Review about Submission id 252538207, entitled „Quantification of residual plasmid DNA and SV40 promoter-enhancer sequences in Pfizer/Biontech and Moderna COVID-19 vaccines from Ontario, Canada

The authors have only partially revised their manuscript based on the comments of 2 reviewers. In my humble opinion, the authors have failed to make adequate changes in their manuscript.

Basically, these authors have investigated a large set of vaccine vials and lots deriving from Biontech/Pfizer (10 vials, 6 lots)and Moderna (22 vials, 10 lots). Their presumption is, that these vaccines contain too much residual DANN, based on their previous publication.

The authors have used three different techniques to quantify residual DNA: (1) qPCR on 3 independent amplicons, Qubit-measurements under various conditions and Nanopore sequencing to obtain an idea about the length distribution of the residual DNA fragments in the measured vaccines vials

In the eyes of the reviewer there is still a problem: the Qubit measurements. As the authors were told by the reviewer in the first round of review, this methodology is unfortunately not suited to make measurements in a situation where the vials should contain a high ratio of RNA to DNA (assumed to be 3000:1). This has been recently demonstrated by clear-cut experiments that these fluorometric quantifications are not able to give reliably results when using vaccine vials (Kaiser et al.,). Otherwise, it will lead to results that differ by a factor of several hundreds from reality, as published by König & Kirchner. The authors themselves have used this technology under different circumstances, namely to measure directly the solution of the vials, to digest enzymatically with RNAse and DNAses in this solution, but they always get very high differences when comparing to their own qPCR data. At least it is now mentioned in the discussion section, that this technique is not giving correct results., of not destryoing the RNA before....although using only 10 minutes for the RNA digest is much too short concerning the amounts of RNA per vial.

Although the authors have realized this problem they are still sticking to these data. These wrong data seems to be so convincing for the authors that the results of their measurements is already mentioned in the abstract instead of using them in the results and discussion section. I also do not understand why the abstract say that the DNA impurities ranged from 371-1548ng/dose and 1130-6280 ng/dose for Bionetc and Moderna, respectively. According to Table 3, the authors have measured by qPCR a range of 0.02-23.72 ng/jab (Biontec) and 0,25-0.72 ng/jab (Moderna). Where are these numbers coming from???

The other problem ist that the authors are citing papers, one of which already flagged by the MDPI Journal for the obvious flaws inside the manuscript (König & Kirchner), and the other from Kämmerer, a german female scientist which is currently in a court trial with her own University (Würzburg) for the false data she is claiming. In addition, Kämmerer and also Kirchner are both anti-vaxx people in Germany, both very well known, and are definitively NOT scientists, as they have decided to sell books (https://www.genimpfstoffe.com) or to found a small association with other anti-vaxx people like Kämmerer has done to make money (https://www.mwgfd.org/). All these people have only in mind to make money out of their „front page stories“, but all of this has nothing to do with reality or science.

By contrast, the authors of the present manuscript are claiming that the paper from Kaiser et al., would contain technical flaws. However, these scientists have at least demonstrated by clear-cut experiments that the Qubit DNA quantification method is giving false data when using it with these mixtures of RNA and DNA. The have done a very convincing experiment with spiking-in additional RNA (commercial MS2-Phage RNA) into a fixed amount of genomic DANN (sheared salmon genomic DANN). Their setting has convincingly shown that the addition of RNA immediately increases the results for all DNA measurements when using the commercial Qubit DANN kits. This was not an experiments that can be mentioned to exhibit technical flaws. It was the killer experiment that demonstrated that the Qubit system should be excluded forever when quantifying specific nucleic acids in all kind of mRNA vaccines

