The Story about the constructed SARS COV-2 Virus - A Review of three Research Groups

Abstract

A literature research on synthetic recombinant SARS Coronavirus was made to answer two questions. Is the SARS CoV-2 virus designed in a laboratory? And why has the SARS CoV-2 such a high mutation rate? A total of 12 research articles, 2 reviews and 10 experimental studies were attributed to three Research Groups, the Wadsworth Center New York, the Vanderbilt Medical Center, and the Chapel Hill North Carolina. The research papers were published between 1991 and 2014.

All 12 research papers reported the successful construction of recombinant SARS Coronaviruses based on RNA reverse genetic and molecular techniques. The Research group from the Medical Center at Vanderbilt University proved how an engineered SARS Coronavirus with an impaired Exonuclease resulted in a progeny virus with high mutation rate.

Furthermore, the review showed that a zoonotic-human transmission was just possible with specific genetic manipulations at the SARS CoV virus genome through selection of virus species for recombination, and targeted manipulation at non-structural virus domains. But importantly, the studies showed that a SARS Coronavirus cross-species infection such as between zoonotic and humans or between different animal species without the exchange of the virus spike protein domain with the host-specific receptor-binding domain (RBD) and additional point mutations was not possible. Therefore, the SARS CoV-2 was deliberately constructed to overcome the receptor limiting factor for animal-human infection.

Interestingly, the review revealed that the study purpose of constructed recombinant SARS CoV changed from the scientific research point of view to vaccine production and development. Competing interests for all reviewed studies by grants from private investors such as the Gates Foundation and vaccine production companies were part of the discussion.

Keywords: SARS CoV-2, Covid19, Spike protein, gene sequencing, Vanderbilt University, University North Carolina, Wadsworth Research Center, New York Health Department, Coronavirus, Bill & Melinda Gates Foundation, WHO, Pfizer, Merck, Novartis, AlphaVax

Introduction

This is an observational study that answered questions based on collected and summarized empirical evidence. A systematic literature research on synthetic recombinant SARS coronaviruses was conducted to answer the following two questions;

1. Was the SARS Coronavirus CoV-2 virus designed in a laboratory?
2. Why has the SARS Coronavirus CoV-2 such a high mutation rate?

Methods

A total of 12 research papers were carefully selected by their quality and research subject.
The articles were attributed to three different research groups based on the author. The articles were reviewed in ascending chronologically order. Reviewed were four studies from the research group by Paul S. Masters at the Wadsworth Center, New York, four studies from the Mark Denison lab at Vanderbilt Medical Center in Nashville, and four studies from authors including Rachel L.Graham, and Barry Rockx by the Chapel Hill, University of North Carolina.

Results
10 out of 12 research articles were experimental studies. Two articles gave an overview of genetic engineering methods with Coronaviruses. The study by Masters and Rottiers published an overview of genetic reverse techniques for constructing Coronavirus recombinants (1).
And the study by Cornelius de Haan and Masters gave a detailed description of the Coronavirus genome manipulation through reverse genetic approaches. Like a recipe, the step-by-step description guidance the researchers through the usage of materials, cell culture and plaque assays as well as infection and electroporation techniques that allowed the successful construction of recombinant chimeric cross-species RNA genome (2). 2 out of 10 experimental studies published their constructed chimeric recombinant RNA gene sequence. Becker et al. and Koetzner et al. assigned their chimeric recombinant gene sequence to the Genbank (3,4).

Wadsworth Center for Laboratories and Research in New york
The first experimental work with coronaviruses started with the coronavirus mouse hepatitis virus (MHV) in 1991. An MHV mutant was found that had ideal properties for the recipient parent virus. Master et al. proved that recombination between an MHV virus and a tailored, synthetic virus species created a stable replicating progeny coronavirus. Thus virus mutant with the first engineered site-specific coronavirus mutants showed different behaviour in thermolability (4).

Kuo and Masters constructed in 1999 a recombinant heterogenous coronavirus between the MHV and the feline infectious peritonitis virus. Through genetic engineering, the spike protein of the MHV virus was replaced by the spike protein of the feline infectious peritonitis virus (fMHV) to create successfully an MHV infection in feline cells. The study concluded that the Coronavirus host cell range was primarily dependant on interactions between spike protein and the virus receptor (5).

