

# Gain of Fiction

## [Gain of Fiction](#)

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*“The only way that the gain of function/bioweapon narrative makes any sense is if the original Latin definition for the word “virus” is used to explain what is happening in this research. In Latin, “virus” means “liquid poison” and what virologists are doing is simply creating a liquid poison in a lab using cell cultures. What they are not doing is creating “infectious agents of a small size and simple composition that can multiply only in living cells of animals, plants, or bacteria” which is the modern definition for the word according to the Britannica...*

[...]

*“What must be realized about the GOF studies and the bioweapon narrative is that these stories are designed to keep people believing in the lies of Germ Theory. This is yet another fear-based tactic utilized by those in power to ensure that the masses are frightened of an invisible enemy that can be unleashed upon the world either accidentally or intentionally at a moments notice.”*

~ Mike Stone, Viroliegy

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## [Gain of Fiction](#)

by [Mike Stone](#), [Viroliegy](#)

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**virus**, infectious agent of small size and simple composition that can multiply only in living cells of animals, plants, or bacteria. The name is from a Latin word meaning “slimy liquid” or “poison.”

<https://www.britannica.com/science/virus>

I have purposefully stayed away from the whole “SARS-COV-2” as a gain of function/bioweapon disinformation campaign as it is obvious to anyone who has ever read any “virus” paper, there is absolutely zero credible evidence for the existence of “SARS-COV-2” or any of these other invisible entities. At no point has any virologist ever properly purified and isolated the particles assumed to be “viruses” directly from a sick patient and then proven them pathogenic in a natural way. As this is a fact that is even admitted by virologists themselves, it should also be obvious that if they can not find the particles assumed to be “viruses” in nature, they can not tinker around and modify these fictional entities in a lab in order to create some sort of contagious bioweapon.

Somehow, this logic escapes many. Even though some have woken to the truth and accepted that “SARS-COV-2” does not exist in nature, they still believe that it must have been developed in a lab and unleashed upon the world in order to create a new contagious disease which is wrecking havoc on the elderly and immunocompromised. What they fail to realize is that there simply is no new disease and that none of the symptoms associated with “SARS-COV-2” are new, unique, or specific. There is zero proof of transmission and/or contagion beyond highly flawed epidemiological studies. There is no new “virus,” no new disease, and no contagious bioweapon. It is pure fiction based upon faulty cell culture and genomic experiments.

Before diving into the experimental evidence presented for

gain of function studies, I figured it would be a good idea to get some background information on what exactly these kinds of studies entail first. From the October 2021 *Nature* article highlighted below, we learn that the gain of function concept earned widespread recognition in 2012 due to a pair of studies which both looked to tweak an [avian influenza "virus"](#) in order to make it transmissible by air between ferrets. Disregarding the contradictory fact that aerosol transmission is supposedly the way an upper respiratory "virus" is supposed to spread, many became concerned that this kind of work may eventually lead to the release of a super "virus" which could result in the next pandemic. These ferret studies were apparently pivotal with bringing virology into the gain of function field, even though it could be easily argued that virology has been performing these kinds of experiments throughout its existence.

The gain of function term refers to any research that improves a pathogen's abilities to cause disease or spread from host to host. This is done by fiddling with [cell culture material](#) in a lab combined with [genomic sequencing](#). They do this either by inserting genetic material into the cell culture or by way of animal models where the animal is said to be genetically altered in some way to be more susceptible to the "viral" material.

The article provides an example where mice were genetically modified to become susceptible to MERS. However, the mice did not become ill upon being challenged with the "virus." Thus, the researchers resorted to passaging the "virus" between mice, which involved infecting a couple of mice, giving the "virus" two days to take hold, and then killing the mice and grinding up the lung tissue to inject into other mice. They repeated these steps at least 30 times which eventually made some mice sick. This process of culturing toxic material, injecting animals with the concoction, killing them and grinding up their remains, and then injecting this emulsified

goop into other animals in an attempt to make them sick is what GOF is all about. While this horrific process is getting recognized today, these kinds of experiments have been a staple of virology since the very beginning:

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### **The shifting sands of 'gain-of-function' research**

"The term first gained a wide public audience in 2012, after two groups revealed that they had tweaked an avian influenza virus, using genetic engineering and directed evolution, until it could be transmitted between ferrets<sup>2,3</sup>. Many people were concerned that publishing the work would be tantamount to providing a recipe for a devastating pandemic, and in the years that followed, research funders, politicians and scientists debated whether such work required stricter oversight, lest someone accidentally or intentionally release a lab-created plague. Researchers around the world voluntarily paused some work, but the issue became particularly politicized in the United States.

US funding agencies, which also support research abroad, later imposed a moratorium on gain-of-function research with pathogens while they worked out new protocols to assess the risks and benefits. But many of the regulatory discussions have taken place out of the public eye.

Now, gain-of-function research is once again centre stage, thanks to SARS-CoV-2 and a divisive debate about where it came from. Most virologists say that the coronavirus probably emerged from repeated contact between humans and animals, potentially in connection with wet markets in Wuhan, China, where the virus was first reported. But a group of scientists and politicians argues that a laboratory origin has not been ruled out. They are demanding investigation of the Wuhan Institute of Virology, where related bat coronaviruses have

been extensively studied, to determine whether SARS-CoV-2 could have accidentally leaked from the lab or crossed into humans during collection or storage of samples.”

“The term GOF didn’t have much to do with virology until the past decade. Then, the ferret influenza studies came along. In trying to advise the federal government on the nature of such research, the US National Science Advisory Board for Biosecurity (NSABB) borrowed the term – and it stuck, says Gigi Gronvall, a biosecurity specialist at the Bloomberg School of Public Health at Johns Hopkins University in Baltimore, Maryland. From that usage, it came to mean any research that improves a pathogen’s abilities to cause disease or spread from host to host.

Virologists do regularly fiddle with viral genes to change them, sometimes enhancing virulence or transmissibility, although usually just in animal or cell-culture models. “People do all of these experiments all the time,” says Juliet Morrison, a virologist at the University of California, Riverside. For example, her lab has made mouse viruses that are more harmful to mice than the originals. If only mice are at risk, should it be deemed GOF? And would it be worrying?

The answer is generally no. Morrison’s experiments, and many others like them, pose little threat to humans. GOF research starts to ring alarm bells when it involves dangerous human pathogens, such as those on the US government’s ‘select agents’ list, which includes Ebola virus and the bacteria responsible for anthrax and botulism. Other major concerns are ‘pathogens of pandemic potential’ (PPP) such as influenza viruses and coronaviruses. “For the most part, we’re worried about respiratory viruses because those are the ones that transmit the best,” says Michael Imperiale, a virologist at the University of Michigan Medical School. GOF studies with those viruses are “a really tiny part” of virology, he adds.”

“Animal research – although fraught with its own set of ethical quandaries – allows scientists to study how pathogens work and to test potential treatments, a necessary precursor to trials in people. That’s what Perlman and his collaborators had in mind when they set out to study the coronavirus responsible for Middle East Respiratory Syndrome (MERS-CoV), which emerged as a human pathogen in 2012. They wanted to use mice, but mice can’t catch MERS.

The rodents lack the right version of the protein DPP4, which MERS-CoV uses to gain entry to cells. So, the team altered the mice, giving them a human-like version of the gene for DPP4. The virus could now infect the humanized mice, but there was another problem: even when infected, the mice didn’t get very ill. “Having a model of mild disease isn’t particularly helpful to understand why people get so sick,” says collaborator Paul McCray, a paediatric pulmonologist also at the University of Iowa.

So, the group used a classic technique called ‘passaging’ to enhance virulence. The researchers infected a couple of mice, gave the virus two days to take hold, and then transferred some of the infected lung tissue into another pair of mice. They did this repeatedly – 30 times<sup>9</sup>. By the end of two months, the virus had evolved to replicate better in mouse cells. In so doing, it made the mice more ill; a high dose was deadly, says McCray. That’s GOF of a sort because the virus became better at causing disease. But adapting a pathogen to one animal in this way often limits its ability to infect others, says Andrew Pekosz, a virologist at the Bloomberg School of Public Health.”

“With all the challenges inherent in GOF studies, why do them? Because, some virologists say, the viruses are constantly mutating on their own, effectively doing GOF experiments at a rate that scientists could never match. “We can either wait for something to arise, and then fight it, or we can anticipate that certain things will arise, and instead

we can preemptively build our arsenals,” says Morrison. “That’s where gain-of-function research can come in handy.”

<https://www.nature.com/articles/d41586-021-02903-x>

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This next source is from 2015. The authors admit that virology is heavily reliant on gain or loss of function studies. They offer an alternative definition for GOF research which is any selection process involving an alteration of genotypes and their resulting phenotypes. Obviously, this definition leans far more into the genomics side of the equation. This is due to the claim that these kinds of studies are used by virologists in order to understand a “viruses” genetic make-up. It is stated that researchers now have advanced molecular technologies, such as reverse genetics, which allow them to produce de novo recombinant “viruses” from cloned cDNA. In other words, they mix genetic material from different sources, poison and/or kill lab animals by injecting them with this toxic soup, and then analyze the resulting mixture using computers so that they can claim that the generated model is a new creation. However, it is admitted that these kinds of mutations happen “naturally” with “viruses” every time a person is infected, thus confirming what we already know: virologists can not sequence the same exact “virus” every time:

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### **Gain-of-Function Research: Background and Alternatives**

**“The field of virology, and to some extent the broader field of microbiology, widely relies on studies that involve gain or loss of function. In order to understand the role of such**

studies in virology, Dr. Kanta Subbarao from the Laboratory of Infectious Disease at the National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH) gave an overview of the current scientific and technical approaches to the research on pandemic strains of influenza and Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) coronaviruses (CoV). As discussed in greater detail later in this chapter, many participants argued that the word choice of “gain-of-function” to describe the limited type of experiments covered by the U.S. deliberative process, particularly when coupled with a pause on even a smaller number of research projects, had generated concern that the policy would affect much broader areas of virology research.