Also their argument that an Ethanol precipitation steps is probably leading to a loss of smaller DNA fragments is ridiculous. I can reassure that there is no loss of smaller DNA when doing an ethanol precipitation correctly. I am working as a scientist for more than 40. We did 40-30 years ago mostly experiments with radioactive labeled probes for specific experiments (Northern blot, Southern Blot, Bacterial colony screening, etc), or even with PNK-kinased radioactive primers (18-22mers) which were then used for primer extension experiments. In all these procedures we had to precipitate the 32P αdATP radioactive-labeled DNA or the 32P γATP labeled very short Oligo's - and I can reassure that I never lost a single count to the supernatant when precipitating these labeled DNA fragments by the normal method of using 3 M NaOAc pH 7.0 and 3 Vol 96% Ethanol – with an afterward step of at least 1h freezing at -80°C
All these arguments of the authors that one lose DNA while doing ethanol precipitions is only making clear that they do not understand what they are doing when making such statements. And citing papers from people who have published the loss of DNA during a standard ethanol precipitation makes things not better, as it only extends the crowd of people not able to perform standard lab experiments.

To my opinion, the data which has been obtained by Q-PCR is correct, because it is cross-validated by the different amplicons, while the data of the Qubit measurement is definitively not. Until the authors are not able to accept this fact, their paper is not acceptable.

Moreover, the authors have done all their works because of the data in the VAERS database. And again, the authors are having the assumption that a high DNA content in these vaccines is responsible for all these adverse (AEs) or severe adverse events (SAEs). But the batch with the highest AEs (lot# FD0810) had the lowest DNA content. As I already mentioned in my first review, this single batch had more AEs and SAEs (941 and 154, respectively) than all the other 15 lots together (a total of 228 Aes and 112 SAEs). So the whole assumption for making this study is not valid anymore, but still ignored by the authors (see Table 1).

The last mistery comes from the diverging results of the three amplicons in their QPCR experiments. The authors assumed that the spike ORF, where RNA is present and complementary to the residual plasmid DNA, may form DNA-RNA hybrids which would then help that these hybrids become less accessible to DNA digestion with DNAse I. It was exactly the opposite, because these DNA amplicon gave 10-times less copies than the SV40 enhancer/promoter element (for lot# FM7380, FN7934a and b; see Table 3) All the other lots gave nearly identical results. So looking to Table 3, we do see 29 times that the DNA content

is much less than allowed by the FDA (<10 ng/jab). Only 3 lots showed 2 ng/jab with the spike amplicon, ~4 ng/jab for the Ori amplicon and ~20 ng/jab for the SV40 promoter-enhancer amplicon. There is no explanantion for this deviating results of the 3 losts, but these data made it to the front page, as well as the wrong results from the Qubit measurements. This is not „good science“ if you have data from the majority of investigated lots, but only the outliers make it to the front page. Instead, the 29 vials give you the impression that side effects are not caused by the residual DNA.

My previous review tried to convince these authors to make scientific interpretations and conclusions with caution, but they refused it again in their revised version. They kept sticking more to their weird results instead of questioning their outliers.

The same is true for the discussion section. Most parts of the discussion section are pure polemic and speculations about everything. Starting wit a setence that all investigated vials contained residual DNA is true, but also must be. Citing in the first paragraph König&Kirchner as well Kämmerer, just tells me that the authors want to fuel the anti-vaxx community. These two papers should not be cited, as Kämmerer has published in a non-peer reviewed Journal and König and Kirchner have been flagged by the MDPI Journal.

The next chapter about Qubit can be completely deleted.

Then the next chapter of the VAERS database and the color of the tap can be deleted as well, because it contains pure speculation.

The next chapter is highlighting the outliers based on the qPCR of the SV40 amplicon, but not on the other 2 amplicons which are not even mentioned.

In the next chapter the authors try to explain why the lot with least DNA inside gives the highest AEs and SAEs, instead of saying that there is unfortunately no correlation between the data in VAERS database and the DNA measured content. Instead the authors speculate now about the chemical modifications of vaccine incredients without demonstrating any evidence for it – but all speculations.

The next chapter is dealing with the potential function of fragmented modRNA as potential inflammatory reasons – again not a single experiment by the authors demonstrating that these molecules do at least exist – all speculations.

