

Since the safety of a vaccine depends on its compliance with the quality criteria, especially those regarding the absence of toxic or potentially toxic contaminations (i. e. of which no human effects are known) it is very important that such compliance is respected in a very strict way. Various studies have posed the issue of the presence of various types of contaminations, both chemical and microbiological, thus raising the question if the vaccines actually comply with the directives imposed by the regulatory bodies, if in turn the regulatroy bodies apply the control for the respect of these directives and if the regulatory bodies have defined with effectve guidelines the criteria for the control and limitation of such contaminations.
In order to answer these questions, Corvelva commissioned a highly qualified center of services, specialized in genomic DNA and RNA sequencing, to carry out the analysis for biological contamination, which should never be present in vaccines.

The study commissioned by Corvelva was based on two types of analysis:

1. **Nucleic acids presence test** (DNA/RNA) of human and animal origin and of microorganisms (viruses, bacteria) using the Next Generation Sequencing method which has allowed to quantify, in a highly specific and accurate way, the sequence of the genetic material contained in the examined vaccines

2. **Verification of the correspondence of the genome sequences** of live and attenuated or inactivated bacteria and viruses present in the vaccines (presence of genetic variants)

Description of the analysis method

Next Generation Sequencing, also known as ***deep sequencing***, generates a single sequence from each DNA fragment, or cDNA, present in a sample. The downstream bioinformatics analysis allows then the differentiation of the origin of the sequence fragments, for example human, bacterial species or a particular virus. This means that mixed biological smples can easily be figures out with this technology, which has now entered the routine of genomic research and diagnostics. Moreover, NGS data make it possible to reconstruct the entire sequence of viral DNA and RNA genomes and bacterial genomes present in the sample and compare it with the reference genomes present in public databases.
The samples examined are shown below along with the results obtained, grouping them by classes of similar vaccines:

** ssRNA: single strand RNA; dsDNA: double strand DNA.*
The underlined terms are made up or contain genetic material (DNA and/or RNA)

Analyzed batches

Batch #1 - 9R009338

Product name:	**Gardasil 9**
Product type:	Human PapillomaVirus vaccine, HPV (strains 6, 11, 16, 18, 31, 33, 45, 52 and 58)
Manufacturer:	MSD Vaccins - manufactured Merck Sharp and Dohme
Composition:	Active substances: 9 non-infectious,hihly purified L proteins of human papilloma virus (strands 6, 11, 16, 18, 31, 33, 45, 52 and 58) in various amounts between 20 and 60 micrograms. L1 proteins are in the form of viral pseudo particles produced on CANADA 3C-5 Saccaromyces cerevisiae cells by recombinant DNA technology. Adsorbed on aluminum adjuvant (Al: 0.5 milligrams); sodium chloride, L-histidine, polysorbate 80, sodium borate.

Requested analysis

Nucleic acids presence test (DNA/RNA) of human and animal origin and of microorganisms (viruses, bacteria), using a metagenomic/metatranscrittomic approach on the Illumina platform of Next Generation Sequencing type.

The comparison of these three vaccines highlight the following critical issues:

Genomic DNA extraction was performed using the Maxwell® 16 Blood DNA Purification Kit sold by Promega and with the automatic extractor Maxwell® 16 IVD (Promega), following the manufacturer's protocol. TNA extraction was performed using the PureLink™ Viral RNA/DNA Mini Kit (Invitrogen) following the manufacturer's protocol.

The starting quantities used for the extractions are as follows:

- DNA extraction: 290 µl of solution for injection
- RNA extraction: 290 µl of solution for injection

The quantification and quality control of the extracted DNA was carried out respectively by the *Qubit 2.0* fluorometer *(Invitrogen, Carlsbad, CA)* and bt the *NanoDrop 1000* spectrophotometer *(Thermo Fisher Scientific, Waltham, USA)*.

Following is the result of DNA quantifications (ND= NanoDrop 1000; QB= Qubit 2.0; HS= dsDNA HS Assay Kit)

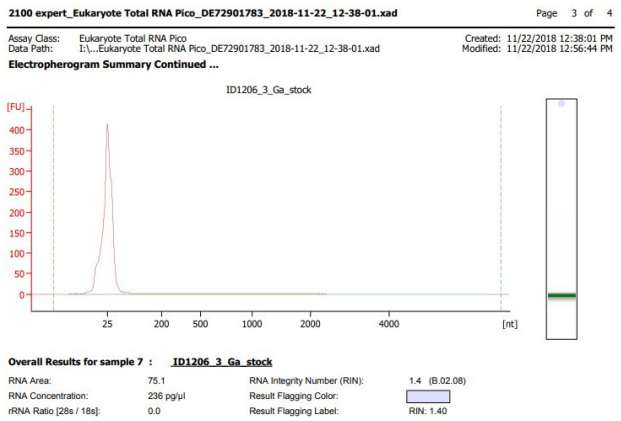
Sample ID	ND A260/280	ND A260/230	QB_HS_ng/µL	volume_µl	Tot_amount_ng
DNA lot 9R009338	1,06	0,41	NA	55	NA

