Review of MS #252538207, entiled „**Quantification of residual plasmid DNA and SV40 enhancer sequences in Pfizer/Biontech and Moderna modRNA COVID-19 vaccines from Ontario, Canada**"
By the 3 co-authors David Speicher, Jessica Rose and Kevin McKernan

The authors describe their experimental efforts to analyze the residual plasmid DNA fragments which are present as "impurity" inside of mRNA vaccines. The aim of the authors is to create a link between the presence and amount of residual DNA fragments and putative side effects. The authors describe in detail their analyses of several batches of Pfizer/Biontech and Moderna vials (32 vials from 16 different lots), by using different methods for the quantification of residual plasmid DNA fragments (Q-PCR and Qubit System), as well as Nanopore sequencing to analyzes the length of the residual plasmid DNA fragments. The conclusion of the authors are: there is too much DNA in these vaccine vials, as the DNA seems to exceed the amount of 10 ng DNA per dose.

Comments

Before I do a review nowadays, I look carefully into the bibliography of authors. In this case we have 3 authors that have contributed to science, one is an experts on viruses (D Speicher), one is working on the topic of genomes and their mutations (K McKernan) and the middle author who has listed many preprints on their orcid account but has only published 2 published paper on her field of expertise, namely data analysis.
The topic of the paper is important, as patient safety in a pandemic situation - where population-wide vaccination campaigns are carried out- is of real importance. Although the WHO have declared the pandemic as ended (5.5.23), we have to analyze carefully the effects of national efforts to fight the pandemic, by using best scientific practice in order to help politicians to make better decisions in the future. In case that bad decisions or bad products have been made, scientific integrity may help to make better decisions in future pandemic situations. Therefore, the topic of the article is of great scientific and political importance. Because of the latter and of what happens world-wide on social media (anti-vaxx movement), it is mandatory for good scientist to find out the truth about this novel class of vaccines.
What most people do not know, mRNA vaccines have been used by the Biontech company over 20 years to treat cancer patients. A recent article in Nature shows the effectiveness and benefits of such mRNA vaccination strategies against pancreatic cancer (all responders survived their cancer). So, this new class of vaccines, which are causing immunological reactions like attenuated viruses, are of great interest.
However, not all classes of modern vaccines are beneficial. First rumours against one novel type of vaccines came up in the first quarter of 2021, shortly after the nation-wide vaccination campaigns have started: problems arose with the adenoviral vector vaccines because they caused a series of severe thrombotic events, followed by the death of a dozen of patients world-wide. This have been first signs of severe side effects, but has immediately led us to avoid these type of vaccines and to use of only the two other available mRNA vaccines from Moderna and Pfizer/Biontech. These vaccines have been applied with ~8 billion of doses worldwide. Based on the data of my own country, side effects of mRNA vaccines can be described easily: the only side observed effect whennusing mRNA vaccines were heart muscle inflammations in 13 (Pfizer Biontech) or 29 patient (Moderna) per million applied doses. All the other less severe severe side effects (headache, etc) were in the range

of 1000 reports per million jabs, however, even this was much less than with other vaccines. Other events like thrombotic events (TTS, ITP), GBS or even death cases related to the vaccination were much lower than statistically expected. The incidence of severe side effects for mRNA vaccines in my country was 0,00000338, which means 3,3 events out of 1 million doses. At least in my country the use of mRNA vaccines has been declared as safe.

Critisisms

1. Unfortunately, I have no acces to the VAERS database data, because the download of the VAERSDataCSVS.zip file from the canadian website remained unzippable. So I could not access the reported data from Canada. But I have to assume that the authors have carefully analyzed these data as depicted in Table 1. I also see the use of 32 vials of 16 different lots of vaccination vials as a positive sign.