The again a discussion about SV40 DNA. It is true that this DNA can be imported in the nucleus, where it will be exposed to hydrolytic enzymes. That integration may accur has not been convincingly demonstrated in published papers, and that this DNA exhibits any oncogenic features also not. We know from hundred of publications that only the SV40 large T-Antigen exhibits oncogenic features, and that infection with SV40 may cause cancer. But there is no evidence that this short piece of DNA is really dangerous. If the authors present experiments where they could show the transfection of this short DNA is causing e.g. loss of contact inhibiton in NIH 3T3 cells, then I will be convinced about their arguments. At the moment, all is speculation and showmaking.

The next chapter is dealing with the fact that Biontec shows always higher lower CT values for the Ori amplicon than the spike amplicon; vice versa with Moderna.
Also here, more experiments are necessary to understand these differences. The authors need to investigate the size distribution in both types of vaccines, or to change other experimental parameters to understand the discrepancies in their experiments. To explain everything with folding of the modRNA or the protection of DNA against DNaseI is not sufficient.

Then again the Qubit method (not a valid method) is being discussed. Delete it.

The next chapter is dealing again with the Kaiser et al. Paper. Again the authors want to show that Qubit measurements are valid. As outlined above – it is not.

The the size distribution in their experiments is discussed (Figure 6). The amplicons which have been used in this paper were 105 bp (ORI), 114 bp (Spike) and 152 (SV40). By looking to Figure 5, where the distribution of the differently sized DNA fragments is shown, it is pretty clear that from 860 reads only less than 100 are smaller than their amplicon – this is roughly 1/9 of all available products. And even if products are smaller, it is by the nature of PCR experiments not impossible that 50 bp fragments when slightly overlapping make larger products. There must no be a full length amplicon-sized DNA fragment to get a PCR product. So the authors are finding any argument to say that there is more DNA than they have measured.

Then the paper of Kaiser et al is again wrongly discredited, in favor of their own false results (see above). When Kaiser et al. used DNase1 digestion they measured afterwards RNA, and vice versa. Therefore even this chpater can be deleted. The discussion about ethnol precipitation can be read above.

The next chapter is again explaining that low amount of DNA – we are talking here about femtomol to picomols are behaving like nanomols, and thus are dangerous. This chapter is really a wizzard-like story. As mentioned above, DNA is very quickly destroyed in cells due to a large bunch of enzymes that kill non-genomic DNA which is not circular before it causes any harm. Only DNA protected by protein may survive a little bit longer. To argue that fragments below 200 bp in length are dangerous is ridiculous.

Their conclusion is also another highlight: billions to hundered of billions...why not saying femtomol to picomol (also picomol is only reached with wrong Qubit measurements). 6x 10e8 molecules are 1 femtomol. Period. To make people afraid with large numbers make this paper not better. As I already outlined in my previous review, nature is working mostly in the nanomolar scale measured by Kd values. So a ligand with its cognate receptor, hormones with their hormone binding nuclear receptors, all interactions are ranged from 1 nmol to several hundred nmol. So why should something with is at least 1000-fold to 1000000-fold less concentrated should make us afraid? It has simply no biological function nor is causing harm. All the discussions about potential inflammatory reactions is stupid, as most people vaccinated with mRNA vaccines suffered for one night about .pressure pain at the injection site, and that’s it. TTP and the more dangerous ITP after vaccination with mRNA vaccines was 20-30 times lower than the natural incidence (data from the German PEI). Therefore, all speculations in here, are really only speculations. There was not a single correlation between

the data of the authors and the VAERS database. This would bring me to the scientific conclusion, that their assumption was wrong, disproving that mRNA vaccines cause any harm because of the contained, residual and fragmented DNA.

Thus, the revised version of the authors have strengthened my opinion that the whole paper is „a mission“ for the „anti-vaxx community“, and not a scientific paper. Since polemic and pure speculations should not be published, I regret to let pass this paper for publication.