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Biosecurity of Synthetic Viruses

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Summary
Synthetic methods in life sciences made rapid progress in the past few years, such as synthetic biology, synthetic genomics and synthetic virology. Synthetic viruses are genetically engineered viruses that are created to get deeper insights into the function of viruses, but they could also be used to develop live vaccines. Synthetic viruses are a small, but increasingly relevant area in virology. The open access to DNA sequence databases of many relevant viruses including smallpox and horsepox and the possibility to order DNA sequences commercially are the main biosecurity issues of synthetic virology. The open databases, the DNA synthesis firms and their clients are in the center of the biosecurity discussion and regulations.
Extinct viruses could already be recreated as synthetic viruses solely on database information and commercially ordered synthetic DNA. The smallpox virus with its very high transmissibility and mortality is extinct and meanwhile new antivirals and vaccines are available which demonstrated their effect during the current monkeypox outbreak. However, the extinct horsepox virus that is closely related to smallpox could be re-created in 2017, i.e., it is technically possible to synthesize poxviruses and it is unlikely that the stockpiled antivirals and vaccines could control a new smallpox pandemic. Technical and financial hurdles for synthetic viruses remain high, but are already lower than in the past. Currently, tacit expert knowledge is still an important factor. Governments and scientific institutions need to detect earliest signs of critical developments. The paper gives a brief overview on synthetic biology, synthetic genomics and virology, the development of synthetic viruses, smallpox-related matters and the biosecurity measures.

Content

1 Background

1.1 Introduction

Synthetic methods in life sciences made rapid progress in the past few years, such as synthetic biology, synthetic genomics and synthetic virology. The key activity of synthetic virology is **to (re-)create viruses by synthesized nucleic acids** (RNA or DNA). The focus is on viruses that already exist and not on creating new viruses. Genetic engineering of viruses can be done in many ways, i.e., that synthetic viruses (viruses created with synthetic DNA) represent only a small portion of genetically modified viruses. This paper will focus on synthetic viruses.

Synthetic viruses are genetically engineered viruses that are created to get deeper insights into the function of viruses, but they could also be used to develop live vaccines. Synthetic viruses are a small, but increasingly relevant area in virology[1].

Synthetic is not the same as artificial. If for example genes of naturally existing viruses (e.g., for capsid and spikes) are combined, the new chimeric virus[2] may be 'artificial', but not the result of synthesized DNA. Furthermore, there are many applications where synthetic oligonucleotides are used or inserted. For example, chimeric viruses are relevant for gene therapy and vaccine production[3].

1.2 Background

Synthetic biology, synthetic genomics and synthetic virology are related research areas. There is no accepted standard definition, but synthetic biology deals with the design and re-design of biological elements. This can reach from small parts (bio-bricks) up to complete biological systems. A key method to achieve these goals is the synthetic genomics which already led to creation of minimal genome cells, artificial chromosomes and synthetic viruses. The design of engineered genetic material and its production can now be separated, i.e., designers and synthesis companies are now in different organizations. A brief overview on synthetic biology is given now.

1.2.1 Minimal genome cell

The minimal genome is the smallest possible genome that allows autonomous life and self-replication. Since 2010, Craig Venter and his team worked to develop a *minimal genome* cell (the first one was called *JCVI-syn1.0* or '*Synthia*' with 901 genes)[4]. *Mycoplasma* was the smallest known autonomous cell type and thus used as model organism since 1984. In 2016, a new cell, called *JCVI-syn3.0*, was created by replacing the genome of *Mycoplasma capricolum* with the genome of *Mycoplasma mycoides*, with removal of unessential deoxyribonucleic acid (DNA). It had only 473 genes, but still the function of 149 genes was unknown[5]. After it was found that a slightly larger genome leads to improved cell growth, a modified minimal cell was created which allowed to reduce the number of genes with unknown function to 30 in the year

[1] A nucleotide is a basic unit of nucleic acids which can ribonucleic or deoxyribonucleic acid (RNA and DNA respectively); nucleotides have bases adenine (A), cytosine (C), guanine (G), and thymine (T). The bases form base pairs: A pairs with T; G pairs with C and the resulting DNA string forms the DNA double helix. The length of a DNA is defined by number of base pairs (bp), in case of more than 1,000 bases, the unit kilobase (kbp) is used, the largest genomes may be expressed as Megabases (Mb), see Garfinkel et al. (2007). Viruses can have a genome consisting of DNA (*DNA viruses* like poxvirus), but can also have and use RNA genomes (*RNA viruses* like coronaviruses).

[2] More detail in Baric 2006

[3] A widely used other approach is the genetic modification of *adeno-associated viruses (AAVs)* for gene therapy. In 2019, more than 250 clinical gene therapy trials were ongoing (Wikipedia, 2022). Modified adenoviruses can be used as carrier for SARS-CoV-2 spikes and then as live vaccines against SARS-CoV-2 without the risk of a coronavirus infection.

[4] Wang/Zhang 2019, p.23

[5] Danchin/Fang 2016

2019[6]. In 2021, a reverse genetics approach determined that seven genes are required together for normal cell division of the minimal cell and the addition of these seven genes for the new *JCVI-syn3A* resulted in a normal morphology while *JCVI-syn3.0* was pleomorph[7].

In 2019, researchers from the *Eidgenössische Technische Hochschule (ETH)* transformed the genome of *Caulobacter crescentus* to a new bacterium *Caulobacter ethensis 2.0*[8] Caulobacter is a well-characterized cell cycle model organism. Initially, *Caulobacter ethensis 1.0 (C. eth-1.0)* was created, but it was not possible to obtain 3- to 4-kb DNA building blocks of *C. eth-1.0* from commercial DNA suppliers due to a multitude of synthesis constraints. The aim of the recoding was to achieve a shorter and easy-to-synthesize genome which was successful for *C. eth-2.0*: the genome was ordered in 436 fragments and 435 of 436 DNA fragments could be synthesized. The new genome bacterium had some functional issues indicating that the recoding affected some gene functions. In total, 432 essential genes of *C. eth-2.0*, corresponding to 81.5% of the design, were equal in functionality to natural genes[9].