In 2005, Masters gave detailed instruction on the RNA manipulation techniques for the coronavirus. By recombining a synthetic donor RNA virus with a recipient parent virus, and altering some characteristics, Masters introduced a general approach for RNA manipulation.
The recombinant gene cDNA fragments were generated into a cDNA transcription vector, and transcribed in vitro as a donor RNA, following electroporation and infection in cells.
But moreover, Masters explained why the coronavirus was the focus of genetic engineering.
The RNA of the coronavirus already showed frequent recombination process during the virus infectious cycle which made genome manipulation easier. Thus mutagenesis at DNA level required no further subcloning steps. The efficient selection of recombinant viruses based on host cell switching was successfully developed within a little time of experimental work. The technology of targeted manipulated RNA was announced for future development of vaccine vectors. Additionally, Masters reported that targeted mutations within the donor coronavirus RNA were incorporated into the recombinant RNA virus progeny (1,2).

In summary, the research group around Masters proved that Coronavirus recombinants with synthetic RNA virus strains were feasible and in the interest of future experimental work. Further, the studies made a first indication that engineered mutations within specific gene domains will be incorporated in progeny virus mutants.

Medical Center at the Vanderbilt University, Nashville
Professor Mark Denison is the director of the Vanderbilt Pediatric Infectious Disease Department, and founder of the Denison Lab where the replication and pathogenesis studies of Coronaviruses went back to 1986 with the first Coronavirus Mouse Hepatitis virus (MHV) studies.
The research in 2001 allowed Denison to visualize a transfer of MHV proteins and virus structures in infected cells through imaging approaches (6).

Graham and Denison gave evidence in 2006 that gene manipulation through deletion of entire protein domains such as the non-structural protein nsp2 resulted in stronger infectivity of the MHV and the SARS CoV virus. Graham concluded that the nsp2 protein domain plays a vital role in virus infection. However, the gene sequence of the new recombinant SARS Coronavirus with deleted nsp2 was not published in the research paper (7).

In 2008, Michelle M. Becker and Mark R. Denison published their pioneer work of engineering the first synthetic bat Sars-like coronavirus with a human Coronavirus spike protein that was cultivated and infected successfully in mice and human cells. Using reverse genetics, Becker and Denison designed in a first step a bat SARS-like Coronavirus genome (BAT-SCoV) from 4 different bat SARS-like Coronavirus gene fragments (Genbank association no. FJ211859). In a second step, Becker replaced the Receptor-binding domain (RBD) in the spike protein of the bat SARS-like Coronavirus with a human RBD from a SARS CoV virus (BAT-SRBD), simulating an in vitro cross-species transmission. In this case, the host accepts the donor coronavirus from another species. The new synthetic RNA strain BAT-SRBD was published in the Genbank, association no. FJ211860 (3) and showed 85% identity with the Spike protein domain of the Wuhan SARS CoV-2. The study of Becker and Denison answered the question if the SARS CoV-2 was replicated in a lab through reverse genetics.

The question why the SARS Coronavirus is a highly mutating virus was answered later by Lance D. Eckerle and Michelle M. Becker in 2009. While small RNA viruses have not enzymatic proteins for error recognition, prevention and repair functions, larger RNA strains such as the Coronavirus showed the existence of such enzymatic proofreading functions called Exonuclease (ExoN).
The research group observed in previous lab experiments with the MHV coronavirus that the nsp14 non-structure protein domain for the Exonuclease (ExoN) is responsible for replication fidelity in large RNA viruses.

In their study, Eckerle and Becker designed a mutant coronavirus from the SARS CoV Urbani (Genbank association no. AY278741) with an inactivated nsp14 (S-ExoN1) and compared the virus growth and mutation rate with the non-manipulated SARS Wildtype (SARS-WT).
Additionally, 4 point mutations were engineered into the S-ExoN1 gene sequence.
The results showed that the manipulated SARS virus with S-ExoN1 had impaired growth and decreased replication fidelity comparing to the wildtype (SARS-WT).