## **TYPES OF GAIN-OF-FUNCTION (GOF) RESEARCH**

Subbarao explained that routine virological methods involve experiments that aim to produce a gain of a desired function, such as higher yields for vaccine strains, but often also lead to loss of function, such as loss of the ability for a virus to replicate well, as a consequence. In other words, **any selection process involving an alteration of genotypes and their resulting phenotypes is considered a type of Gain-of-Function (GoF) research**, even if the U.S. policy is intended to apply to only a small subset of such work.

**Subbarao emphasized that such experiments in virology are fundamental to understanding the biology, ecology, and pathogenesis of viruses and added that much basic knowledge is still lacking for SARS-CoV and MERS-CoV.** Subbarao introduced the key questions that virologists ask at all stages of research on the emergence or re-emergence of a virus and specifically adapted these general questions to the three viruses of interest in the symposium (see [Box 3-1](#)). To answer these questions, **virologists use gain- and loss-of-function experiments to understand the genetic makeup of viruses and the specifics of virus-host interaction. For instance,**



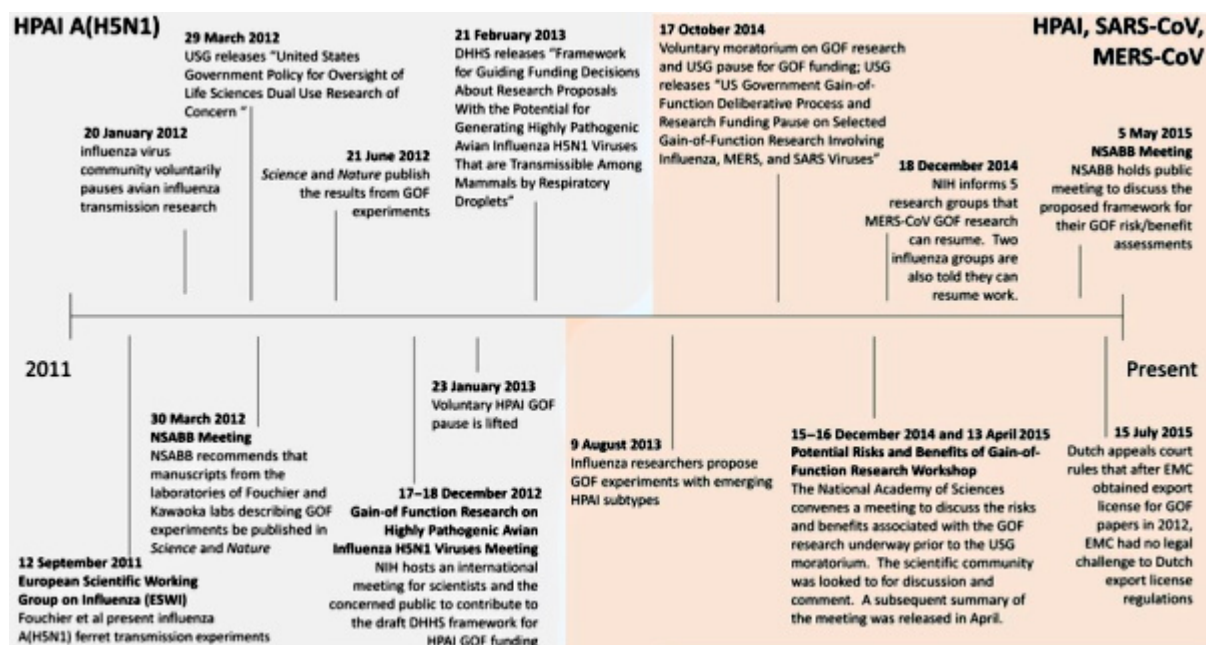
researchers now have advanced molecular technologies, such as reverse genetics, which allow them to produce de novo recombinant viruses from cloned cDNA, and deep sequencing that are critical for studying how viruses escape the host immune system and antiviral controls. Researchers also use targeted host or viral genome modification using small interfering RNA or the bacterial CRISPR-associated protein-9 nuclease as an editing tool.

During Session 3 of the symposium, Dr. Yoshihiro Kawaoka, from the University of Wisconsin-Madison, classified types of GoF research depending on the outcome of the experiments. **The first category, which he called “gain of function research of concern,” includes the generation of viruses with properties that do not exist in nature.** The now famous example he gave is the production of H5N1 influenza A viruses that are airborne-transmissible among ferrets, compared to the non-airborne transmissible wild type. **The second category deals with the generation of viruses that may be more pathogenic and/or transmissible than the wild type viruses but are still comparable to or less problematic than those existing in nature.** Kawaoka argued that the majority of strains studied have low pathogenicity, but mutations found in natural isolates will improve their replication in mammalian cells. **Finally, the third category, which is somewhere in between the two first categories, includes the generation of highly pathogenic and/or transmissible viruses in animal models that nevertheless do not appear to be a major public health concern.** An example is the high-growth A/PR/8/34 influenza strain found to have increased pathogenicity in mice but not in humans. During the discussion, Dr. Thomas Briese, Columbia University, further described GoF research done in the laboratory as being a **“proactive” approach to understand what will eventually happen in nature.**”

“Imperiale explained that, with respect to the GoF terminology, whenever researchers are working with RNA

viruses, GoF mutations are naturally arising all the time and escape mutants isolated in the laboratory appear “every time someone is infected with influenza.” He also commented that the term GoF was understood a certain way by attendees of this symposium, but when the public hears this term “they can’t make that sort of nuanced distinction that we can make here” so the terminology should be revisited.”

<https://www.ncbi.nlm.nih.gov/books/NBK285579/>



Hopefully the above two sources have shown that GOF studies are nothing more than the exact same cell culture experiments utilizing the exact same genomic sequencing technologies and tricks that virologists have always used. The only difference is that they are combining different culture supernatant and genetic materials together into one in order to create a brand new synthetic computer-generated sequence. At no point in time are any purified/isolated particles ever used in these studies. In fact, there are [no EM images](#) of the new “virus” of any kind. It should therefore not be surprising that we can

see the exact same pattern of unscientific methods and illogical reasoning in GOF studies as found in any of the original “virus” papers.

Seeing as to how the 2012 avian flu studies brought GOF research to the forefront, it seemed ideal to step into this area a bit more to see what actually transpired. The main study presented as evidence of GOF research was led by a man named Ron Fouchier. If that name sounds familiar, that’s because it should. Fouchier was involved in the 2003 [“SARS-COV-1” study](#) which proclaimed the satisfaction of Koch’s Postulates for proving a microorganism causes disease yet it failed miserably by not only not being able to satisfy Koch’s four original Postulates, but also [Thomas River’s six revised Postulates](#) made strictly for virology. In other words, it was an epic fail.

In Fouchier’s 2012 avian flu GOF study, he attempted to make the H5N1 “virus” infectious through the air. This was done through a process involving cell culturing combined with genetic engineering as well as passaging the material through numerous ferrets. Sounds familiar to the mice example from before, correct? You also see this same process with the early [polio](#) and [influenza](#) studies as well as in many other virology papers. The main difference is the genomic narrative and the use of modern technology such as reverse genetics to claim the insertion of specific genes.

Highlights from the below paper provide an overview of what was done during this study. It details how the material was collected from a flu strain in Indonesia, genetically altered in a Petri dish, and then transferred to ferrets in a series of experiments using the “wildtype” strain along with different modified strains. Fouchier and Co. were repeatedly unsuccessful in their endeavors of infecting ferrets until they started passaging the “virus” in the animals by injecting them with the cultured soup, grinding up their lung tissues, and injecting other ferrets in the same manner. They repeated

this process 6 times and then changed up the experiment by switching to nasal turbinates for the last 4 passage attempts. The only illness said to be achieved via airborne exposure was a loss of appetite, lethargy, and ruffled fur. Upon sequencing the “viruses,” there were only two amino acid switches shared by all six “viruses.” There were several other mutations, but none that occurred in all six airborne “viruses.” In other words, they could not sequence the same “virus” at any point:

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### **Fouchier study reveals changes enabling airborne spread of H5N1**

**“A study showing that it takes as few as five mutations to turn the H5N1 avian influenza virus into an airborne spreader in mammals—and that launched a historic debate on scientific accountability and transparency—was released today in Science, spilling the full experimental details that many experts had sought to suppress out of concern that publishing them could lead to the unleashing of a dangerous virus.**

**In the lengthy report, Ron Fouchier, PhD, of Erasmus Medical Center in the Netherlands and colleagues describe how they used a combination of genetic engineering and serial infection of ferrets to create a mutant H5N1 virus that can spread among ferrets without direct contact.**

They say their findings show that H5N1 viruses have the potential to evolve in mammals to gain airborne transmissibility, without having to mix with other flu viruses in intermediate hosts such as pigs, and thus pose a risk of launching a pandemic.”