Escherichia coli (*E. coli*) is a well-characterized model organism in genetic studies. Various research groups work on re-design and semi-synthetic genomes for *E. coli*[10].

1.2.2 Synthetic DNA for Data Storage

A new research area evaluates synthetic DNA as a relatively stable storage medium. The DNA is then produced by synthesis and assembly and later on analyzed and decoded by sequencers. Many DNA sequencing systems code the nucleotides adenine, thymine, cytosine, and guanine (A, T, C, and G) of the DNA as bit combination - A is coded as 00, C as 01, G as 10, and T as 11. Even hundreds of Gigabytes result in a DNA piece that looks like a very small and thin piece of a hair. This allows covert data transportation in a practically invisible and undetectable manner. Researchers from Harvard University were able to insert coded DNA into an *Escherichia coli* bacterium[11].

Researchers of the *University of Washington* were encoding computer malware into a DNA segment. When this part of the DNA ran through a sequencer with an analysis program, the code infected the computer and the attackers were able to get control over the attached computer. The experiment was quite complicated, but it showed that it is possible to intrude companies that work with DNA by sending maliciously encoded DNA[12].

1.2.3 Synthetic genomes

Another area is *synthetic genomes*[13]. Synthetic genomes are typically created by synthesis of oligonucleotides which then are assembled (stuck together) to a genome, as it is still difficult to produce very long error-free DNA pieces at once. Genomes of larger organisms are organized in units called chromosomes, e.g., 46 Chromosomes (23 pairs) for human beings.

The first step were *Yeast Artificial Chromosomes (YAC)* and *Bacterial Artificial Chromosomes (BAC)*. They were developed as part of the *Human Genome Project (HGP)* in the 1990ies as structures where larger DNA sequences can be stored and copied (cloned), when the yeast cell or bacterium replicates itself. The replication increases the amount of available DNA which

[6] Lachance et al. 2019
[7] Pelletier et al. 2021
[8] Venetz et al. 2019
[9] Venetz et al. 2019
[10] Venter et al. 2022
[11] NATO 2021
[12] Ney et al. 2017
[13] Wang/Zhang 2019, p.23

facilitates analysis by sequencing. As BACs were more stable, they replaced the YACs as main tool. A widely used BAC is a ring chromosome, the F-plasmid, in *Escherichia coli* bacteria which can take up several hundred thousand base pairs of DNA from another organism/source[14].

In 2013, synthetic genomics was used to develop vaccine viruses against influenza. The DNA sequences of the influenza antigens *Hemagglutinin (HA or H)* and *Neuraminidase (NA or N)* were synthesized. These sequences together with genetically improved influenza virus backbones that coded for the other viral genes *Madin-Darby Canine Kidney (MDCK) cells* were transfected, resulting in synthetic influenza viruses that yielded the most vaccine antigen[15].

In the same year, the first synthetic RNA vaccine was developed[16]. The researchers obtained the sequence of the seasonal H7N9 influenza virus genome from the *Global Initiative for Sharing All Influenza Data (GISAID)* on Day 1 of the study. One day later, synthetic DNA oligonucleotides could already be ordered. Here, the DNA sequence for the H7 protein was selected and transcribed into RNA. Already on Day 8, a messenger RNA (mRNA) vaccine coding for the H7 antigen was ready. Once injected, the mRNA is translated by the patient cells into the H7 protein which is sufficient to activate the immune system. By this, the patient is protected against infection with the *H7N9 influenza* virus. However, this first vaccine was tested only in mice, not in humans. Since 2021, human mRNA vaccines are well-established against coronavirus SARS-CoV 2 (*Pfizer/BionTech* and *Moderna*).

In 2017, a new method was developed, *the transformation-associated recombination (TAR) cloning* in yeast. The key advantage is the use of smaller DNA fragments during the procedure: before assembly, each fragment can be genetically modified as needed which is easier than modifying the whole genomic virus at once. Furthermore, the use of shorter fragments principally allows the use of synthetic DNA instead of natural fragments, because the length of the DNA is still a technical hurdle for synthetic DNA[17]. In other words, TAR could be used as platform technology for synthetic viruses.

In 2017, a *Herpesvirus* genome-containing BAC was cut into 11 overlapping fragments[18]. In short, each fragment was combined with a genetic element, the *cloning vector*, that allowed cloning in yeast and could be enzymatically removed thereafter. The fragments could then be assembled in yeast cells to a complete genome. Thereafter, the complete genome was extracted and put into mammalian *Vero* cells to produce fully infectious viruses. In a human *cytomegalovirus (CMV)* study, the same procedure was successfully executed with 16 overlapping DNA fragments of the human CMV strain *Toledo*[19].

A new area are coronaviruses. SARS-CoV viruses are RNA viruses which are in the first step transformed into the complementary DNA (cDNA) for further action and it was shown that further steps could theoretically be done with either natural or synthetic DNA[20].