2. However, when I start reading the manuscript, I felt somehow uncomfortable, because the complete manuscript does not exploited the topic in here in an unbiased fashion. Many comments in the introduction already pinpoint to certain opinions or assumption of the authors, even before they presented their experimental data. They are saying e.g. in the introduction section that parts of the vector are “oncogenic” (page 4, line 59). This is an overstatement, although this sequence of concern originates from a known oncovirus (SV40). I have to remind the authors that there is not a single paper out in the literature which has ever confirmed that this short DNA sequence (SV40promoter/enhancer) has ever caused cancer in a single human being. If you take the large T Antigen of SV40, cells become immortalized (which is per se not oncogenic), but may lead over time to certain brain tumors or lymphomas. But the Large T Antigen is a known oncoprotein, which is not the case for a simple DNA element. I can clearly say this, because I am an expwert in the field of oncology for more than 33 years. Since the pandemic, I am also working in the field of SARS-CoV-2.
The authors then describe very clearly the production process, on how Moderna and Pfizer/Biontech are and have produced their drugs. At least from the rapporteur report world file, we know that Biontech has changed the production process (process 1 and process 2) when switching from their clinical trails to gross production. But also here, the introduction makes a big mistake as the authors say that there is “a large increase in residual DNA” (page 4, line 69). This is only an opinion, but has no experimental source.
So my first recommendation would be to change the introduction section in a way to make the whole paper less biased.

3. The next point from my side is the Materials and methods section. Here the first sentence already describes a biased assumption. mRNA vaccines can only be produced in such large amounts when IVT is being used. This process requires linearized plasmids which can be transcribed by the T7 RNA Polymerase. Of course these templates needs to be destroyed afterwards in a way that the remaining residual DNA fragments can be tolerated in the many vaccinated persons. I have to mention that other vaccines are on the market which contain more than 1 µg DNA per dose, without harming vaccinated person, just to make the point that DNA may per se not harmful. Therefore, I suggest to use one term throughout the manuscript, e.g. “ residual plasmid DNA fragements” I would not use other terms like “Inpurities”. Impurities are usually used to describe protein contamination in vaccines that are causing strongly inflammatory reactions. This term is not applicable for mRNA vaccines, but for many other vaccines on the market.

4. What I found quite interesting in Table 1 on page 8 is the first lot# FD0810, which is – according to the VAERS data base - the batch causing the most “adverse side effects” (941 AES) and also the most “severe adverse side effects” (154 SAES). This single lot has more events listed than all other 15 lots investigated here (15 lots with a total of 228 AES and 112 SAES). So I was expecting that this single lot is really the worst candidate for a poor production process. However, it turned out later in the manuscript that this lot does not contain the highest amount of DNA; by contrast, it was the lot which seemed to have the lowest amount of residual plasmid DNA fragments. If I would be an author on this manuscript, I have stopped working on this paper as this clearly shows that the assumption about high DNA amounts in vaccines as the putative agent causing severe side effects is simply not true. However, I havn’t found any comment in the manuscript, e.g. in the discussion section, mentioning this clearly. I think it is worth to do so, as it clearly argues in favor that the assumed hypothesis is probably wrong, and that the residual plasmid DNA fragments have nothing to do with the described side affects.

5. There is an important point. The authors already argued in their introduction section that the produced mRNA (they call it “modRNA”) may bind to residual DNA, and thus, protect these RNA/DNA hybrids against the hydrolytic activity of the used DNaseI during the production process (see page 5, lines 80/81). However, also this assumption was in the end not true, because the qPCR data shown in Table 3 display lower values when the Spike amplicon was analyzed, specially when comparing to the number of Ori amplicons of SV40 promoter/enhancer amplicons that could not form RNA/DNA hybrids. Also here, I am missing a clear-cut conclusion in the discussion section because the initial assumption turned out to be wrong. The Spike amplicon scored always less when Biontech samples were analyzed. On the other hand, there is not a single explanation given why the SV40 amplicon scored so high in the other lots FM7380, FN7934, FX4343, GK0932 and HD9867. Also here, lot FD0810 scored with the lowest amount of amplified DNA, although showing the highest AES and SAES in Table 1.