### **Indonesian H5N1 strain used**

**Fouchier’s team started with an H5N1 virus collected in Indonesia and used reverse genetics to introduce mutations**

that have been shown in previous research to make H5N1 viruses more human-like in how they bind to airway cells or in other ways. Avian flu viruses prefer to bind to alpha2,3-linked sialic acid receptors on cells, whereas human flu viruses prefer alpha2,6-linked receptors. In both humans and ferrets, alpha2,6 receptors are predominant in the upper respiratory tract, while alpha 2,6 receptors are found mainly in the lower respiratory tract.

The amino acid changes the team chose included N182K, Q222L, and G224S, the numbers referring to positions in the virus's HA protein, the viral surface molecule that attaches to host cells. Q222L and G224S together change the binding preference of H2 and H3 subtype flu viruses, changes that contributed to the 1957 and 1968 flu pandemics, according to the report. And N182K was found in a human H5N1 case.

The scientists created three mutant H5N1 virus strains to launch their experiment: one containing N182K, one with Q222L and G224S, and one with all three changes, the report explains. They then launched their lengthy series of ferret experiments by inoculating groups of six ferrets with one of these three mutants or the wild-type H5N1 virus. Analysis of samples during the 7-day experiment showed that ferrets infected with the wild-type virus shed far more virus than those infected with the mutants.

In a second step, the team used a mutation in a different viral gene, PB2, the polymerase complex protein. The mutation E627K in PB2 is linked to the acquisition by avian flu viruses of the ability to grow in the human respiratory tract, which is cooler than the intestinal tract of birds, where the viruses usually reside, according to the report.

The researchers found that this mutation, when added to two of the HA mutations (Q222L and G224S), did not produce a virus that grew more vigorously in ferrets, and the virus did not spread through the air from infected ferrets to uninfected

ones.

## The passaging step

Seeing that the this mutant failed to achieve airborne transmission, the researchers decided to “passage” this strain through a series of ferrets in an effort to force it to adapt to the mammalian respiratory tract—the move that Fouchier called “really, really stupid,” according to a report of his initial description of the research at a European meeting last September.

They inoculated one ferret with the three-mutation strain and another with the wild-type virus and took daily samples until they euthanized the animals on day 4 and took tissue samples (nasal turbinates and lungs). Material from the tissue samples was then used to inoculate another pair of ferrets, and this step was carried out six times. For the last four passages, the scientists used nasal-wash samples instead of tissue samples, in an effort to harvest viruses that were secreted from the upper respiratory tract.

The amount of mutant virus found in the nasal turbinate and nose swab samples increased with the number of passages, signaling that the virus was increasing its capacity to grow in the ferret upper airway. In contrast, viral titers in the samples from ferrets infected with the wild-type virus stayed the same.

The next step was to test whether the viruses produced through passaging could achieve airborne transmission. Four ferrets were inoculated with samples of the “passage-10” mutant virus, and two ferrets were inoculated with the passage-10 wild strain. Uninfected ferrets were placed in cages next to the infected ones but not close enough for direct contact.

The ferrets exposed to those with the wild virus remained uninfected, but three of the four ferrets placed near those harboring the mutant virus did get infected, the researchers

**found.** Further, they took a sample from one of the “recipient” ferrets and used it to inoculate another ferret, which then transmitted the virus to two more ferrets that were placed near it.

**Thus, a total of six ferrets became infected with the mutant virus via airborne transmission. However, the level of viral shedding indicated the airborne virus didn’t transmit as efficiently as the 2009 H1N1 virus does.**

**In the course of the airborne transmission experiments, the ferrets showed signs of illness, including lethargy, loss of appetite, and ruffled fur. One of the directly inoculated ferrets died, but all those infected via airborne viruses survived.**

**When the scientists sequenced the genomes of the viruses that spread through the air, they found only two amino acid switches, both in HA, that occurred in all six viruses: H103Y and T156A. They noted several other mutations, but none that occurred in all six airborne viruses.**

**“Together, these results suggest that as few as five amino acid substitutions (four in HA and one in PB2) may be sufficient to confer airborne transmission of [highly pathogenic avian flu] H5N1 virus,” the researchers wrote.**

**In further steps, the researchers inoculated six ferrets with high doses of the airborne-transmissible virus; after 3 days, the ferrets were either dead or “moribund.” “Intratracheal inoculations at such high doses do not represent the natural route of infection and are generally used only to test the ability of viruses to cause pneumonia,” the report notes.”**

**<https://www.cidrap.umn.edu/news-perspective/2012/06/fouchier-study-reveals-changes-enabling-airborne-spread-h5n1>**



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While the proceeding article did an excellent job of providing the main points from Fouchier's 2012 GOF study, I wanted to showcase relevant highlights directly from the paper to flesh out the methods used even further. Here you will see that Fouchier's team claimed that they genetically modified A/H5N1 "virus" by site-directed mutagenesis and subsequent serial passage in ferrets. They used Influenza "virus" A/Indonesia/5/2005 (A/H5N1) which they said was isolated from a human case of HPAI "virus" infection. This was passaged once in embryonated chicken eggs which was followed by a single passage in Madin-Darby Canine Kidney (MDCK) cells. All eight gene segments were amplified by reverse transcription polymerase chain reaction and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000. They then used the QuickChange multisite-directed mutagenesis kit to introduce the desired amino acid substitutions. Site-directed mutagenesis is a synthetic process utilizing PCR to make artificial changes in a DNA sequence. They then took their synthetically-created cultured soup and experimented on ferrets while manipulating the methods until they achieved the results that they desired.

At no point in the paper was a "virus" of any kind ever purified and isolated. At no point were any electron microscope images of the newly mutated "viruses" ever shown. The only "evidence" of an airborne strain is genomic sequencing data from consensus genomes which did not match up. Fouchier and Co. even admitted that airborne transmission could be tested in a second mammalian model system such as guinea pigs, but even this would still not provide conclusive evidence that transmission among humans would occur. They also stated that the mutations they had identified needed further testing to determine their effect on transmission in other A/H5N1 "virus" lineages, and that further experiments are



needed to quantify how they affect “viral” fitness and “virulence” in birds and mammals. In other words, their study only told them that they could create mutated genomes and not that they created more “virulent viruses” that are transmissible by air:

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### **Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets**

“Highly pathogenic avian influenza A/H5N1 virus can cause morbidity and mortality in humans but thus far has not acquired the ability to be transmitted by aerosol or respiratory droplet (“airborne transmission”) between humans. To address the concern that the virus could acquire this ability under natural conditions, we genetically modified A/H5N1 virus by site-directed mutagenesis and subsequent serial passage in ferrets. The genetically modified A/H5N1 virus acquired mutations during passage in ferrets, ultimately becoming airborne transmissible in ferrets. None of the recipient ferrets died after airborne infection with the mutant A/H5N1 viruses. Four amino acid substitutions in the host receptor-binding protein hemagglutinin, and one in the polymerase complex protein basic polymerase 2, were consistently present in airborne-transmitted viruses. The transmissible viruses were sensitive to the antiviral drug oseltamivir and reacted well with antisera raised against H5 influenza vaccine strains. Thus, avian A/H5N1 influenza viruses can acquire the capacity for airborne transmission between mammals without recombination in an intermediate host and therefore constitute a risk for human pandemic influenza.

Influenza A viruses have been isolated from many host species, including humans, pigs, horses, dogs, marine mammals, and a wide range of domestic birds, **yet wild birds in the orders**

**Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and waders) are thought to form the virus reservoir in nature (1).** Influenza A viruses belong to the family Orthomyxoviridae; these viruses have an RNA genome consisting of eight gene segments (2, 3). Segments 1 to 3 encode the polymerase proteins: basic polymerase 2 (PB2), basic polymerase 1 (PB1), and acidic polymerase (PA), respectively. These proteins form the RNA-dependent RNA polymerase complex responsible for transcription and replication of the viral genome.”

Since the late 1990s, HPAI A/H5N1 viruses have devastated the poultry industry of numerous countries in the Eastern Hemisphere. To date, A/H5N1 has spread from Asia to Europe, Africa, and the Middle East, resulting in the death of hundreds of millions of domestic birds. In Hong Kong in 1997, the first human deaths directly attributable to avian A/H5N1 virus were recorded (11). **Since 2003, more than 600 laboratory-confirmed cases of HPAI A/H5N1 virus infections in humans have been reported from 15 countries (12).** Although limited A/H5N1 virus transmission between persons in close contact has been reported, sustained human-to-human transmission of HPAI A/H5N1 virus has not been detected (13–15). Whether this virus may acquire the ability to be transmitted via aerosols or respiratory droplets among mammals, including humans, to trigger a future pandemic is a key question for pandemic preparedness. Although our knowledge of viral traits necessary for host switching and virulence has increased substantially in recent years (16, 17), **the factors that determine airborne transmission of influenza viruses among mammals, a trait necessary for a virus to become pandemic, have remained largely unknown (18–21).** Therefore, investigations of routes of influenza virus transmission between animals and on the determinants of airborne transmission are high on the influenza research agenda.