In 2020, a research group obtained SARS-CoV-2 and used the synthetic genomics TAR method described above. The virus genome was analyzed and for practical purposes (length of DNA) divided finally into 14 fragments which were then ordered at synthesis companies. But of these. two larger fragments could not be produced and delivered, but this could be resolved by

[14] Shiyuza/Kouros-Mehr 2001
[15] Venter et al. 2022
[16] Hekele 2013
[17] Vashee et al. 2017
[18] Oldfield 2017
[19] Vashee et al. 2017. A similar study was conducted for *Autographa californica nucleopolyhedrovirus* in insect cells (Koster et al. 2022)
[20] Thi Nu Thao et al. 2020

utilizing DNA sequences from an infected patient and further steps[21]. Finally, all fragments could be assembled in yeast cells resulting in fully functional SARS-CoV2 viruses. The same group showed that **this method could be also used for other RNA-viruses** like *Flaviviruses* and *Pneumoviruses*. Furthermore, it was shown that **the assembly works with DNA from different sources** such as viral isolates, cloned viral DNA, clinical samples or synthetic DNA.[22]

In two further studies published in 2021, SARS-CoV-2 viruses were engineered by reverse genetics. In a Chinese study, seven plasmids with SARS-CoV-2 complementary (cDNA) fragment clones were amplified in bacteria, then assembled into a genome-length cDNA, then transcribed *in vitro* into RNA which was brought into mammalian *Vero E6* cells by electroporation to gain recombinant RNA viruses for further studies. The publication included all 110 steps including recommendations for troubleshooting during the experiment[23]. In a Japanese study, ten 10 SARS-CoV-2 complementary (cDNA) fragment clones with linker fragments were assembled by a *circular polymerase extension reaction (CPER)*; the resulting circular genome was transfected into *VeroE6/TMPRSS2* cells[24]. The construction of infectious clones resulted in high titers of recombinant SARS-CoV-2. The advantage of this method is that it is **bacterium-free**, i.e., at no point bacterial genomes are needed anymore.

These viral technology platforms allow to rapidly engineer viruses with desired mutations to study the virus *in vitro* and *in vivo*, for live-attenuated vaccine development and modification by reporter genes and may facilitate the creation of synthetic viruses in future.

The rapid technical progress of DNA synthesis and new assembly methods also allows a full synthesis of natural *Yeast (Saccharomyces cerevisiae)* chromosomes. In the *Synthetic Yeast Genome Project* "Sc2.0", complete natural *Saccharomyces cerevisiae* chromosomes could be artificially reconstructed[25].Via stepwise and systematic replacement of the native yeast genome with 30–40 kb steps (12 DNA fragments of 2–4 kb), the project is close to the completion of the largest synthetic genome of the 16 natural yeast chromosomes to reshape and minimize the genome[26]. Also, 16 natural chromosomes of *S. cerevisiae* were successfully fused into a single chromosome; the artificial cell still had similar cellular functions[27].

1.2.4 Synthetic virology

The creation, re-creation or modification of viruses, the use of helper viruses or of viruses with reporter genes are meanwhile well-established in virological research.

The key activity of synthetic virology is **to (re-)create viruses by synthesized nucleic acids** (RNA or DNA). The focus is on viruses that already exist and not on creating new viruses.

A driver of this development is **reverse genetics**. While in classic genetics researchers evaluate a certain phenotype (appearance) to find the genetic basis, the reverse genetics analyses genetic sequences to evaluate the impact on phenotype. This includes genetic engineering of specific nucleic acid sequences and then of virus genomes. Genetic engineering of viruses can be done in many ways, i.e., synthetic viruses (viruses created with synthetic DNA) represent only a small portion of genetically modified viruses.

The general research strategy is as follows: A naturally existing virus is sequenced, i.e., the exact order of nucleotides in the viral genome is evaluated. Modern *Next Generation*

[21] Thi Nu Thao et al. 2020
[22] Thi Nu Thao et al. 2020
[23] Xie et al. 2021
[24] Torii et al, 2021
[25] Sun 2022
[26] Koster et al. 2022
[27] Shao 2018, Wang/Zhang 2019

Sequencers (NGS) analyze the viral DNA in a massive parallel manner, i.e., the DNA is cut off in small snippets which are analyzed at the same time; then, the computer combines the sequence pieces to the correct whole DNA sequence (computational synthesis)[28].

However, for many relevant viruses, this initial sequencing step is not needed anymore. The automatization of desoxyribonucleic acids has made substantial progress and it should be noted that in 2020 the full-length genome sequences of 9,240 different viruses, including the smallpox virus, were publicly available in an online database maintained by the *US National Institutes of Health (NIH)*[29]. The database information makes it **possible to re-create extinct viruses as synthetic viruses** which is a biosecurity issue.

As it is still challenging to synthesize long DNA pieces of more than several hundred base pairs, usually short pieces of the sequence are ordered as oligonucleotides ("oligos") which can then be assembled (stuck together) by specialized enzymes such as polymerases and ligases[30]. The synthetic oligonucleotides can be produced by and ordered from commercial companies[31]. However, larger laboratories can conduct the DNA synthesis with specialized machines on their own, i.e., the commercial companies are not the only way to get synthetic DNA.

A virus is of course more than 'naked' DNA. The synthesized DNA can be put into the cells (transfection, e.g., by electroporation where electricity mediates DNA intake) which then produce the full virus with capsid, membranes, spikes etc. In special cases, e.g., for the recreation of the horsepox virus, helper viruses are needed to support the final synthesis. However, the helper virus is <u>not</u> part of the final product. Once the synthetic virus is ready, it can infect cells and replicate on its own.

Within the so-called *Gain-of-Function research*, genetically modified viruses (or modified animals for studies of virus infections) are created to get insights into the function and modifications may be done to enhance viral properties, but this research area is different from synthetic viruses [32].

2 Synthetic Viruses

The creation of synthetic viruses initially started with small viruses. The first virus assembled from synthetic oligonucleotides was *poliovirus*, an RNA virus with 7,500 bp length in 2002[33]. A full-length *poliovirus* complementary DNA (cDNA) was chemically synthesized, which could then be enzymatically transcribed to RNA. In 2003, the bacteriophage phiX174, a virus that infects bacteria, was synthesized[34].