6. I was also checking for the math behind: the PCR amplicon are pretty small, ranging from 105 bp (ori amplicon), 114 bp (spike amplicon) to 152bp (SV 40 amplicon). A single amplimer of these 3 amplicons would have a mass of 0.115 ag, 0.125 ag and 0.166 ag, respectively. Multiplicated with the template “copy numbers/dose” is ending with similar estimated “ng amount/dose” as listed in Table 3. My numbers are sometimes either slightly smaller or bigger (±10), but the range of identified ng/dose is nearly identical.

7. Figure 2 is showing now the estimated “ng of DNA per dose” and compares it with the adverse events of the VAERS data base. This was for me one of the most important Figures in the present manuscript, as it clearly tells the reader that there is no correlation between reported side effects and the content of identified DNA. Wow! This Figure destroys all the hope of vaccine-damaged persons to receive any compensation, either from the company or the government. The question is now, what caused these side effects in affected persons? It will never be the residual DNA. FD0810 contains the same amount of DNA as FX4343, but the complaints were 941 vs. 5. Although here, if I would be the author of the paper, I have immediately corrected my personal assumption and have stopped working on this paper. It must be something else causing this high amount of reported side effects, and even if the amount of DNA was slightly higher than the allowed 10ng/does, the reported side effects

dropped to 64 or 50. Any explanation for this? Any discussion on this in the discussion section: NO!

8. Then comes the chapter about Qubit measurements. Here, the authors made nearly the same mistake as the paper of Kirchner and König, which is highly disputed in the field. Assuming that a vaccine dose contains ~30 µg mRNA and less than 10 ng of residual DNA fragments (while 50 µg mRNA and much less than 10 ng DNA in case of the Moderna vaccine), Qubit measurements are giving wrong results. And it doesn't matter whether Triton X100 is being used (paper from König & Kirchner), or like here, the LNPs were heated up to liberate the LNP-contained nucleic acids. Qubit Kits, or any other kit using fluorescent dyes are not useful for the correct quantification of DNA. This has been nicely demonstrated by the recent paper from Kaiser et al. in the Vaccine Journal.
Here, the authors realized the dramatic increase in the amount of measured DNA when using the Qubit system and whole nucleic acid liberated from the LNP's. The more than 3000-fold difference between RNA and DNA makes the real DNA content unmeasurable. Since the authors are citing the Kaiser paper, why don't they have repeated their experiment by using RNase in combination with ethanol precipitation step in order to get rid of the RNA before measuring the DNA content? The use of RNase A for only 10 minutes doesn't solve the problem, because the hydrolyzed free RNA nucleotides are still in the solution and will compete for the fluorescent dyes as well. Only a purification step (Ethanol precipitation and rinsing the pellet with 70% ethanol will get rid of mono-nucleotides) will help to get the right concentrations by using this particular method. This needs to be either done, or I would leave the complete Qubit section out of the manuscript, as it is more confusing non-expert readers than helping them to understand the problem. Thus, all the data around the Qubit measurements, including Figures 3- 5 are not useful, rather misleading.

9. Also Figure 6 is misleading, as Figure 2 had already clarified that the amount of residual DNA have nothing to do with the reported adverse side effects. To now use these few ng of residual plasmid DNA fragments (mostly lower than the allowed 10 ng) to draw a relation with reported side effects by using Pearson correlations is not valid. Also here the lines, which are putative predicted by a computer program are not valid.

10. Coming to Figure 7 which displays data form the nanopore sequencing. I found it quite interesting that this graphs clearly shows that roughly ¼ of the DNA contained in the samples was probably to small to get amplified in all three amplicons (Spike, ori or SV40). I am counting 230 reads for fragments below 145 bp, but 635 reads for fragments that were above that size, and thus, residual plasmid DNA fragment length should not be a problem for the 3 chosen amplicons. Based on their own analysis, a ratio of 3 out of 4 residual plasmid DNA fragments should allow a successful PCR amplification. However, in the discussion section, this fact seems to be ignored because the authors now talk about 100-fold discrepancy between what has been amplified experimentally and "reality"(see line 505). Based on the Nanopore experiments, I would use only a correction factor of 133% or the multiplication of all measurements by a factor of 1,33 to get the correct amount of residual DNA. So I would kindly ask the authors to really look critically to their own data, and to not make overestimations about the real DNA content. I suggest that the authors trust their data on Q-PCR, because the PCR data seemed quite robust although using 3 different amplicons. Vice versa, I would not trust the Qubit data, because of the known and published problems with that method.