The mutant virus clones from infected cells had not only retained the engineered point mutations but also showed additional 100 new point mutations compared to 7 new point mutations in the wildtype. Eckerle and Becker answered the question why the SARS CoV-2 has such a high mutation rate. Through synthetic manipulation of the nsp14 Exonunclease and additional engineered point mutations within the nsp14 protein domain, the SARS CoV virus genome becomes unstable and shows increase mutations with every replication cycle. Unfortunately, the paper didn't publish the genome sequence of the SARS CoV with impaired S-ExoN1 protein (8).

In 2010, Mark J Gadlage and Mark R. Denison described an experimental scenario with the MHV virus and human coronavirus mutants. Targeted deletion of specific gene sequences called cleavage sites (CS) at the non-structural proteins nsp1-3 resulted in an alteration of the virus protein processing and virus replication. The order and number of the cleavage sites differentiate between the human coronavirus genotype (HcoV-229E) and the more clinical severe SARS CoV virus.
With exchange and/or deletion of the CS, the virus showed changes in the infectivity.
A transfer between the HcOv-229E and the more clinical SARS CoV can be achieved by gene manipulation. The genetic sequence of the Coronavirus mutants with manipulated CS was not published (9).

In Summary, the Coronavirus Research group at the Vanderbilt Medical Center revealed the following findings:

a) Gene manipulation by deletion of specific gene sequences in the non-structural proteins nsp2 and nsp2-3 changes the infectivity of the SARS Coronavirus.
b) Gene manipulation by an exchange in the spike protein domain allows a recombinant cross-species infection.
c) Gene manipulation by deletion of the Exonuclease protein and additional engineered point mutations within the non-structural protein domains resulted in high mutation rate in progeny SARS Coronaviruses with every replication cycle.

All three findings showed that a synthetic SARS CoV-2 with higher infectivity was designed through engineered cross-species recombination. Also, targeted RNA mutation impaired the natural RNA error prevention and repair function of the SARS CoV-2 and resulted in virus progenies with high mutation rates.

Chapel Hill at University of North Carolina
Additionally to the previous research groups, Barry Rockx and Timothy Sheahan published in 2007 their results about synthetic recombinant SARS CoV viruses. Using reverse genetics, Barry Rockx constructed 5 SARS CoV virus strains with the backbone from the SARS CoV Urbani (GenBank accession no. AY27841) and replaced the spike protein domain each with 3 different human and 2 different zoonotic spike protein domains. The human SARS CoV spike protein domains were taken from CUHK-W1 (GenBank accession no. AY278554), the GZ02 (GenBank accession no. AY390556) with synthetic side-specific mutagenesis that has been previously created in the author's laboratory (GenBank accession no. AY304486), and the Hc/SZ/61/03 (GenBank accession no. AY515512).

The zoonotic spike proteins were taken from the Himalayan civet (A031G) and a racoon dog (GenBank accession no. AY687358). All human recombinants showed growth and infection in human epithelial cells. The human recombinant with a synthetic side-specific mutagenesis GZ02 and the palm civet recombinant showed infection and death in mice. They acquired six mutations for the development of their lethal phenotype. However, the recombinant with raccoon dog spike protein showed no infection and growth. All gene sequences of the synthetic recombinant viruses were not published (10).

While their previous study, Rockx and Sheahan reported that a synthetic recombinant SARS CoV virus with the spike protein from the palm civet wasn't able to enter human cell cultures.
A follow-up study with constructed cell lines expressing civet (cACE2) and human (hACE2) ACE2 receptors in delayed Brain tumour cells (DBT) showed that the cell entry barrier was overpowered by the receptor expression in the constructed cell lines.

Cells expressing cACE2 supported the recombinant palm civet SARS CoV with infecting successfully civet cells but not human cells. However, virus mutants with targeted point mutations in the palm civet spike protein domain enhanced growth and infection in the hACE2 humane cells but at the same time not in the civet cACE2 cells. The transmission of a SARS civet recombinant virus in human cells required targeted point mutations close proximity but outside of the receptor-binding domain (RBD) of the spike protein domain to promote a rearrangement of the spike protein binding interface for cell entry (11).