**The viruses that caused the major pandemics of the past**

century emerged upon reassortment (that is, genetic mixing) of animal and human influenza viruses (22). However, given that viruses from only four pandemics are available for analyses, we cannot exclude the possibility that a future pandemic may be triggered by a wholly avian virus without the requirement of reassortment. Several studies have shown that reassortment events between A/H5N1 and seasonal human influenza viruses do not yield viruses that are readily transmitted between ferrets (18–20, 23). In our work, we investigated whether A/H5N1 virus could change its transmissibility characteristics without any requirement for reassortment.

We chose influenza virus A/Indonesia/5/2005 for our study because the incidence of human A/H5N1 virus infections and fatalities in Indonesia remains fairly high (12), and there are concerns that this virus could acquire molecular characteristics that would allow it to become more readily transmissible between humans and initiate a pandemic. **Because no reassortants between A/H5N1 viruses and seasonal or pandemic human influenza viruses have been detected in nature and because our goal was to understand the biological properties needed for an influenza virus to become airborne transmissible in mammals,** we decided to use the complete A/Indonesia/5/2005 virus that was isolated from a human case of HPAI A/H5N1 infection.

**We chose the ferret (*Mustela putorius furo*) as the animal model for our studies. Ferrets have been used in influenza research since 1933 because they are susceptible to infection with human and avian influenza viruses (24).** After infection with human influenza A virus, ferrets develop respiratory disease and lung pathology similar to that observed in humans. Ferrets can also transmit human influenza viruses to other ferrets that serve as sentinels with or without direct contact (fig. S1) (25–27).”

Human-to-human transmission of influenza viruses can occur through direct contact, indirect contact via fomites

(contaminated environmental surfaces), and/or airborne transmission via small aerosols or large respiratory droplets. The pandemic and epidemic influenza viruses that have circulated in humans throughout the past century were all transmitted via the airborne route, in contrast to many other respiratory viruses that are exclusively transmitted via contact. **There is no exact particle size cut-off at which transmission changes from exclusively large droplets to aerosols. However, it is generally accepted that for infectious particles with a diameter of 5 mm or less, transmission occurs via aerosols.** Because we did not measure particle size during our experiments, we will use the term “airborne transmission” throughout this Report.”

**“Using a combination of targeted mutagenesis followed by serial virus passage in ferrets, we investigated whether A/H5N1 virus can acquire mutations that would increase the risk of mammalian transmission (34).** We have previously shown that several amino acid substitutions in the RBS of the HA surface glycoprotein of A/Indonesia/5/2005 change the binding preference from the avian  $\alpha$ -2,3-linked SA receptors to the human  $\alpha$ -2,6-linked SA receptors (35). A/Indonesia/5/2005 virus with amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S (numbers refer to amino acid positions in the mature H5 HA protein; N, Asn; Q, Gln; L, Leu; G, Gly; S, Ser) in HA display attachment patterns similar to those of human viruses to cells of the respiratory tract of ferrets and humans (35). Of these changes, we know that together, Q222L and G224S switch the receptor binding specificity of H2 and H3 subtype influenza viruses, as this switch contributed to the emergence of the 1957 and 1968 pandemics (36). N182K has been found in a human case of A/H5N1 virus infection (37).

Our experimental rationale to obtain transmissible A/H5N1 viruses was to select a mutant A/H5N1 virus with receptor specificity for  $\alpha$ -2,6-linked SA shed at high titers from the

URT of ferrets. Therefore, we used the QuickChange multisite-directed mutagenesis kit (Agilent Technologies, Amstelveen, the Netherlands) to introduce amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S in the HA of wild-type (WT) A/Indonesia/5/2005, resulting in A/H5N1HA N182K, A/H5N1HA Q222L,G224S, and A/H5N1HA N182K,Q222L,G224S. Experimental details for experiments 1 to 9 are provided in the supplementary materials (25). For experiment 1, we inoculated these mutant viruses and the A/H5N1wildtype virus intranasally into groups of six ferrets for each virus (fig. S3). Throat and nasal swabs were collected daily, and virus titers were determined by end-point dilution in Madin Darby canine kidney (MDCK) cells to quantify virus shedding from the ferret URT. Three animals were euthanized after day 3 to enable tissue sample collection. All remaining animals were euthanized by day 7 when the same tissue samples were taken. Virus titers were determined in the nasal turbinates, trachea, and lungs collected post-mortem from the euthanized ferrets. Throughout the duration of experiment 1, ferrets inoculated intranasally with A/H5N1wildtype virus produced high titers in nose and throat swabs—up to 10 times more than A/H5N1HA Q222L,G224S, which yielded the highest virus titers of all three mutants during the 7-day period (Fig. 1). However, no significant difference was observed between the virus shedding of ferrets inoculated with A/H5N1HA Q222L, G224S or A/H5N1HA N182K during the first 3 days when six animals per group were present. Thus, of the viruses with specificity for  $\alpha$ -2,6-linked SA, A/H5N1HA Q222L,G224S yielded the highest virus titers in the ferret URT (Fig. 1).

As described above, amino acid substitution E627K in PB2 is one of the most consistent host-range determinants of influenza viruses (29–31). For experiment 2 (fig. S4), we introduced E627K into the PB2 gene of A/Indonesia/5/2005 by site-directed mutagenesis and produced the recombinant virus A/H5N1HA Q222L,G224S PB2 E627K. The introduction of E627K in PB2 did not significantly affect virus shedding in ferrets,

because virus titers in the URT were similar to those seen in A/H5N1HA Q222L,G224S-inoculated animals [up to  $1 \times 10^4$  50% tissue culture infectious doses (TCID<sub>50</sub>)] (Mann-Whitney U rank-sum test,  $P = 0.476$ ) (Fig. 1 and fig. S5). When four naïve ferrets were housed in cages adjacent to those with four inoculated animals to test for airborne transmission as described previously (27), A/H5N1HA Q222L,G224S PB2 E627K was not transmitted (fig. S5).

Because the mutant virus harboring the E627K mutation in PB2 and Q222L and G224S in HA did not transmit in experiment 2, we designed an experiment to force the virus to adapt to replication in the mammalian respiratory tract and to select virus variants by repeated passage (10 passages in total) of the constructed A/H5N1HA Q222L,G224S PB2 E627K virus and A/H5N1wildtype virus in the ferret URT (Fig. 2 and fig. S6). In experiment 3, one ferret was inoculated intranasally with A/H5N1wildtype and one ferret with A/H5N1HA Q222L,G224S PB2 E627K. Throat and nose swabs were collected daily from live animals until 4 days postinoculation (dpi), at which time the animals were euthanized to collect samples from nasal turbinates and lungs. The nasal turbinates were homogenized in 3 ml of virus-transport medium, tissue debris was pelleted by centrifugation, and 0.5 ml of the supernatant was subsequently used to inoculate the next ferret intranasally (passage 2). This procedure was repeated until passage 6.

From passage 6 onward, in addition to the samples described above, a nasal wash was also collected at 3 dpi. To this end, 1 ml of phosphate-buffered saline (PBS) was delivered dropwise to the nostrils of the ferrets to induce sneezing. Approximately 200 ml of the “sneeze” was collected in a Petri dish, and PBS was added to a final volume of 2 ml. The nasal-wash samples were used for intranasal inoculation of the ferrets for the subsequent passages 7 through 10. We changed the source of inoculum during the course of the experiment, because passaging nasal washes may facilitate the selection of

viruses that were secreted from the URT. Because influenza viruses mutate rapidly, we anticipated that 10 passages would be sufficient for the virus to adapt to efficient replication in mammals.

Virus titers in the nasal turbinates of ferrets inoculated with A/H5N1wildtype ranged from  $\sim 1 \times 10^5$  to  $1 \times 10^7$  TCID50/gram tissue throughout 10 serial passages (Fig. 3A and fig. S7). In ferrets inoculated with A/H5N1HA Q222L,G224S PB2 E627K virus, a moderate increase in virus titers in the nasal turbinates was observed as the passage number increased. These titers ranged from  $1 \times 10^4$  TCID50/gram tissue at the start of the experiment to  $3.2 \times 10^5$  to  $1 \times 10^6$  TCID50/gram tissue in the final passages (Fig. 3A and fig. S7). Notably, virus titers in the nose swabs of animals inoculated with A/H5N1HA Q222L,G224S PB2 E627K also increased during the successive passages, with peak virus shedding of  $1 \times 10^5$  TCID50 at 2 dpi after 10 passages (Fig. 3B). These data indicate that A/H5N1HA Q222L,G224S PB2 E627K was developing greater capacity to replicate in the ferret URT after repeated passage, with evidence for such adaptation becoming apparent by passage number 4. In contrast, virus titers in the nose swabs of the ferrets collected at 1 to 4 dpi throughout 10 serial passages with A/H5N1wildtype revealed no changes in patterns of virus shedding.

**Passaging of influenza viruses in ferrets should result in the natural selection of heterogeneous mixtures of viruses in each animal with a variety of mutations: so-called viral quasi-species (38).** The genetic composition of the viral quasi-species present in the nasal washes of ferrets after 10 passages of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform (Roche, Woerden, the Netherlands) (tables S1 and S2). The mutations introduced in A/H5N1HA Q222L,G224S PB2 E627K by reverse genetics remained present in the virus population after 10 consecutive passages at a



**frequency >99.5%** (Fig. 4 and table S1). **Numerous additional nucleotide substitutions were detected in all viral gene segments of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K after passaging**, except in segment 7 (tables S1 and S2). Of the 30 nucleotide substitutions selected during serial passage, **53% resulted in amino acid substitutions**. **The only amino acid substitution detected upon repeated passage of both A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K was T156A** (T, Thr; A, Ala) in HA. This substitution removes a potential N-linked glycosylation site (Asn-X-Thr/Ser; X, any amino acid) in HA and was detected in 99.6% of the A/H5N1wildtype sequences after 10 passages. T156A was detected in 89% of the A/H5N1HA Q222L,G224S PB2 E627K sequences after 10 passages, and the other 11% of sequences possessed the substitution N154K, which removes the same potential N-linked glycosylation site in HA.