In 2005, the *1918 Spanish Flu influenza virus* strain which caused millions of deaths was recreated by reverse genetics. The researchers identified eight coding segments of the old virus

[28] White 2021
[29] Wikipedia entry for Synthetic Virology 20 Nov 2022
[30] The authors vary in their estimates, Craig et al. 2022 state that Oligonucleotides 60-100 bp in length can be easily synthesized, but for fragments ranging from 200-2000 bp, shorter oligonucleotides need to be assembled.
[31] Garfinkel et al. 2007
[32] In the broadest definition, gain of function describes the gain of new functions by organisms through genetic changes, which can naturally occur or by experimental genetic modification. This includes e.g., cancer cells, plant cells and microorganism like bacteria that become resistant against antibiotics. Only a small part of the Gain-of-Function research deals with viruses and again, only a subset of this is the *Gain-of-Function research of concern (GOFROC) on enhanced potentially pandemic pathogens (ePPPs)* that could be harmful for humans. Here, influenza and coronaviruses are main research targets and in the center of the public debate and regulations, because they cause pandemics by airborne infections. In principle, synthetic viruses could also serve as organisms for gain-of-function research, but overall, this is a different research area.
[33] Chen et al. 2021
[34] White 2021

from a publication, synthesized them and put them into the genome of a common influenza virus[35]. They were able to produce the 1918 virus then in human kidney cell cultures[36]

In 2008, it was possible to extend the capacity to synthesize full-length viral genomes to about 30 kb and used for the design, synthesis, and recovery of a recombinant bat SARS-like coronavirus (SCoV)[37]. As the virus could not be cultivated at that time, the researchers had to create an assumed consensus sequence of 4 virus variants[38], also they required some elements from SARS-CoV. The complementary DNA (cDNA) was then synthesized commercially and later on transcribed to RNA (as the virus is an RNA-virus). Despite these limitations, the new virus could be cultivated and was infectious both in cultured cells and in mice[39].

Already in 2017, a researcher was able to re-create an extinct virus, the *horsepox virus (HPVX)*, by synthetic DNA and other measures. While this was done with the good intention to have a new technology platform for safer smallpox vaccines for humans, scientists were concerned about the future consequences, see Section 4 below. However, as assessed by the *PLOS Dual Use Research of Concern (DURC) Committee*, "the study did not provide new information specifically enabling the creation of a smallpox virus, but uses known methods, reagents and knowledge that have previously been used in the synthesis of other viruses (such as influenza and polio viruses)"[40].

The *horsepox* was not observed anymore since the 1980ies and was apparently extinct[41]. However, the genome sequence was still available for the HPXV strain MNR-76 [GenBank accession DQ792504][42]. Based on this, ten synthesized overlapping 10–30 kb DNA fragments could be ordered and obtained from a commercial company. As the available *horsepox virus* genome was not sequenced to the end, end pieces from *vaccinia virus (VACV)* were added instead, so called inverted terminal repeats (ITRs; 'hairpins'). *Vaccinia virus* is a poxvirus used for vaccination against smallpox and a close relative of horsepox and smallpox. Due to these additions, the new virus was not 100% horsepox, but a (bit of a) chimeric virus, the *synthetic chimeric HPXV (scHPXV)* [43]. Then, cells that were already infected with a helper virus, the *Shope fibroma virus (SFV)*, that helped to activate the poxvirus DNA after transfection. The reason for this step was that poxviruses do not grow if the DNA is transfected into cells, but it was known that a cell infected with one poxvirus can reactivate the DNA of a second virus[44]. The resulting *scHPXV* virus was less virulent (harmful) in mice than vaccinia virus, but provided like a vaccine 100% protection in mice that were infected with lethal doses of vaccinia virus[45]. The study showed that **extinct viruses can be recreated as synthetic virus solely on database information and commercially ordered synthetic DNA**.

The manufacturer, *Tonix Pharmaceuticals Holding Corp*. now develops TNX-801 as a live virus vaccine based on synthesized *horsepox (sHPXV)*. Also, they **meanwhile fully synthesized the vaccinia virus (VACV)** as well: it was assembled using synthetic DNA to develop another potential smallpox vaccine candidate, TNX-1200. TNX-1200 was based on a complete genome sequence of a laboratory isolate of VACV [Genbank Accession #

[35] Li et al. 2021
[36] Tumpey et al. 2005
[37] Venter et al. 2022
[38] Becker et al. 2008
[39] Becker et al. 2008
[40] Thiel 2018
[41] Thiel 2018
[42] Noyce et al. 2018. This was based on a genetic sequence isolated from horses in Mongolia in 1976 and the last real sample is stored at the *US Center for Disease Control and Prevention (CDC)*, Rourke et al. 2020
[43] Noyce et al. 2018
[44] As mentioned by Thiel 2018, this was not the first use of helper viruses. For example, the *fowlpox virus* is routinely used to launch replication from naked *Vaccinia virus* genomic DNA
[45] Noyce et al. 2018

MN974380] which was very similar to the published sequence of VACV strain used for the widely used smallpox vaccine ACAM2000®.[46] TNX-801 was able to protect cynomolgus macaques infected with monkeypox.

3 Smallpox (Variola major)

3.1 Background

3.1.1 Introduction

While the potential harm caused by modified influenza and corona viruses remains disputed, the smallpox virus with its very high transmissibility and mortality is still a major biothreat and bioweapon. The virus is extinct and meanwhile antivirals such as tecovirimat and new vaccines are available which demonstrated their effect during the current monkeypox outbreak.

However, it has been demonstrated in 2017 that the recreation of extinct poxviruses is technically possible and it is unlikely that the stockpiled drugs and vaccines could control a smallpox pandemic.

Smallpox (Variola major) virus was officially declared to be extinct in 1980 after an intense global vaccination and surveillance campaign[47]. From that moment on, the vaccination against smallpox virus (which historically was the first vaccination ever) was cancelled, because the vaccination used at that time had still severe side-effects. There was a significant proportion of children who suffered from brain inflammation (encephalitis) or heart inflammation (perimyocarditis), both diseases with a high risk for long-term damage or even death. On the other hand, this meant that from that after stop of vaccination the world population was not protected against poxviruses anymore. From the very beginning, there was a debate what would happen if poxviruses return.