Agnihothram and Becker repeated in 2014 their recombinant human-zoonotic studies with the SARS Coronavirus. This time, they used a bat Coronavirus (BTCoV-HKU5) strain as the backbone of the virus and replaced the spike protein domain with a human SARS CoV (BT-CoV-HKU5-SE) through reverse genetic techniques, comparable with Becker and Graham's study from the Vanderbilt University. For cell entry in mice, an additional mouse-adapted strain was constructed which showed more virulence (BT-CoV-HKU5-SE MA).

Finally, targeted mutations in the non-structural proteins nsp13 and nsp14 (ExoN) with enzymatic proofreading function showed also increased virulence in mice. The synthetic recombinant SARS Coronavirus showed growth and infection in mice and human lung epithelial cells. New insights revealed that the bat Coronavirus backbone requires additional gene modification in the spike protein domain in order to passage mice cells. Unfortunately, the synthetic recombinant genome was not published (12).

To Summarize, the research group around Rockx, Sheahan gave more comprehensive details about dependants of the spike protein domain with the human receptor for virus infection. Constructed cross-species recombinants resulted in gene point mutations. Virus transmission between zoonotic and human cells required not only exchange in the spike protein domain but depending on the host species targeted mutations in specific gene sequences were necessary for increasing infection ability of the virus. This clearly answered the question if the SARS CoV-2 was constructed.

2 out of 10 experimental studies mentioned competing interest statements. The study by Lance D. Eckerle was funded by grants from the NIH and the Vanderbilt University (8). Although the author declared no competing interests, the conflict of interests for this study will be discussed in the next section. The study by Becker and Graham declared a conflict of interest because the contributor to the research study, Robert E. Johnston from the North Carolina Vaccine Institute, is an equity holder for PharmaVax and Executive Director of Global Vaccine Inc. (3,13). Except for the study by Graham and Becker who was privately funded, all 9 experimental studies were supported by grants from the National Institute of Health (NIH).

Discussion

Experience with cross-species Coronavirus transmissions was available throughout 10 years of experimental studies with coronavirus species such as the MHV or Bats-SARS-like Coronavirus. And the knowledge about the construction of stable independent virus progenies from recombination between Coronavirus genomic RNA and tailored synthetic RNA species which resulted in high rate of progeny mutants were already available in the studies from 1991 to 2001 (4,5,6). Years before the first SARS CoV virus outbreak started in 2002-2003 (14).

All 12 research studies revealed that SARS Coronaviruses are strictly species-specific in cell growth and infection. Particularly, the genetic differences between the virus spike protein and the human receptor domain (ACE2) create a major limiting factor and prevent cross-species transmissions. But as Paul Masters from the Wadsworth Center in New York already reported in 2005, a recipe with the right coronavirus species selection with targeted cross-species recombination and engineered mutations can change a temperature-sensitive and thermolabile coronavirus phenotype into “intentionally constructed lethal mutants”(1).

Interestingly, the study interest changed from gene expression, gene function and understanding of the Coronavirus cell interactions in 1991-2001 (4,6) to mainly vaccine development in the next years. While in 1991 Paul Master's recombinant studies were focused on interactions on the molecular level, in 2005 Masters changed the purpose of his recombinant studies in favour of vaccine development. “Second, the ability to genetically rearrange coronavirus genomes provides a critical safety asset, because it will allow the construction of vaccine or vector viruses...” (1).

The urge for vaccine development could be explained by the first SARS pandemic outbreak in 2002-2003. However, Masters mentioned already in 2001, specific gene deletion in the MHV virus for the benefit of vaccine development (15).

The study by Barry Rockx in 2007 went even one step further. While Rockx observed severe lung infection in aged mice induced by one of the human recombinant SARS Coronavirus strains and the palm civet recombinant strain, he concluded from laboratory experiment with 12 month aged mice that vaccines protect elderly populations against future zoonotic SARS CoV virus strains (10). From today's perspective, the author's conclusion resembled the announcement of a vaccine distribution for the elderly population rather than an objective statement based on scientific observations.