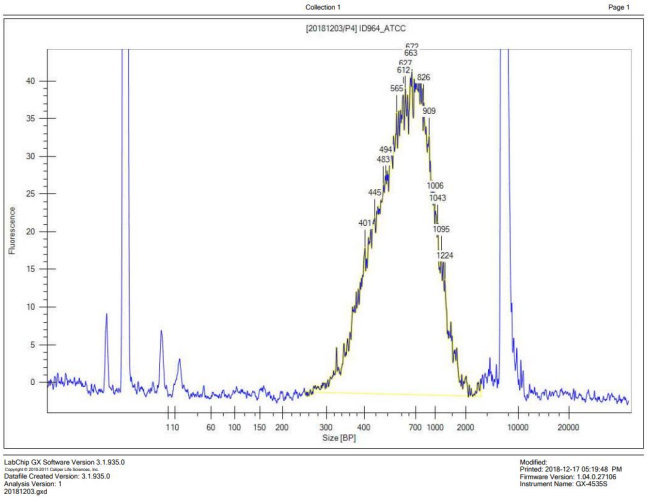
The measurement of the concentration by *QuBit* fluorimeter showed that the DNA contained in Gardasil 9 batch 9R009338, **is not quantifiable by standard fluorimetric methods.**

The quantification and quality control of the extracted RNA was performed by *Agilent 2100 Bioanalyzer* using the *Agilent RNA 6000 Pico Kit* (Agilent Technologies, Santa Clara, CA). Below are the concentration values and the RIN (RNA Integrity Number) measured by Bioanalyzer:

Sample ID	Bioanalyzer_pico_totale_ng/µL	RIN	volume_ul	Tot_amount_ng
RNA lot 9R009338	0,236	1,4	37	8,732

The amount of **RNA** contained in the vial of Gardasil 9 batch 9R009338 **was found to be 8.732 ng** thus calcutated: 0,236 ng/µl (concentration determined by Bionalyzer) x 37 (volume of final resuspension of RNA after extraction, expresses in microliters) x 2 (total volume della solution for iniection/volume used for extraction). The RIN of 1,4 indicates a degraded RNA. The trace appears flat considering the very low amount of RNA contained in the sample.

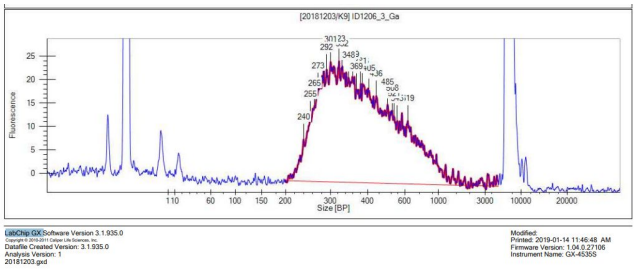




Preparation of DNA-seq library with Illumina technology
The *Ovation® Ultralow System V4 1–96 kit (Nugen, San Carlos, CA)* was used to prepare the libraries following the manufacturer's instructions, starting from a quantity of less than 1ng of genomic DNA. The final library was quantified with the *Qubit 2.0* fluorometer (*Invitrogen, Carlsbad, CA*) and tested for quality using the *Agilent 2100 Bioanalyzer, High Sensitivity DNA Analysis kit* (*Agilent technologies, Santa Clara, CA*).
Here follows the layout of the obtained library:

Preparation of RNA-seq library with Illumina technology
RNA-seq libraries were prepared by using the *Ovation® RNA-Seq System V2 kit (Nugen, San Carlos, CA)* to prepare the cDNA, and the *Ovation® Ultralow System V4 1–96 kit* to prepare the library starting from 10ng of cDNA. The final library was quantified with the *Qubit 2.0* fluorometer (*Invitrogen, Carlsbad, CA*) and tested for quality using the *Caliper GX system* (*PerkinElmer, Waltham, MA*).