3.1.2 The Smallpox Disease

Historically, the term smallpox was used to differentiate it from 'great-pox' which is Syphilis, a bacterial infection. There are two different *Variola* strains. *Variola major* causes the regular smallpox disease with a mortality rate of 30% while *Variola minor*, also known as *Alastrim virus*, causes a mild smallpox form with 1% mortality rate. The *Alastrim virus* is also extinct[48]. When researchers discuss 'Variola' today, they refer to the *Variola major* virus.

Overall, poxviruses can infect vertebrae (*Chordopoxvirinae*) and insects (*Enteropoxvirinae*)[49]. *Chordopoxvirinae* are divided into 4 genus, the *Molluscipox*, *Orthopox*, *Parapox* and the *Yatapox*. In principle, 14 poxviruses can infect humans. One of them, the *molluscipox* virus causes a still existing and common dermatological disease called *Molluscum contagiosum*, which is not fatal. Then, there are seven *Orthopox* viruses (vaccinia virus, variola, monkeypox, cowpox, buffalopox, cantagalo and aracatuba), five *Parapox* viruses (orf, paravaccinia, bovine popular stomatitis, deerpox and sealpox), and one *Yatapox* virus (tanapox)[50].

In practice, *molluscum contagiosum* is most frequent, followed by the current monkeypox outbreak. *Vaccinia virus* infections are occasionally reported. Other infections are rare. Smallpox infections are not reported anymore, but are the most important biothreat.

[46] Tonix 2020
[47] DiEuliis/Berger/Gronvall 2017
[48] Parker et al. 2008. The authors vary with respect to the estimated smallpox mortality, e.g., 16% or 40%, but many authors estimate 30%.
[49] Harrison et al. 2004
[50] Parker et al. 2008

Variola and *molluscum contagiosum* are the only poxviruses that only exist in humans and that do not have animal reservoirs. Note that 'monkeypox' only means that usually monkeys are infected, but the animal reservoir are squirrels[51].

Poxviruses are the largest known animal viruses with approximately 200 distinct genes and a genome size or more than 180,000 base pairs (180 kbp). They are DNA viruses that do not replicate in the core of cell, but completely in the cytoplasm. At least partially, poxviruses damage their hosts by overactivation and confusion of the immune system. Immune cells communicate with immune hormones, the so-called cytokines and related molecules. The *Variola genome* carries homologues of human cytokines (known as **virokines**) and of cytokine-receptors (**viroreceptors**) which both mimic their human equivalents and massively disturb the normal immune functions. This affects a broad range of cytokines like interleukins, interferons and tumor necrosis factor-alpha as well as chemokines and growth factors, which indicates that poxviruses damage and kill humans at least partially by a cytokine storm[52].

Officially, frozen Variola samples are safely stored at only two locations in the United States and Russia, this is the *Center for Disease Control and Prevention CDC* in Atlanta and the *State Center of Virology and Biotechnology VECTOR* in Koltsovo, but e.g., in 2014 the United States NIH found forgotten frozen poxviruses in their own archive[53].

The smallpox infection starts with a respiratory infection (and respiratory droplets then spread the infection to others). Then, the virus spreads in the infected person in the blood (viremia). It takes 7 to 19 days from respiratory infection to first symptoms of fever, malaise, headache and backache. The last phase begins with a cutaneous rash and subsequent lesions with 0.5 to 1 cm diameter, where papules develop into vesicles and then into pustules which cover the entire body, which then dry in 2 to 3 weeks and may lead to pox scars[54].

The current strategy for response to a smallpox outbreak following confirmed diagnosis of human smallpox relies on isolation and treatment of confirmed cases and focused vaccination of contacts of confirmed and suspected cases (ring vaccination) as an early post-exposure vaccination may still have protective effects.

From the biosecurity perspective, **the long incubation period in combination with the high transmissibility (quick and easy spread of infection) is a key problem**, because when medics and authorities realize what is going on, the outbreak area is probably already too large for an effective containment. Also, governments do not have the necessary stockpiles for a mass vaccination.

3.2 Treatment and Vaccination

3.2.1 Vaccination (Active immunization)

For military prevention, the United States set up in 1998 the *Military Vaccination Programs* under the direction of the *Military Vaccine Agency*, originally known as MILVAX program[55]. As a consequence, all military personnel on the Korean peninsula staying longer than 15 days and staff of the *U.S. Central Command* must be vaccinated with smallpox vaccine.

A smallpox variant, the *Vaccinia Virus (VACV)* was incrementally attenuated by cultivation and mutation but was still providing immunity against smallpox viruses. Originally, the *Dryvax* vaccine was used, then a new vaccine called ACAM 2000 was developed as a clonal, cell culture-derived vaccine. However, a study in 2005 found similar side-effects to the original vaccine which indicated that the side-effects were related to the virus, but not to the way of

[51] Harrison et al. 2004
[52] Schwantes, A, Süzer, Y., Sutter G. 2010, p.705
[53] DiEuliis/Berger/Gronvall 2017, p.2
[54] Harrison et al. 2004, Parker et al., 2008
[55] Anderson 2008

vaccine production[56]. To decrease the still existing risk of perimyocarditis and other side effects, another variant was cultivated, the *Modified Vaccinia Ankara (MVA)* Strain which lost the ability to self-replicate in human cells[57]. Meanwhile the MVA vaccine from *Bavarian Nordic* is available as Imvamune® or Jynneos™[58].