Michelle M. Becker articulated in 2008 that vaccine development was required for the protection against future emerging zoonotic pathogens. While the study declared a conflict of interest because the contributor in the study, Robert E. Johnston, was the founder of two private vaccine production and distribution companies, Alphavax and Global Vaccine Inc., Becker published in her study the following statement: " ...the current panel of SARS-CoV vaccines may provide significant protection against other SARS-like CoVs that emerge from zoonotic pools by natural recombination or are deliberately designed to cross-species" (3). The question arises why vaccines against deliberately designed cross-species transmissions are required? Not only was this study directly supported by Robert Johnston's, founder of AlphaVax, but also indirectly by the Gates Foundation who funded AlphaVax (16,17). Results and conclusion based on studies by private investors are without any scientific basis and contradict the observations made during the research.

Although Timothy Sheahan demonstrated how several obstacles had to be overpowered in simulating a cross-species infection, by constructing recombinants in designed ACE2 receptor cell lines and additionally targeted point mutations, he concluded that natural evolution occurred through repeated transfer of cross-species transmission which justified the development of cross-reactive antibody vaccines for the benefit of the public health (11).

While Sheahan made not a conflict of interest statement, the support for this research came from the NIH grant (R01 AI059136 and AI059443) which was funded by the Gates Foundation (18). But the competing interest of the author appeared in another experimental study, where Sheahan demonstrated the expression of the SARS CoV spike protein in a Venezuelan Equine Encephalitis Virus replicon using reverse genetics techniques for the purpose of vaccine testing. The study was supported by AlphaVax Inc which was as previously mentioned funded by the Gates Foundation (19).

The bat-human recombinant study by Denison from 2014 gave 61 search results for the word vaccine. And a section marked in red declared that the significance of the study purpose lied in control over newly emerging viruses. However, this study purpose fades under a strong focus on vaccine development. The author articulated over three times that constructed RNA genomes of different Coronavirus species provided a strategy for vaccine development against future zoonotic coronaviruses. Nevertheless, convincing the reader to product development such as vaccine production by repeated expression of future Coronavirus threats is based on business methods rather than scientific conclusions.

While this was not a vaccine study, the author took the opportunity to make a general recommendation on the vaccine content based on results of his in-vitro study that vaccines against future novel coronaviruses should contain the spike protein or a spike protein chimeric epitope from different coronaviruses (12). As previously discussed the spike protein domain is species-specific for the SARS coronavirus and requires multiple gene manipulation for interaction with the human

receptor domain. Thus, the discussion about chimeric epitopes from different virus species would certainly have been applicable and highly preferable.

The author, Agnihothram did not state competing interests. However, there were two conflicts of interests in the study. While Michelle M. Becker was mentioned as a co-author, it is noteworthy to mention that she was already appointed to a US federal office position in 2011 (20). Furthermore, the study was fully funded by the NIH who received grants in 2013 of over 24 million US Dollars from the Gates Foundation (21).

To summarize, all reviewed experimental studies were from high-profile Scientists working for renowned research institutions. But the studies lacked visibility. Just two articles published their synthetic designed genome sequences. And also just two authors mentioned competing interest statements.

None of the experimental studies was independent studies in the interest of the public or for scientific research purpose because they were directly or indirectly funded by private investors. Nine studies received funds from the NIH. Where the NIH received by 2020 a total of 490 million US Dollars in grants (22).

Where the experimental studies received all grants from the NIH, research institutions such as Chapel Hill from the University of North Carolina and the Vanderbilt University received additional grants from pharmaceutical companies and again from the Gates Foundation. The Vanderbilt University received grants from pharmaceutical companies such as Pfizer, Novartis, and Merck (23). The Gates Foundation awarded the Vanderbilt Medical Center by 2020 1,6 million US Dollars and the Vanderbilt University another additional 18 million US Dollars (24). The Chapel Hill University North Carolina received 143 million US Dollars worth of grants from the Gates Foundation (25).

The Covid-19 pandemic was not the result of human carelessness or human excesses as the WHO promotes on their website (26). When medical research is infiltrated by private business investors, and pharmaceutical companies who seek a maximum return on their investment or when academic research institutions exploit researchers for commercial profit then the purpose of medical research changes to the opposite. Instead of using advance knowledge to improve health worldwide, a destructive alien for the sole aim of global destruction and human exploitation was constructed (27).

In the author's view, that nature won't benefit from crossing its own created barriers between animal and human transmission. But it is in human's nature to destroy itself through destructive behaviour. This behaviour can't be discovered in any other species, not even that of a zoonotic virus.

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