Here follows the layout of the obtained library:



Preparation of a DNA-seq library with Illumina technology from a mix of genomic DNA of a metagenomic community of known composition
In order to validate the workflow from the preparation of the library to the data analysis, an ATCC standard – i.e a mix of genomic DNA of known composition, 20 Strain Staggered Mix Genomic Material, ATCC® MSA-1003 ™ - was used to build a library with the Ovation® Ultralow System V4 1-96 kit starting from 10ng of DNA.
Here follows the track on Caliper GX (PerkinElmer, Waltham, MA) of the obtained library:

Sequencing

The libraries were sequenced on Illumina HiSeq 2500 instrument with 125bp reads and 'paired-end' parameter, according to Illumina standard indications. Version 1.8.2 of the Illumina CASAVA pipeline was used to process the raw sequences.
9,194,982 Illumina paired-end sequences were produced, corresponding to 4,597,491 fragments/reads identifying the DNA-seq library; 12,495,858 Illumina paired-end sequences of 6,247,929 identifying fragments/reads for the RNA-seq library; 9,938,490 Illumina paired-end sequences of 4,969,245 identifying fragments/reads for the DNA-seq library constructed from the DNA of the Mix Genomic Material standard, ATCC® MSA-1003.

Bioinformatic analysis
Cleaning sequences (trimming)
The sequences of the adapters – i.e. 'artificial' oligonucleotide sequences that are introduced during the Illumina library preparation – as well as low- quality reads of DNA bases were removed using the ERNE[1] and Cutadapt[2] softwares.

Identification of organisms of origin of DNA and cDNA / RNA sequences with Kraken software
The metagenomic analysis was performed using the Kraken[3] software on the 'Human-Virus-Bacteria_25mer' database (https://ccb.jhu.edu/software/kraken/).
Kraken is a system for assigning taxonomic labels to short DNA readings. It does this by examining the *k*-mers within a read and querying a database with those *k*-mers.
The presence of DNA and RNA is expressed on the basis of the number of reads/fragments and the percentage of reads/fragments out of the total of the reads/fragments produced, attributed by the public databases to the various organisms.

The sequences classified with Kraken software were also confirmed manually with the BLAST software (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Gardasil 9 Batch 9R009338 – DNA analysis

DNA Seq total fragments/reads	**4,597,491**		

Classification		**n° reads**	**% reads**
Bacteria		2405197	54%
Homininae (Homo sapiens)		411670	9%
Murinae (Mus musculus)		400972	9%
Viruses*		1253	0,03%
Unassigned		1378399	30%

*Viruses classification		**n° reads**	**% reads**
dsDNA viruses		1215	0,03%
	Human papillomavirus virus-L1 fragment	752	0,02%
	Myoviridae (phages)	350	0,008%
	Molluscum contagiosum virus	113	0,002%
Retroviridae		38	0,0008%
	Murine leukemia virus	37	0,0008%
	Human endogenous retrovirus K	1	0,00001%

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Gardasil 9 Batch 9R009338 – RNA analysis

RNA Seq total fragments/reads	**6,247,929**		

Classification		**n° reads**	**% reads**
Bacteria		736256	15%
Artificial sequences (synthetic construct)		540799	10%
Saccharomyces		2216355	35%
Viruses*		61254	1%
Unassigned		2693265	43%

***Viruses classification**		**n° reads**	**% reads**
dsDNA viruses		**53184**	**1%**
	Human papillomavirus virus-L1 fragment	53184	0,9%
dsRNA viruses		**7595**	**0,1%**
	Saccharomyces cerevisiae virus L-BC (La)	7582	0,1%
ssRNA viruses		**469**	**0,007%**
	Saccharomyces 20S RNA narnavirus	469	0,007%
Ortervirales		**6**	0,0001%
	Equine infectious anemia virus	3	0,00005%
	Murine leukemia virus	3	0,00005%

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Results of the DNA-seq analysis performed with the Kraken software on a genomic standard of known composition (20 Strain Staggered Mix Genomic Material, ATCC® MSA-1003 ™)

DNA Seq total fragments/reads	4,969,245		

Classification	n° reads	% reads	% declared by ATCC
Acinetobacter baumannii	10735	0,2%	0,18%
Actinomyces odontolyticus	2	0,00004%	0,18%
Bacillus cereus	176327	3,5%	1,8%
Bacteroides vulgatus	1088	0,02%	0,02%
Bifidobacterium adolescentis	489	0,01%	0,02%
Clostridium beijerinckii	123609	2,5%	1,8%
Cutibacterium acnes	6528	0,13%	0,18%
Deinococcus radiodurans	745	0,02%	0,02%
Enterococcus faecalis	704	0,01%	0,02%
Escherichia coli	929837	19%	18%
Helicobacter pylori	4738	0,1%	0,18%
Lactobacillus gasseri	4491	0,1%	0,18%
Neisseria meningitidis	9820	0,19%	0,18%
Porphyromonas gingivalis	578294	12%	18%
Pseudomonas aeruginosa	152307	3%	1,8%
Rhodobacter sphaeroides	1135927	23%	18%
Staphylococcus aureus	72598	1,5%	1,8%
Staphylococcus epidermidis	634940	13%	18%
Streptococcus agalactiae	31622	0,6%	1,8%
Streptococcus mutans	526420	11%	18%
Unassigned	14248	0,3%	0%

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