3.2.2 Immunoglobulins (Passive immunization)

Intramuscular administration of *vaccinia immune globulin (VIG)*, a product derived from the pooled blood plasma of vaccinated individuals, is indicated for treatment of generalized vaccinia, progressive vaccinia (vaccinia necrosum), eczema vaccinatum and certain auto-inoculations, i.e., complications which may occur during vaccination with *Vaccinia virus*. In 2005, the *US Food and Drug Administration (FDA)* approved the manufacture of new stocks of VIG by *DynPort Vaccine Company*[59]. Due to limited number of cases, the effect of VIG could not be tested in large studies.

3.2.3 Antivirals

Cidofovir was the first antiviral licensed for treatment of molluscipoxvirus infection in AIDS patients and was recommended by the U.S. *Center for Disease Control and Prevention CDC* for use in treating complications with existing smallpox vaccines. It is an acyclic nucleoside phosphonate that blocks the DNA polymerase (poxvirus enzyme E9).[60]

In 2018, the *U.S. Food and Drug Administration (FDA)* approved a new and highly selective antiviral tecovirimat (TPOXX®; ST-246) that inhibits the orthopoxvirus protein VP37 or F13L, which is necessary for membrane envelopment of *intracellular mature virus (IMV)* particles, because only enveloped viruses can be released from the cell[61]. The antiviral was tested with the *FDA Animal Efficacy Rule*, i.e., given to animals to treat the respective poxvirus disorders, as it was of course impossible to test it in humans with smallpox infections. This is possible, because the poxviruses of the tested animals are closely related and the results then can be used to assess the efficacy in humans. It was approved for the treatment of symptomatic smallpox.

However, when orthopoxviruses are passaged in the presence of either tecovirimat or cidofovir in cell culture, they can become drug-resistant against both drugs by mutations in E9 and VP37/F13L[62].

3.3 The Monkeypox outbreak from 2022

Monkeypox is an orthopox infection that can infect monkeys and humans. During the 2022, the mortality of the infection was very low. The course of the infection is however similar to smallpox: after an incubation phase of 7 to 14 days fever and (in contrast to smallpox) a lymphadenopathy (swollen lymph nodes) appears, followed by rash and lesions[63].

In 1970, first monkeypox infection was reported in a child in the Democratic Republic of the Congo (DRC), but the disease remained rare, the next three decades less than 1,000 cases were observed. From September 2018 to November 2021, sporadic human monkeypox cases were reported outside Africa, but all related with travels to Nigeria[64]. Since early May 2022 and as of 7 July, the *European Centre for Disease Prevention and Control (ECDC)* noted 7 553 cases of monkeypox in non-endemic countries, thereof 4,908 cases in the *European Union/European*

[56] Parker et al. 2008
[57] Zitzmann-Roth et al. 2015
[58] Russo et al. 2021
[59] Parker et al. 2008
[60] Harrison et al., 2004, Parker et al., 2008
[61] Russo et al. 2021
[62] Xiang/White 2022
[63] Xiang/White 2022
[64] Xiang/White 2022

Economic Area (EU/EEA). In contrast to the past, the most of the cases have been detected primarily among men who have sex with men[65].

According to ECDC data, most of the currently detected cases are in males between 18-50 years who have therefore not received the smallpox vaccine, which confers cross-protection to monkeypox. According to older studies, it is estimated to be up to 85% effective in preventing monkeypox infections.[66]

Tecovirimat and brincidofovir (an orally bioavailable lipid conjugate of cidofovir) are two antivirals that have been approved in the U.S. for treating smallpox. While tecovirimat was effective against monkeypox in humans as well, brincidofovir showed only poor efficacy, i.e., tecovirimat is the first choice in treatment of monkeypox infections. Non-replicating *Modified Vaccinia Ankara (MVA)* vaccines (IMVAMUNE®, JYNNEOS™) are apparently protective against monkeypox infections as well because of the similarity between the MPXV and the smallpox virus[67]. The USA and European Union have approved MVA vaccines in 2022 also for monkeypox[68]. MVA is administered as a subcutaneous injection with a second dose given at least 28 days after the first, but the first dose already provides protection while the second dose boosts the duration of protection.[69]

4. Biosecurity

4.1 Introduction

Synthetic methods in life sciences made rapid progress in the past few years, such as synthetic biology, synthetic genomics and synthetic virology. The key activity of synthetic virology is **to (re-)create viruses by synthesized nucleic acids** (RNA or DNA). The benefits are a deeper understanding of virus genomes, structure and function and also the possibility to create new vaccines rapidly.

The open access to DNA sequence databases of many relevant viruses including smallpox and horsepox and the possibility to order DNA sequences commercially are main biosecurity issues of synthetic virology. The open databases, the DNA synthesis firms and their clients are in the center of the biosecurity discussion. However, larger laboratories can conduct the DNA synthesis with specialized machines on their own, i.e., the commercial companies are not the only way to get synthetic DNA.

None of the biosecurity matters that will be discussed in this section are specific or limited to smallpox, but smallpox is always mentioned in biosecurity discussions.

4.2 The Smallpox debate

The main reason is the high **mortality of smallpox which is estimated around 30% of infected cases together with a lack of immunity** as the mass vaccination was stopped when the virus was declared to be extinct.

Furthermore, **the long incubation period of smallpox in combination with the high transmissibility (quick and easy spread of infection) is a key problem**, because when medics and authorities realize what is going on, the outbreak area is probably already too large for an effective containment. Also, governments do not have the necessary stockpiles for a mass vaccination.

[65] ECDC 2022
[66] ECDC 2022
[67] Xiang/White 2022
[68] CHMP 2022
[69] Xiang/White 2022

Given the recent experience with monkeypox, the smallpox vaccination of the elderly still has a protective effect. This would mean that a smallpox outbreak would primarily affect people below 50 years with a mortality of up to 30% which would have devastating effects on the demographic structure of the affected country. It would not only reduce the population size, but also lower the future growth rates. In addition, the World Bank data show that expected birth rates are steadily declining in the long term, and by 2050 will have approached or even fallen below the stability factor of 2.16 children/pair[70] in most regions, i.e., the regeneration of populations is increasingly difficult.