In experiment 4 (see supplementary materials), we investigated whether airborne-transmissible viruses were present in the heterogeneous virus population generated during virus passaging in ferrets (fig. S4). **Nasal-wash samples, collected at 3 dpi from ferrets at passage 10, were used in transmission experiments to test whether airborne-transmissible virus was present in the virus quasi-species**. For this purpose, nasal-wash samples were diluted 1:2 in PBS and subsequently used to **inoculate six naïve ferrets intranasally**: two for passage 10 A/H5N1wildtype and four for passage 10 A/H5N1HA-Q222L,G224S PB2 E627K virus.

The following day, a naïve recipient ferret was placed in a cage adjacent to each inoculated donor ferret. These cages are designed to prevent direct contact between animals but allow airflow from a donor ferret to a neighboring recipient ferret (fig. S1) (27). **Although mutations had accumulated in the viral genome after passaging of A/H5N1wildtype in ferrets, we did not detect replicating virus upon inoculation of MDCK cells with swabs collected from naïve recipient ferrets**



after they were paired with donor ferrets inoculated with passage 10 A/H5N1wildtype virus (Fig. 5, A and B). In contrast, we did detect virus in recipient ferrets paired with those inoculated with passage 10 A/H5N1HA Q222L,G224S PB2 E627K virus. Three (F1 to F3) out of four (F1 to F4) naïve recipient ferrets became infected as confirmed by the presence of replicating virus in the collected nasal and throat swabs (Fig. 5, C and D). A throat-swab sample obtained from recipient ferret F2, which contained the highest virus titer among the ferrets in the first transmission experiment, was subsequently used for intranasal inoculation of two additional donor ferrets. Both of these animals, when placed in the transmission cage setup (fig. S1), again transmitted the virus to the recipient ferrets (F5 and F6) (Fig. 6, A and B). A virus isolate was obtained after inoculation of MDCK cells with a nose swab collected from ferret F5 at 7 dpi. The virus from F5 was inoculated intranasally into two more donor ferrets. One day later, these animals were paired with two recipient ferrets (F7 and F8) in transmission cages, one of which (F7) subsequently became infected (Fig. 6, C and D).

We used conventional Sanger sequencing to determine the consensus genome sequences of viruses recovered from the six ferrets (F1 to F3 and F5 to F7) that acquired virus via airborne transmission (Fig. 4 and table S3). All six samples still harbored substitutions Q222L, G224S, and E627K that had been introduced by reverse genetics. Surprisingly, only two additional amino acid substitutions, both in HA, were consistently detected in all six airborne-transmissible viruses: (i) H103Y (H, His; Y, Tyr), which forms part of the HA trimer interface, and (ii) T156A, which is proximal but not immediately adjacent to the RBS (fig. S8). Although we observed several other mutations, their occurrence was not consistent among the airborne viruses, indicating that of the heterogeneous virus populations generated by passaging in ferrets, viruses with different genotypes were transmissible. In addition, a single transmission experiment is not

sufficient to select for clonal airborne-transmissible viruses because, for example, the consensus sequence of virus isolated from F6 differed from the sequence of parental virus isolated from F2.

Together, these results suggest that as few as five amino acid substitutions (four in HA and one in PB2) may be sufficient to confer airborne transmission of HPAI A/H5N1 virus between mammals. The airborne-transmissible virus isolate with the least number of amino acid substitutions, compared with the A/H5N1 wildtype, was recovered from ferret F5. This virus isolate had a total of nine amino acid substitutions; in addition to the three mutations that we introduced (Q222L and G224S in HA and E627K in PB2), this virus harbored H103Y and T156A in HA, H99Y and I368V (I, Ile; V, Val) in PB1, and R99K (R, Arg) and S345N in NP (table S3). Reverse genetics will be needed to identify which of the five to nine amino acid substitutions in this virus are essential to confer airborne transmission.

During the course of the transmission experiments with the airborne-transmissible viruses, ferrets displayed lethargy, loss of appetite, and ruffled fur after intranasal inoculation. One of eight inoculated animals died upon intranasal inoculation (Table 1). In previously published experiments, ferrets inoculated intranasally with WTA/Indonesia/5/2005 virus at a dose of  $1 \times 10^6$  TCID<sub>50</sub> showed neurological disease and/or death (39, 40). It should be noted that inoculation of immunologically naïve ferrets with a dose of  $1 \times 10^6$  TCID<sub>50</sub> of A/H5N1 virus and the subsequent course of disease is not representative of the natural situation in humans. Importantly, although the six ferrets that became infected via respiratory droplets or aerosol also displayed lethargy, loss of appetite, and ruffled fur, none of these animals died within the course of the experiment. Moreover, previous infections of humans with seasonal influenza viruses are likely to induce heterosubtypic immunity that would offer

some protection against the development of severe disease (41, 42). It has been shown that mice and ferrets previously infected with an A/H3N2 virus are clinically protected against intranasal challenge infection with an A/H5N1 virus (43, 44).

**After intratracheal inoculation (experiment 5; fig. S9), six ferrets inoculated with  $1 \times 10^6$  TCID50 of airborne-transmissible virus F5 in a 3-ml volume of PBS died or were moribund at day 3. Intratracheal inoculations at such high doses do not represent the natural route of infection and are generally used only to test the ability of viruses to cause pneumonia (45), as is done for vaccination-challenge studies. At necropsy, the six ferrets revealed macroscopic lesions affecting 80 to 100% of the lung parenchyma with average virus titers of  $7.9 \times 10^6$  TCID50/gram lung (fig. S10). These data are similar to those described previously for A/H5N1 wildtype in ferrets (Table 1). Thus, although the airborne-transmissible virus is lethal to ferrets upon intratracheal inoculation at high doses, the virus was not lethal after airborne transmission.”**

**“Although our experiments showed that A/H5N1 virus can acquire a capacity for airborne transmission, the efficiency of this mode remains unclear. Previous data have indicated that the 2009 pandemic A/H1N1 virus transmits efficiently among ferrets and that naïve animals shed high amounts of virus as early as 1 or 2 days after exposure (27). When we compare the A/H5N1 transmission data with that of reference (27), keeping in mind that our experimental design for studying transmission is not quantitative, the data shown in Figs. 5 and 6 suggest that A/H5N1 airborne transmission was less robust, with less and delayed virus shedding compared with pandemic A/H1N1 virus.**

**Airborne transmission could be tested in a second mammalian model system such as guinea pigs (59), but this would still not provide conclusive evidence that transmission among humans would occur. The mutations we identified need to be tested for their effect on transmission in other A/H5N1 virus lineages**

**(60), and experiments are needed to quantify how they affect viral fitness and virulence in birds and mammals. For pandemic preparedness, antiviral drugs and vaccine candidates against airborne-transmissible virus should be evaluated in depth. Mechanistic studies on the phenotypic traits associated with each of the identified amino acid substitutions should provide insights into the key determinants of airborne virus transmission. Our findings indicate that HPAI A/H5N1 viruses have the potential to evolve directly to transmit by aerosol or respiratory droplets between mammals, without reassortment in any intermediate host, and thus pose a risk of becoming pandemic in humans. Identification of the minimal requirements for virus transmission between mammals may have prognostic and diagnostic value for improving pandemic preparedness (34)."**

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4810786/#!po=70.4819>

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From the Supplementary Materials:

## **Materials and methods**

### **Viruses**

"Influenza virus A/Indonesia/5/2005 (A/H5N1) was isolated from a human case of HPAI virus infection and passaged once in embryonated chicken eggs followed by a single passage in Madin-Darby Canine Kidney (MDCK) cells. All eight gene segments were amplified by reverse transcription polymerase chain reaction and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 (63-64). Mutations of interest (N182K, Q222L, G224S in HA and E627K in PB2) were introduced in reverse genetics vectors using the QuikChange multi-site-directed mutagenesis kit (Aligent, Amstelveen, The Netherlands) according to the instructions of

the manufacturer. Recombinant viruses were produced upon transfection of 293T cells and virus stocks were propagated and titrated in MDCK cells as described (63).