11. The discussion is starting with a wrong statement. The work presented in this study shows very clearly that QPCR and Qubit data are differing very much. Only the Qubit data are consistent with other pre-published and published data, however, these data are always wrong. To cite these wrong data from others does not make these data better, but simply shows that the 3 authors are unable to reflect their own data in the context of the present literature. And the literature has conclusively shown that Qubit data are senseless for the analysis of mRNA LNP's, and does only give correct results wehen the present RNA is eliminated.

12. That Moderna vaccines gives the highest Qubit results is quite clear: Moderna vaccines contain the highest mRNA content(50µg vs 30µg in Pfizer/Biontech), which increases the Qubit results. No self- reflection on their own data!

13. That the mRNA is protecting the DNA fragments is pure speculation when you compare the data in Table 3 for all 3 amplicons. In that case the data obtained with the spike amplicon should be always higher. Not the case for Pfizer/Biontech samples.

14. Also the next paragraph lines 433 to 461 contains mostly pure speculations.

15. the section on dsRNA is also a pure speculation.

16. The discussion about the integration of SV40 sequences, present only in the Pfizer Biontech plasmid, is also pure speculation. If the authors wish to analyze this, incubate LNP's from the vaccine vials in cell culture with mammalian cells and analyze for Putative integration of such sequences into the genomic DNA. At least one of the 3 authors is a specialist for such analysis. If such experiments are not performed, just avoid such speculations. Stick to your data!

16. Avoid the assumption that the QPCR data is probably 100-fold too low. This is not the case. See my point under no. 10.

17. I also suggest to stop speculations about losing DNA in ethanol precipitation experimentss. If the authors want to argue about potential losses when using this technique, then they should perform experiments with exact DNA amounts and to demonstrate that experimentally by using a proper ethanol precipition to demonstrate such losses. I am working now for more than 40 years in molecular labs around the world, I can clearly testify that I have measured personally the efficiency of ethanol precipitation of nucleic acids (DNA, RNA, etc), and I can clearly tell from my own experience that I can even precipitate a 20mer oligo quantitatively (100% efficiency) when the precipitation and centrifugation steps are done accordingly. In addition, the Kaiser paper presented nearly identical data on the DNA content in vaccines as the authors of this manuscript when looking to the Q-PCR data. Thus, questioning the Qubit method in its abilities to measure DNA in the presence of a 3000-fold excess of RNA would be the right thing to do. Instead the authors make senseless statements to substantiate their own - but wrong - Qubit measurement data.

18. In their conclusion they are talking about billions of DNA molecules. Please rephrase this to into femtomolar concentrations (6 x 10^8 molecules are 1 femtomol, our body usually responds to molecules in the nanomolar range!). You don't need to make people afraid of

DNA. Think about that our human body is producing every day ~3 mg uric acid from purine nucleotides deriving from our nutrition. If you calculate the amount of DNA which we are eating, the risk to get gene modified by nutrition is very much higher than from any vaccine that has ever been given to a human being.

19. To my opinion, the authors have successfully shown that the DNA content in these mRNA vaccines are either near or lower than the allowed amounts of residual plasmid DNA fragment in vaccine doses. Avoid the Qubit stuff, as it has been shown that this assay makes wrong measurements. If the authors don't believe the reviewer, then please repeat the procedure as published by Kaiser et al. (destroy LNPs, destroy RNA, make ethanol precipitation and measure the residual DNA) and look by yourself for correct DNA measurements when the mRNA is missing. In that case you will find the same data as in your QPCR experiments. Best learning is doing an experiment. I also suggest to make the necessary and requested changes throughout the paper. I will be happy to review the revised manuscript again.