The coronavirus pandemics have shown that it is not possible to isolate countries from a global pandemic or in case of a smallpox pandemic, when the isolations measures are effective, it is probably already too late to stop spread. Also, while vaccines against coronavirus were developed rapidly, the global distribution was slow and poor. A global defense strategy against smallpox is therefore unrealistic. But this means that smallpox as bioweapon would backfire and result in self-damage, i.e., from the military perspective it is a **counterproductive weapon**.

With respect to bio-terrorism, its potential to reduce population size and growth rates may make it nevertheless interesting. In movies, the idea that for whatever reasons too many or the wrong people live on Earth is a common story, e.g., James Bond *Moonraker*, Lara Croft *The cradle of life* or more recently *Kingsmen*. In reality, various actors are concerned that the population growth could de-stabilize states (e.g., in the *Youth bulge* theory[71]), could lead to food and water shortages (e.g., the theory of *Malthus*) or damage environment (*Neo-Malthusianism*)[72]. Meanwhile, the debate has shifted from population growth to the *ecological footprint*, because not only the population, but also the individual wealth and consumption is growing rapidly[73]. Researchers did not yet note an increasing interest in synthetic virus technology in respective discussion fora[74], but nevertheless smallpox should not be considered as a past biothreat.

However, it has been demonstrated in 2017 that the recreation of extinct poxviruses is technically possible and it is unlikely that the stockpiled drugs and vaccines could control a smallpox pandemic. In 2006, a journalist at *The Guardian* was able to obtain a fragment of the Smallpox virus (*Variola major*) via having the DNA sent to their apartment[75]. Evans reported the technical methods for the chemical synthesis of horsepox virus with 212,000 base pairs with $100,000 in 2018[76].

As one horsepox virus sample from 1976 is still archived by the U.S. *Center of Disease Control and Prevention (CDC)*, it would have been possible for the manufacturer of the synthetic horsepox, Tonix, to ask the CDC to provide the virus. Instead, it was apparently easier and cheaper to recreate the virus as a synthetic virus[77].

However, the experts expected this already. Prior to the meeting of the *WHO Independent Advisory Group on Public Health Implications of Synthetic Biology Technology Related to Smallpox*, a *Scientific Working Group (SWG) on Synthetic Biology and Variola Virus and Smallpox* met in April 2015 and concluded that "there is now the capability to recreate the

[70] In simple terms, a population can only be kept stable if a pair has an average of 2.16 or more children (five pairs with 2 children, every sixth pair with 3 children), because there are always some children dying due to infections, cancer, hereditary diseases, accidents or crimes so that a birth rate just over 2.1 children ensures that two parents are replaced by 2 surviving children.
[71] Weisflog 2017
[72] For a full overview and details, refer to Merchant 2022
[73] Diamond 2005
[74] Nature 2021
[75] NATO 2021
[76] Sun et al. 2022
[77] Rourke 2020

variola virus, the causative agent of smallpox [….] by multiple institutions and persons, including those with malicious intent."[78]

Governments need to detect earliest signs of critical developments. Recreated viruses cause biosecurity concerns due to their pandemic potential[79].

4.3 Risk mitigation

4.3.1 Legal and organizational measures

The possibility to order DNA sequences and the open databases are the key biosecurity problem for synthetic viruses[80].

The core of the risk mitigation in the US is a guidance for handling of synthetic DNA; the *United States Department of Health and Human Services (HHS) 2010 Screening Framework Guidance for Providers of Synthetic Double-Stranded DNA*[81]. When receiving an order for synthetic dsDNA, the U.S. Government recommends that providers perform customer screening and sequence screening. If either customer screening or sequence screening raises any concerns, providers should perform follow-up screening. If follow-up screening does not resolve the concerns, authorities may be asked for advice, like the *FBI Field Office Weapons of Mass Destruction (WMD) Coordinator*. The guidance particular addresses sequences of Select Agents (dangerous pathogens under the *Select Agent Rule SAR*); toxins or any other sequence of concern (SOC). A point that was critically discussed is the threshold length of 200 nucleotides for a check, as meanwhile shorter sequences (oligos) are standard. In other words, there is a risk that malicious actors order a lot of oligos that cannot be directly identified as parts of a dangerous virus. On other hand, a marking of shorter sequences may lead to a lot of false positive results and overload the system with false alerts[82].

The advances in synthetic genomics and virology require an update of this guidance, this is currently ongoing and expected to be completed in 2023. The *2022 Screening Framework Guidance for Providers and Users of Synthetic Oligonucleotides (Proposed Revised Guidance)* expands the guidance to users of synthetic DNA and it is planned to expand the guidance to DNA and RNA. In addition to documentation of orders and users, a transfer to any other individuals not listed in the original order, should be documented as well such as through a *Material Transfer Agreement (MTA)* or another sample tracking process. For sequences of concern (SOCs), records of SOCs and their transfers should be retained retained for at least 8 years. Manufacturers should provide a data logging function to maintain a record of the oligos synthesized on the equipment. The order batch size should be considered as well to identify orders of small oligonucleotides that could be assembled into larger sequences of concern[83].

The guidance is still voluntary. The authorities do not want to overload actors with bureaucratic measures. The approach is "know your client"[84], i.e., to be aware of uncommon orders from persons or institutions without legitimate needs. A part of this strategy are provider and scientific organizations that engage their members to adhere to high standards.

The Members of the *International Gene Synthesis Consortium (IGSC)*, an umbrella organization of 18 leading synthesis companies, have agreed on a harmonized screening protocol to guard against the delivery of concerning DNA sequences and to ensure compliance

[78] WHO 2015
[79] DiEuliis/Berger/Gronvall, 2017; Kupferschmidt, 2017
[80] Wang/Zhang 2019, Chen et al. 2022
[81] HHS 2010
[82] Diggans/Leproust 2019
[83] HHS 2022
[84] Nature 2021

with the above-mentioned HHS Guidance[85]. The IGSC has a *Regulated Pathogen Database*. To avoid "venue shopping" where a bad actor could submit a problematic order to multiple companies in the hope of finding a company whose screening system will permit the order, the IGSC members alert each other[86].