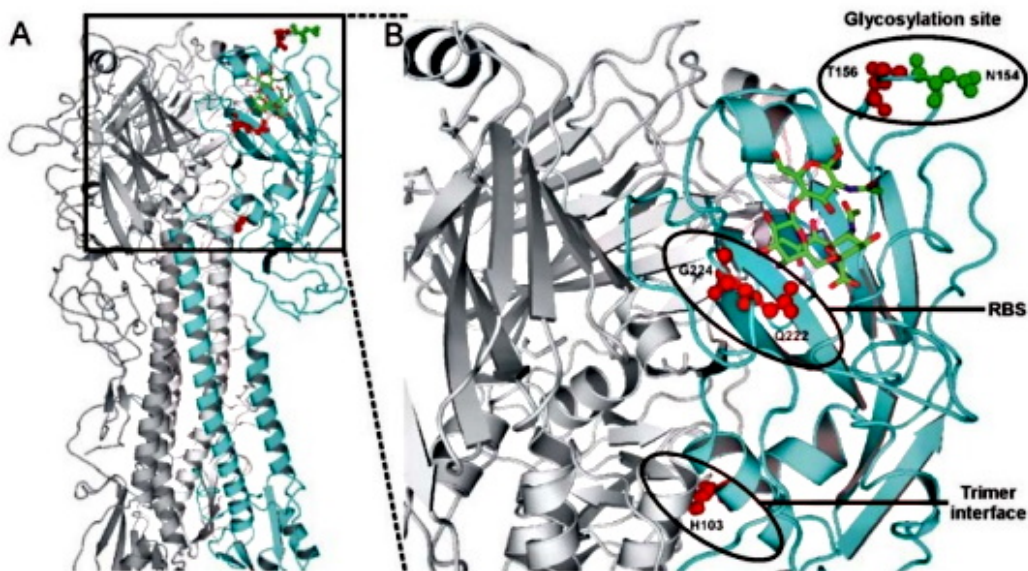
## **Cells**

MDCK cells were cultured in Eagle's minimal essential medium (EMEM, Lonza Benelux BV, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM Hepes (Lonza), and non-essential amino acids (MP Biomedicals Europe, Illkirch, France). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM, Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2mM glutamine, 1mM sodium pyruvate, and non-essential amino acids.

## **Virus titration in MDCK cells**

Virus titrations were performed as described previously (27). Briefly, MDCK cells were inoculated with tenfold serial dilutions of virus preparations, homogenized tissues, nose swabs, and throat swabs. Cells were washed with PBS one hour after inoculation and cultured in 200µl of infection media, consisting of EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, 1.5mg/ml sodium bicarbonate, 10mM Hepes, non-essential amino acids, and 20 µg/ml trypsin (Lonza). Three days after inoculation, supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of virus replication in the cells. Infectious virus titers were calculated from four replicates each of the homogenized tissue samples, nose swabs, and throat swabs and for ten replicates of the virus preparations by the method of Spearman-Kärber (65)."

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**Fig. S8.** Cartoon representation of a model of the trimer structure HA of A/Indonesia/5/2005 (68) (PDB ID: 1JSM). One monomer is colored cyan for clarity. The structure of the three-

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Cartoon representation...aren't they all?

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NEWS FEATURE | 27 October 2021

## The shifting sands of 'gain-of-function' research

The mystery of COVID's origins has reignited a contentious debate about potentially

- The term "Gain of Function" first gained a wide public audience in 2012, after two groups revealed that they had tweaked an avian influenza "virus," **using genetic engineering and directed evolution**, until it could be transmitted between ferrets
- Most virologists say that the "coronavirus" **probably**



**emerged** from repeated contact between humans and animals, potentially in connection with wet markets in Wuhan, China, where the “virus” was first reported

- However, a group of scientists and politicians argues **that a laboratory origin has not been ruled out**
- The term GOF didn't have much to do with virology **until the past decade when the ferret influenza studies came along**
- From that usage, it came to mean **any research that improves a pathogen's abilities to cause disease or spread from host to host**
- Virologists regularly fiddle with “viral” genes to change them, sometimes enhancing virulence or transmissibility, **although usually just in animal or cell-culture models**
- Other major concerns are ‘**pathogens of pandemic potential**’ (PPP) such as influenza “**viruses**” and “**coronaviruses**”
- “For the most part, we're worried about respiratory “**viruses**” because those are the ones that transmit the best,” says Michael Imperiale, a virologist at the University of Michigan Medical School
- He added that GOF studies with those “viruses” are “**a really tiny part**” of virology
- Perlman and his collaborators set out to study the “coronavirus” responsible for Middle East Respiratory Syndrome (MERS-CoV), which emerged as a human pathogen in 2012
- They wanted to use mice, **but mice can't catch MERS**
- The rodents lack the right version of the protein DPP4, which MERS-CoV uses to gain entry to cells and **so the team altered the mice, giving them a human-like version of the gene for DPP4**
- The “virus” could now infect the humanized mice, but there was another problem: **even when infected, the mice didn't get very ill**
- So, the group used a classic technique called

**'passaging'** to enhance "virulence"

- The researchers infected a couple of mice, gave the "virus" two days to take hold, **and then transferred some of the infected lung tissue into another pair of mice**
- They did this **repeatedly – 30 times** and by the end of two months, the "virus" had evolved to replicate better in mouse cells
- In so doing, **it made the mice more ill**; a high dose was deadly
- Some virologists say **"viruses" are constantly mutating on their own**, effectively doing GOF experiments **at a rate that scientists could never match**

### 3 Gain-of-Function Research: Background and Alternatives

#### Publication Details

The field of virology, and to some extent the broader field of microbiology, widely relies on studies that involve gain or loss of function. In order to understand the role of such studies in virology, Dr. Kanta Subbarao from the Laboratory of Infectious Disease at the National Institute of Allergy and

- The field of virology, and to some extent the broader field of microbiology, **widely relies on studies that involve gain or loss of function**
- Any selection process **involving an alteration of genotypes and their resulting phenotypes** is considered a type of Gain-of-Function (GoF) research
- Subbarao emphasized that such experiments in virology are **fundamental to understanding the biology, ecology, and pathogenesis of "viruses"** and added that **much basic knowledge is still lacking for "SARS-CoV" and "MERS-CoV"**
- Virologists use gain- and loss-of-function experiments **to understand the genetic makeup of "viruses"** and the specifics of "virus-host" interaction



- Researchers now have advanced molecular technologies, such as reverse genetics, **which allow them to produce de novo recombinant “viruses” from cloned cDNA** (*i.e. they are synthetic lab creations*)
- Researchers also use targeted host or **“viral” genome modification using small interfering RNA or the bacterial CRISPR-associated protein-9 nuclease** as an editing tool
- Dr. Yoshihiro Kawaoka, from the University of Wisconsin-Madison, classified types of GoF research depending on the outcome of the experiments:
  1. The first category is “gain of function research of concern,” includes the generation of **“viruses” with properties that do not exist in nature**
    - The now famous example he gave is the production of H5N1 influenza A “viruses” that are airborne-transmissible among ferrets, compared to the non-airborne transmissible wild type
  2. The second category deals with the generation of “viruses” that may be more pathogenic and/or transmissible than the wild type “viruses” **but are still comparable to or less problematic than those existing in nature** (*which is odd considering no “viruses” have been found in nature...*)
    - Kawaoka argued that the majority of strains studied have low pathogenicity, but mutations **found in natural isolates** (*there are no natural isolates*) will improve their replication in mammalian cells
  3. The third category, which is somewhere in between the first two categories, includes the generation of highly pathogenic and/or transmissible “viruses” **in animal models that nevertheless do not appear to be a major public health concern**
    - An example is the high-growth A/PR/8/34

influenza strain found to have increased pathogenicity in mice but not in humans

- Dr. Thomas Briese, Columbia University, further described GoF research done in the laboratory **as being a “proactive” approach to understand what will eventually happen in nature**
- GoF mutations **are naturally arising all the time** and escape mutants isolated in the laboratory appear “every time someone is infected with influenza.”
- *In other words, they can never sequence the same “virus” every time so what they do in the lab in GoF studies is no different than how they culture and “isolate viruses” in order to sequence the genomes in the first place*

The screenshot shows the top portion of the CIDRAP website. On the left is the CIDRAP logo, which consists of a stylized 'C' made of colorful geometric shapes. To the right of the logo are social media icons for email, Facebook, Twitter, YouTube, and RSS, followed by a 'Contact Us' button. Below the logo is the text 'Center for Infectious Disease Research and Policy' and a navigation menu with links for 'Ongoing Programs', 'About Us', and 'DONATE NOW'. A dark navigation bar contains a home icon and three menu items: 'News & Perspective', 'Infectious Disease Topics', and 'Antimicrobial Stewardship'. Below this is a 'FEATURED NEWS TOPICS' section with links for 'COVID-19', 'Flu Vaccines Roadmap', 'MERS-CoV', and 'Chronic Wasting Disease'. The main headline reads 'Fouchier study reveals changes enabling airborne spread of H5N1'. Below the headline is a sub-headline: 'Filed Under: Avian Influenza (Bird Flu); Biosecurity Issues; Bioterrorism; Dual-Use Research; Pandemic Influenza'. The author information is 'By: Robert Roos | Jun 21, 2012'. At the bottom of the header are social sharing icons for Facebook, Twitter, LinkedIn, Email, and Print & PDF.

