Polizisten ins Krankenbett?

Die Polizisten schützen uns - jetzt schützen wir unsere Polizisten

Vorwort:

Polizisten sind Angestellte des Volkes. Als Gefahrenabwendungs- und Strafverfolgungsbehörde soll die Polizei das Volk vor Schaden bewahren. Dies bedingt ein gegenseitiges Vertrauensverhältnis. Die einzigen, die dieses Vertrauen stören, sind Staatsdiener und Politiker in anderen Behörden/Institutionen, die die Bevölkerung belügen.

In einem guten Unternehmen kümmern sich Arbeitgeber und Angestellte umeinander und stellen sich aufeinander ein. So wie unsere Polizisten uns vor den alltäglichen und nicht alltäglichen Gefahren schützen, wollen wir als Bürger unsere Polizisten vor Schaden durch lügende Behörden schützen.

Bürger und Polizei kommen aus denselben Reihen und gehören zusammen. Wir stehen eindeutig auf derselben Seite und dürfen uns nicht durch kriminelle Energien aus anderen Behörden auseinanderbringen lassen.

Wir brauchen unsere Polizisten!
Wir wollen unsere Polizisten gesund!

Auch und gerade die Polizisten müssen vor Korruption in Behörden geschützt werden

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Pandemie-Impfung: [Liebe Polizisten, vergiftet euch nicht!]

Alle diskutieren darüber, ob der Nutzen der Impfung das Risiko überwiegt. Diese Fragestellung missachtet die Tatsache, dass die Impfung biologisch gar keinen Nutzen haben, sondern nur Schaden anrichten kann.
Die Frage kann also nur lauten:

Wie groß wird der Schaden sein, den die Impfung anrichtet?

- Er wird aufgrund der NANO-Partikel im Impfstoff enorm sein. Die Chance auf Langzeitschäden und Tod ist gegeben.
- Über die möglichen schweren und schwersten Impfschäden wird vor der Impfung entgegen der gesetzlichen Verpflichtung nicht aufgeklärt; sie werden verschwiegen, wodurch die Einwilligung zur Impfung erschlichen und die Impfung selbst rechtlich eine gefährliche Körperverletzung (§ 224 StGB) ist.
- Die Behörden wollen u.a. Polizisten zuerst impfen.
- Dies wird nicht die Aufrechterhaltung der öffentlichen Ordnung, sondern ihre Zerstörung durch zu großen körperlichen Ausfall der Polizeikräfte bewirken.

JEDER nimmt körperlichen Schaden.
Für einige wird der Schaden lebenslang oder gar der vorzeitige Tod sein.

Wird die öffentliche Ordnung zusammengebrochen, ist die gesamte Bevölkerung in großer Gefahr.

Die Impfung kann aufgrund ihrer Wirkung auf den lebenden Organismus nur den Nutzen haben, die öffentliche Ordnung zum Zusammenbruch zu bringen.

So, wie es in den Pandemieplänen „vorhergesagt“ wird

Das Virus zur Pandemie

Leider muss ich Ihnen mitteilen, dass der öffentliche Gesundheitsdienst einschließlich der Gesundheitsämter und des Landesamtes für eine Beweisführung für oder gegen die Existenz von Viren nicht zuständig ist.
Leider kann ich Ihnen auch keine zuständige Behörde nennen.

(Dr. Dr. Heinz Rinder,
Leiter des Sachgebiets Infektiologie,
Bayerisches Landesamt für Gesundheit und
Lebensmittelsicherheit, 16.03.2005)

Eine umfassende Erstbeschreibung des Neuen Influenza-Erregers werden Sie allerdings nicht vorfinden. Es handelt sich ja lediglich um einen neuen Stamm des Influenza-Erregers. Das humanpathogene Influenza-Virus als solches wurde schon 1933 durch Andrewes, Smith und Laidlaw vom National Institute for Medical Research, London, isoliert und beschrieben. Es war seither Gegenstand unzähliger Forschungsaktivitäten und entsprechender Veröffentlichungen. Aktuelle Forschungsaktivitäten in Bezug auf den neuen Erreger sind vor allem auf dessen Besonderheiten gerichtet.

(Judith Petschelt, Ref. Presse- und
Öffentlichkeitsarbeit des RKI)
Edit 2022: Siehe Anlage

Der Grund, weshalb die Impfung keinen biologischen Nutzen haben kann, ist:

Das Virus, gegen das angeblich geimpft wird, existiert nicht.

Die Regierung und die Gesundheitsbehörden behaupten die Existenz von Influenza-Viren in dem nachgewiesenen Wissen, dass sie keinen wissenschaftlichen Beweis für die Existenz der Viren haben.

Sie handeln gegen das Gesetz, die Bevölkerung - und damit auch die Polizisten - wahrheitsgemäß informieren und vor Wissenschaftsbetrug und Körperverletzung schützen zu müssen.

Die Nachweisverfahren der Viren im Labor sind nicht geeicht, was auch in jedem Testbeipackzettel steht, da die Viren nie isoliert und nachgewiesen wurden. Alle sog. Virustests sind so angelegt, dass sie beliebig positiv oder negativ gedeutet werden können.