The *International Genetically Engineered Machine (iGEM)* is a science and technology competition for college students in the field of synthetic biology with strict security policies. The *International Union of Biochemistry and Molecular Biology* and the *International Union of Microbiological Societies (IUMS)* established respective *Codes of Ethics* against misuse of microbial knowledge, research, and resources[87].

Costs for gene synthesis products go down, but biosecurity screenings require expensive expert judgments which result in a competitive disadvantage to companies who do not perform those checks. Another issue is that synthesis is meanwhile international business which makes oversight difficult. On the other hand, *do-it-yourself (DIY)* researchers (also known as 'biohackers') express concerns that there are also hundreds of smaller local synthesis companies which are also difficult to control[88].

4.3.2 Technical measures

Technical measures like automated screening may facilitate and accelerate the security check of ordered sequences. Commercial suppliers of synthetic DNAs perform *Basic Local Alignment Search Tool (BLAST)* searches to find regions of local similarity between nucleotide or protein sequences to any known sequences of concern[89].

The *Intelligence Advanced Research Projects Activity (IARPA)* is developing the *Functional Genomic and Computational Assessment of Threats (Fun GCAT)*[90]. Fun GCAT is the computational analysis of DNA and answers three questions per sequence: What organism does it come from? What biological functions does it have? How dangerous is it? Neural networks and other bioinformatic tools are used to learn the common patterns of sequences with similar origins and functions, resulting in a 500 times higher computational efficiency over state-of-the-art and stable performance also for short (<50 base pairs) sequences. The U.S. Intelligence Community can now conduct relevant missions from rapid screening of very large datasets to field-based, targeted analysis[91].

Some governments have restricted exports of certain partly or entirely automated Nucleic acid assemblers and synthesizers[92].

Also, it is possible to embed security into the machines, similar to photocopy machines that block copies of paper money. The assembly machine of *Synthetic Genomics DNA, Inc. (now Codex DNA)* automatic blocks assembly of non-approved sequences[93].

Technical and financial hurdles for synthesis are still high, but already lower than in the past. Costs decrease while quality increases by advances in assembly and error-correction methods. While many researchers argue that the costs and complexity are still an effective hurdle for malicious actor from outside, as the lab facilities would be expensive to assemble and operate, and difficult to acquire without being noticed, the DYI researcher Zayner states that his company sells a pretty complete molecular biology lab for $1,600 with profit. This is caused

[85] Nature 2021
[86] Diggans/Leproust 2019
[87] Sun et al. 2022
[88] Nature 2021
[89] Nature 2021
[90] IARPA 2022
[91] IARPA 2022
[92] NATO 2021
[93] Venter 2021

by a growing second-hand-market for laboratory equipment. Further, Zayner believes that the technical complexity of making plasmid DNA and of cultivating and transfecting *Vero* or HEK293 cells is overestimated[94].

4.3.3 The knowledge barrier

But even if the technical and financial hurdles are taken, the most critical thing is the tacit expert knowledge, i.e., the informal, unwritten sum of experiences how to handle things and to do troubleshooting. This is for example relevant for the assembly of a genome of a larger number of oligos into a complete viral genome and also for the next step, i.e., the generation of a replicating virus as these steps are neither error-free nor trivial[95]. And even for experts, there are still limitations as scientists are not yet in a position to design functional proteins that do not exist in nature[96].

In summary, the organizational, technical, financial and knowledge hurdles could currently only be taken with the help of an expert, i.e., an **insider threat**. As shown by the *anthrax* attacks after 9/11, this threat is not theoretical. How long would an experienced researcher with financial support need to synthesize something of concern?

An analysis of the U.S. *Center of Disease Control and Prevention (CDC)* reported 727 incidents of theft, loss or release of select agents and toxins in the USA between 2004 and 2010, and 11 laboratory-acquired infections[97] On the other hand, Burki argued that serious events are extremely rare, events that resulted in an infection in the community are virtually unknown[98].

However, while the above-described Fun GCAT project of IARPA may significantly improve detection of problematic sequences, governments and scientific institutions should pay attention to any researcher in the area of poxviruses and synthetic viruses who suddenly changes habits or lifestyle, shows signs of psychiatric disorders or starts to express radical views.

A new challenge to the control of sensitive information is the emergence of preprint servers that allow the posting of research findings before peer review[99]. Here, researchers and publishers have to pay attention to findings that could be used in dual-use settings.

5 Concluding remarks

The open access to DNA sequence databases of many relevant viruses including smallpox and horsepox and the possibility to order DNA sequences commercially are the main biosecurity issues of synthetic virology. The open databases, the DNA synthesis firms and their clients are in the center of the biosecurity discussion and regulations.

Extinct viruses could already be recreated as synthetic viruses solely on database information and commercially ordered synthetic DNA. The smallpox virus with its very high transmissibility and mortality is extinct and meanwhile new antivirals and vaccines are available which demonstrated their effect during the current monkeypox outbreak. However, the extinct horsepox virus that is closely related to smallpox could be re-created in 2017, i.e., it is technically possible to synthesize poxviruses and it is unlikely that the stockpiled antivirals and vaccines could control a new smallpox pandemic. Technical and financial hurdles for synthetic viruses remain high, but are already lower than in the past. Currently, tacit expert knowledge is still an important factor. Governments and scientific institutions need to detect earliest signs of critical developments.

[94] Nature 2021
[95] Nature 2021
[96] Carter et al. 2014
[97] Henkel et al. 2012
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[99] Musunuri et al. 2021

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