- A 2012 study supposedly showed that it takes as few as five mutations to turn the H5N1 avian influenza “virus” into an airborne spreader in mammals—and **this launched a historic debate on scientific accountability and transparency**
- In the lengthy report, Ron Fouchier, PhD, of Erasmus Medical Center in the Netherlands and colleagues describe how **they used a combination of genetic engineering and serial infection of ferrets to create a mutant H5N1 “virus” that can spread among ferrets without direct contact**

- Fouchier's team **started with an H5N1 "virus" collected in Indonesia and used reverse genetics to introduce mutations** that have been shown in previous research to make H5N1 "viruses" more human-like in how they bind to airway cells or in other ways
- The **amino acid changes the team chose** included N182K, Q222L, and G224S, the numbers referring to positions in the "virus's" HA protein, the "viral" surface molecule that attaches to host cells
- The scientists created three mutant H5N1 "virus" strains to launch their experiment: **one containing N182K, one with Q222L and G224S, and one with all three changes**
- They then launched their lengthy series of ferret experiments **by inoculating groups of six ferrets with one of these three mutants or the wild-type H5N1 "virus"**
- Analysis of samples during the 7-day experiment showed that ferrets infected with the wild-type "virus" **shed far more "virus"** than those infected with the mutants
- In a second step, the team **used a mutation in a different "viral" gene, PB2, the polymerase complex protein**
- The researchers found that this mutation, **when added to two of the HA mutations (Q222L and G224S)**, did not produce a "virus" that grew more vigorously in ferrets, and the "virus" did not spread through the air from infected ferrets to uninfected ones
- Seeing that the this mutant failed to achieve airborne transmission, the researchers decided to **"passage" this strain through a series of ferrets in an effort to force it to adapt to the mammalian respiratory tract**
- This was the move that Fouchier called **"really, really stupid"** (*are we sure he wasn't referring to the whole study?*)
- They inoculated one ferret with the three-mutation strain and another with the wild-type "virus" **and took daily samples until they euthanized the animals on day 4 and took tissue samples (nasal turbinates and lungs)**

- Material from the tissue samples was then **used to inoculate another pair of ferrets, and this step was carried out six times**
- For the last four passages, the scientists **used nasal-wash samples instead of tissue samples**, in an effort to harvest “viruses” that were secreted from the upper respiratory tract
- *In other words, they completely changed the source material from tissue to nasal secretions more than halfway through the experiment*
- It was said that the amount of mutant “virus” found in the nasal turbinate and nose swab samples **increased with the number of passages** while “viral” titers in the samples from ferrets infected **with the wild-type “virus” stayed the same**

#### **Quick Sidenote From the Supplementary Materials:**

“After inoculation with A/H5N1wildtype, **virus titers in the nasal turbinates were variable but high, ranging from  $1.6 \times 10^5$  to  $7.9 \times 10^6$  TCID50/gram tissue (panel A)**, with no further increase observed with repeated passage. After inoculation with A/H5N1HA Q222L,G224S PB2 E627K, **virus titers in nasal turbinates averaged  $1.6 \times 10^4$  in the first three passages,  $2.5 \times 10^5$  in passage four to seven and  $6.3 \times 10^5$  TCID50/gram tissue in the last three passages, suggestive of improved replication and virus adaptation**. In the lungs, no apparent adaptation was observed for animals inoculated with either virus. **Virus titers in lungs were highly variable; presumably it was a matter of chance whether the virus reached the lower airways.**”

In other words, the “wildtype virus” titers remained and stayed high while the “mutant virus” started low and elevated throughout passaging yet was still underneath the amount seen in the “wildtype” strain. They also note that finding “virus” in the lungs was a “matter of chance” with either “virus.”

## End Quick Sidenote.

- The next step was to test whether the “viruses” **produced through passaging** could achieve airborne transmission so four ferrets were **inoculated with samples of the “passage-10” mutant “virus,”** and two ferrets were inoculated with the passage-10 wild strain
- Uninfected ferrets were placed in cages next to the infected ones but not close enough for direct contact
- The ferrets exposed to those with the wild “virus” remained uninfected, but three of the four ferrets placed near those harboring the mutant “virus” did get infected (*“infected” meaning they found “viral” RNA*)
- Thus, a total of six ferrets became “infected” with the mutant “virus” via airborne transmission
- However, **the level of “viral” shedding** indicated the airborne “virus” didn’t transmit as efficiently as the 2009 H1N1 “virus”
- In the course of the airborne transmission experiments, the ferrets showed signs of illness, **including lethargy, loss of appetite, and ruffled fur** (*no consideration is given to the fact that the animals were caged, tortured, and experimented on*)
- One of the directly inoculated ferrets died, **but all those infected via airborne “viruses” survived**
- When the scientists sequenced the genomes of the “viruses” that spread through the air, **they found only two amino acid switches, both in HA, that occurred in all six “viruses:”** H103Y and T156A
- They noted several other mutations, **but none that occurred in all six airborne “viruses”**
- *In other words, once again they were unable to sequence the exact same genome in the samples from each ferret*
- In further steps, the researchers **inoculated intratracheally six ferrets with high doses** of the airborne-transmissible “virus;” after 3 days, the ferrets were either dead or “moribund”

- They stated: “Intratracheal inoculations at such high doses **do not represent the natural route of infection** and are generally used only to test the ability of viruses to cause pneumonia”

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## Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets

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- Highly “pathogenic” avian influenza A/H5N1 “virus” can cause morbidity and mortality in humans **but thus far has not acquired the ability to be transmitted by aerosol or respiratory droplet (“airborne transmission”) between humans**
  - To address the concern that the “virus” **could acquire this ability under natural conditions**, the researchers **genetically modified** A/H5N1 “virus” by site-directed mutagenesis and **subsequent serial passage in ferrets**
  - *In other words, in order to test whether the “virus” could mutate naturally, they mutated it synthetically...*
  - The **genetically modified** A/H5N1 “virus” **acquired mutations during passage** in ferrets, ultimately becoming airborne transmissible in ferrets (*all “viruses” acquire mutations every time they are sequenced as no “viral” genome is ever the same as the original*)
  - **None of the recipient ferrets died** after airborne infection with the mutant A/H5N1 “viruses”
  - Wild birds in the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and

waders) **are thought to form the “virus” reservoir in nature**

- Since 2003, more than 600 **laboratory-confirmed cases** of HPAI A/H5N1 “virus” infections in humans have been reported from 15 countries
- Although **limited** A/H5N1 “virus” transmission between persons in close contact has been reported, **sustained human-to-human transmission of HPAI A/H5N1 “virus” has not been detected**
- Whether this “virus” may acquire the ability to be transmitted via aerosols or respiratory droplets among mammals, including humans, **to trigger a future pandemic is a key question for pandemic preparedness**
- **The factors that determine airborne transmission of influenza “viruses” among mammals, a trait necessary for a “virus” to become pandemic, have remained largely unknown**
- The “viruses” that caused the major pandemics of the past century **emerged upon reassortment (that is, genetic mixing) of animal and human influenza “viruses”**
- However, **given that “viruses” from only four pandemics are available for analyses**, they cannot exclude the possibility that a future pandemic may be triggered by a wholly avian “virus” without the requirement of reassortment
- **No reassortants** between A/H5N1 “viruses” and seasonal or pandemic human influenza “viruses” **have been detected in nature** and their goal was **to understand the biological properties needed** for an influenza “virus” to become airborne transmissible in mammals
- They chose the ferret (*Mustela putorius furo*) as the animal model for the studies as **ferrets have been used in influenza research since 1933** because they are susceptible to infection with human and avian influenza “viruses”
- There is **no exact particle size cut-off** at which transmission changes from exclusively large droplets to

aerosols

- It is **generally accepted** that for infectious particles with a diameter of 5 μm or less, transmission occurs via aerosols
- The researchers used the QuickChange multisite-directed mutagenesis kit to introduce amino acid substitutions in the HA of wild-type “virus”
- For experiment 1, they **inoculated these mutant “viruses” and the A/H5N1 wildtype “virus” intranasally into groups of six ferrets** for each “virus”
- Throat and nasal swabs were collected daily, and “virus” titers **were determined by end-point dilution in Madin Darby canine kidney (MDCK) cells** to quantify “virus” shedding from the ferret URT
- When four naïve ferrets were housed in cages adjacent to those with four inoculated animals **to test for airborne transmission** as described previously, A/H5N1HA Q222L,G224S PB2 E627K **was not transmitted**
- Because the mutant “virus” harboring the E627K mutation in PB2 and Q222L and G224S in HA **did not transmit in experiment 2**, they designed an experiment **to force the “virus” to adapt** to replication in the mammalian respiratory tract and to select “virus” variants **by repeated passage (10 passages in total)** of the constructed A/H5N1HA Q222L,G224S PB2 E627K “virus” and A/H5N1 wildtype “virus” in the ferret URT
- In experiment 3, one ferret was **inoculated intranasally** with A/H5N1 wildtype and one ferret with A/H5N1HA Q222L,G224S PB2 E627K
- Throat and nose swabs were collected daily from live animals until 4 days postinoculation (dpi), at which time the animals were euthanized **to collect samples from nasal turbinates and lungs**
- The **nasal turbinates were homogenized in 3 ml of “virus-transport” medium**, tissue debris was pelleted by centrifugation, and 0.5 ml of the **supernatant was subsequently used to inoculate the next ferret**



## **intranasally (passage 2)**

- This procedure **was repeated until passage 6**
- From passage 6 onward, in addition to the samples described above, **a nasal wash was also collected at 3 dpi**
- To this end, **1 ml of phosphate-buffered saline (PBS) was delivered dropwise** to the nostrils of the ferrets to induce sneezing
- Approximately 200 ml of the “sneeze” was collected in a Petri dish, **and PBS was added to a final volume of 2 ml**
- The **nasal-wash samples were used for intranasal inoculation** of the ferrets for the subsequent passages **7 through 10**
- They **changed the source of inoculum during the course of the experiment**, because passaging nasal washes may facilitate the selection of “viruses” that were secreted from the URT
- Because influenza “viruses” mutate rapidly, they **anticipated (*i.e. guessed arbitrarily*) that 10 passages would be sufficient** for the “virus” to adapt to efficient replication in mammals
- The genetic composition of the “viral” quasi-species present in the nasal washes of ferrets after 10 passages of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K **was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform**
- The mutations introduced in A/H5N1HA Q222L,G224S PB2 E627K **by reverse genetics** remained present in the “virus” population after 10 consecutive passages at a frequency >99.5%
- **Numerous additional nucleotide substitutions were detected in all “viral” gene segments** of A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K after passaging, except in segment 7
- Of the 30 nucleotide substitutions selected during serial passage, **53% resulted in amino acid substitutions**
- The **only amino acid substitution detected upon repeated**

**passage of both A/H5N1wildtype and A/H5N1HA Q222L,G224S PB2 E627K was T156A**

- In experiment 4, nasal-wash samples, collected at 3 dpi from ferrets at passage 10, **were used in transmission experiments** to test whether airborne-transmissible “virus” was present in the “virus” quasi-species
- For this purpose, **nasal-wash samples were diluted 1:2 in PBS** and subsequently used to inoculate six naïve ferrets **intranasally**
- **Although mutations had accumulated in the “viral” genome after passaging** of A/H5N1wildtype in ferrets, they **did not detect replicating “virus” upon inoculation of MDCK cells** with swabs collected from naïve recipient ferrets after they were paired with donor ferrets inoculated with passage 10 A/H5N1wildtype “virus”
- In contrast, they did detect “virus” in recipient ferrets **paired with those inoculated with passage 10 A/H5N1HA Q222L,G224S PB2 E627K “virus”**
- Three out of four naïve recipient ferrets became “infected” **as confirmed by the presence of replicating “virus”** in the collected nasal and throat swabs (*in other words, they saw CPE in a cell culture and claimed “virus” was present*)
- A “virus isolate” was obtained **after inoculation of MDCK cells** with a nose swab collected from ferret F5 at 7 dpi
- They used conventional Sanger sequencing **to determine the consensus genome sequences** of viruses recovered from the six ferrets that acquired “virus” via airborne transmission and all six samples still harbored substitutions Q222L, G224S, and E627K that had been **introduced by reverse genetics**
- *In other words, they created consensus sequencing through alignment to reference genomes using computer software and algorithms from unpurified material*
- They **observed several other mutations** for which their occurrence was not consistent among the airborne “viruses,” indicating that of the **heterogeneous “virus”**

**populations** generated by passaging in ferrets, "viruses" with **different genotypes** were transmissible

- *In other words, they were unable to sequence the exact same "virus" genome every time...and if that wasn't clear*

- In addition, a single transmission experiment is not sufficient to select for clonal airborne-transmissible "viruses" because, for example, **the consensus sequence of "virus" isolated from F6 differed from the sequence of parental "virus" isolated from F2**
- Together, they claim that these results **suggest** that as few as five amino acid substitutions (four in HA and one in PB2) **may be sufficient to confer airborne transmission** of HPAI A/H5N1 "virus" between mammals
- During the course of the transmission experiments with the airborne-transmissible "viruses," ferrets displayed **lethargy, loss of appetite, and ruffled fur** after intranasal inoculation
- It should be noted that inoculation of immunologically naïve ferrets with a dose of  $1 \times 10^6$  TCID<sub>50</sub> of A/H5N1 "virus" and the subsequent course of disease **is not representative of the natural situation in humans**
- Importantly, although the six ferrets that became "infected" via respiratory droplets or aerosol also displayed lethargy, loss of appetite, and ruffled fur, **none of these animals died within the course of the experiment**
- After **intratracheal** (*in the throat*) inoculation, six ferrets inoculated with  $1 \times 10^6$  TCID<sub>50</sub> of airborne-transmissible "virus" F5 **in a 3-ml volume of PBS died or were moribund at day 3**
- Intratracheal inoculations at such high doses **do not represent the natural route of infection** and are generally used only to test the ability of "viruses" to cause pneumonia, **as is done for vaccination-challenge studies**

- Although the airborne-transmissible “virus” is lethal to ferrets **upon intratracheal inoculation at high doses**, the “virus” was **not lethal after airborne transmission**
- *They openly admit that the route of injection and the amount of toxic culture goo injected causes the severity of disease, which does not require the “virus” as an explanation*
- They state that although experiments showed that A/H5N1 “virus” can acquire a capacity for airborne transmission, **the efficiency of this mode remains unclear**
- They pointed out that their experimental design for studying transmission **is not quantitative** (*i.e. they do not know how much “virus” is required for airborne transmission and assume it occurs via PCR results*)
- They airborne transmission could be tested in a second mammalian model system such as guinea pigs, **but this would still not provide conclusive evidence that transmission among humans would occur**
- The mutations they identified need to be tested for their effect on transmission in other A/H5N1 “virus” lineages, **and experiments are needed to quantify how they affect “viral” fitness and “virulence” in birds and mammals**
- Their findings indicate that HPAI A/H5N1 “viruses” **have the potential to evolve** directly to transmit by aerosol or respiratory droplets between mammals, **without reassortment in any intermediate host**, and thus pose a risk of becoming pandemic in human
- *Of course, the only place reassortment occurs is in a lab so they never need a host...*
- Identification of the minimal requirements for “virus” transmission between mammals **may have prognostic and diagnostic value** for improving pandemic preparedness
- Influenza “virus” A/Indonesia/5/2005 (A/H5N1) was isolated from a human case of HPAI “virus” infection

and **passaged once in embryonated chicken eggs followed by a single passage in Madin-Darby Canine Kidney (MDCK) cells**

- All eight gene segments were **amplified by reverse transcription polymerase chain reaction and cloned** in a modified version of the bidirectional reverse genetics plasmid pHW2000
- Mutations of interest were **introduced in reverse genetics vectors** using the QuikChange multi-site-directed mutagenesis kit
- Recombinant “viruses” were **produced upon transfection of 293T cells and “virus” stocks were propagated and titrated in MDCK cells**
- MDCK cells (*canine*) were cultured in Eagle’s minimal essential medium supplemented with:
  1. 10% fetal calf serum (FCS)
  2. 100 IU/ml penicillin
  3. 100 µg/ml streptomycin
  4. 2 mM glutamine
  5. 1.5 mg/ml sodium bicarbonate
  6. 10 mM Hepes
  7. Non-essential amino acids
- 293T cells (*human embryonic kidney*) were cultured in Dulbecco modified Eagle’s medium supplemented with:
  1. 10% FCS
  2. 100 IU/ml penicillin
  3. 100 mg/ml streptomycin
  4. 2mM glutamine
  5. 1mM sodium pyruvate
  6. Non-essential amino acids
- For “virus” titrations, **MDCK cells were inoculated with tenfold serial dilutions of “virus” preparations, homogenized tissues, nose swabs, and throat swabs**
- Cells were washed with PBS one hour after inoculation and **cultured in 200µl of infection media**, consisting of EMEM supplemented with:
  1. 100 U/ml penicillin

2. 100 µg/ml streptomycin
  3. 2mM glutamine
  4. 1.5mg/ml sodium bicarbonate
  5. 10mM Hepes
  6. Non-essential amino acids
  7. 20 µg/ml trypsin
- Three days after inoculation, supernatants of infected cell cultures were tested for agglutinating activity **using turkey erythrocytes as an indicator of “virus” replication in the cells**
  - Infectious “virus” titers **were calculated** from four replicates each of the homogenized tissue samples, nose swabs, and throat swabs and for ten replicates of the “virus” preparations by the method of Spearman-Kärber

The only way that the gain of function/bioweapon narrative makes any sense is if the original Latin definition for the word “virus” is used to explain what is happening in this research. In Latin, “virus” means “liquid poison” and what virologists are doing is simply creating a liquid poison in a lab using cell cultures. What they are not doing is creating “infectious agents of a small size and simple [composition](#) that can multiply only in living cells of [animals](#), [plants](#), or [bacteria](#)” which is the modern definition for the word according to the Britannica. The only way the liquid poison can potentially harm one is through injection. Cell cultured soup is not transmitted through the air nor is it infectious and/or contagious. In other words, GOF studies are not creating “viruses” in the modern sense of the word and can only be considered as such if viewed through the original Latin lens.

What must be realized about the GOF studies and the bioweapon narrative is that these stories are designed to keep people believing in the lies of Germ Theory. This is yet another fear-based tactic utilized by those in power to ensure that the masses are frightened of an invisible enemy that can be

unleashed upon the world either accidentally or intentionally at a moments notice. There will be figureheads who appear to be on the side of truth, questioning the natural existence of “SARS-COV-2,” challenging the safety of the vaccines, promoting alternative therapies, etc. who will also continue to push the idea that “viruses” exist and can be manipulated in a lab. These people are the Pied Pipers leading those who are going astray back into the fold. There is no need to create a “virus” bioweapon when all that was needed to control the masses is a PCR test and some well-designed propaganda.

To anyone who may have been taken in by this GOF/Bioweapon narrative, remember that there is no evidence of any purified and isolated “viral” particles ever coming directly from human samples that are then proven pathogenic in a natural way. Virology does not dispute this. If they can not find a “virus” in nature, they can not create one in a lab. That is truly all you need to know.

**[Connect with Mike Stone at Viroliegy](#)**

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