Regierung und Behörden berufen sich auf einen internationalen Glauben, bezeichnen diesen als Konsens anerkannter Wissenschaftler, der dogmatisch als unanfechtbar gilt, obwohl das Gesetz wissenschaftlich publizierte Nachweise der Viren fordert.
Das Infektionsschutzgesetz fordert das IST, die tatsächliche Existenz eines Erregers, nicht den Glauben daran.

Hier handeln Regierung und Behörden eindeutig zum Schaden der Bevölkerung, und kämpfen nicht gegen Viren, sondern gegen die eigene Bevölkerung incl. der Polizisten.

Die Wirklichkeit: [Was wirklich auf den Körper einwirkt]

Die Symptome sind echt, die echten Ursachen werden aber hinter unbewiesenen Virusdefinitionen und Infektionsdefinitionen versteckt. Fast alle glauben, mit der Infektions- und Virenhypothese die Antwort schon gefunden zu haben, so dass tatsächlich existierende Faktoren unberücksichtigt bleiben:

1. Der zunehmende Mangel ab Herbst an Wärme und Luftfeuchte führen zu Umbau- und Aufbauvorgängen in den Atemorganen, Nervensystem und Muskulatur, um den Körper auf die neuen Umweltbedingungen einzustellen.

Um- und Aufbauvorgänge gehen im Körper IMMER mit sehr leichten bis sehr schweren Symptomen wie Husten (Atemorgane), Kopf- und Gliederschmerzen (Nervensystem und Muskulatur) und oft mit Fieber einher.

2. Der zunehmende Mangel ab Herbst an Licht kann zu Mangel an Hormonen und Vitaminen führen, konkret z.B. Vitamin D, was zu Knochen(haut)schmerzen bei Knochenaufbau und generell zu einer erhöhten Sterblichkeit im Herbst führt.

3. Luftdruckabfall (Hoch/Tiefdruckgebiete) führt zur Freisetzung von Gasen im Blut und Gewebe, wie bei zu schnellem Auftauchen, was zu Schmerzen, Benommenheit und üblichen Kränkeleien führt.
Deswegen gibt es viele wetterfühlige Menschen, die besonders an Wund- und Bruchstellen bei Luftdruckabfall (=Wetterumschwung) Schmerzen empfinden.

Diese Vorgänge erschweren ALLE anderen Krankheiten und führen IMMER zu einer erhöhten Sterblichkeit (besonders nach großen Anstrengungen, Kriegen etc.) und werden wider besseres Wissen als viral verursacht behauptet.

Erstellt für die klein-klein-Bewegung durch
Christoph Hannemann, Dr. Stefan Lanka, Karl Krafeld
ViSdPR: Karl Krafeld, Albrechtstraße 17, 44137 Dortmund

Edit 21.08.2022: Auch 2022 verweist das RKI noch auf die unzureichenden britischen Tierversuche von 1933 und hat keine umfassende Erstbeschreibung des Influenza-Virus oder seiner angeblichen neuen Stämme veröffentlicht mit Dokumentation der Arbeitsschritte (Isolation mit Trennung der viralen Struktur von allen Zellbestandteilen, Aufreinigung, biochemische Charakterisierung, Negativkontrollen usw.) und behauptet Grippe-Symptome als viral verursacht.

Die Tierärztin Dr. med. vet. Barbara Kahler kommentiert in einem Video vom 30.07.2022 den Schriftwechsel zwischen dem RKI und impfen-nein-danke und die unzureichende britische Tierstudie von 1933, die das Influenzavirus indirekt nachweisen soll:
https://odysee.com/@impfen-nein-danke:b/Kahler_RKI_kein_Influenzavirus:d

Der Begriff „Isolation“ wird irreführend verwendet und meint gerade nicht die Trennung von allen fremden Bestandteilen.

Die britische Studie (keine des RKI) „A Virus Obtained from Influenza Patients“ von Andrewes, Smith und Laidlaw (1933), enthält keinen „Materials & Methods“-Abschnitt, keine Dokumentation von Kontrollen und Studiendesign, und kann darum nicht als wissenschaftlich bezeichnet werden.

Damit sich die Leser selbst ein Bild machen können, haben wir die Studie, auf die sich das RKI auch 2022 noch beruft, verlinkt und anliegend eingefügt.

Auf schriftliche Anfragen der Aufklärerin Christine Massey bei bisher 180 Institutionen (und weiterlaufend) in 30 Ländern konnte keine Gesundheitsbehörde bisher ein Virusisolat zu irgendeinem Virus vorzeigen:

https://www.fluoridefreepeel.ca/fois-reveal-that-health-science-institutions-around-the-world-have-no-record-of-sars-cov-2-isolation-purification/

https://odysee.com/@impfen-nein-danke:b/mike_adams_christine_massey_keine_virusnachweise:c

Indirekt hat das RKI damit zugegeben, auch 2022 noch keinen hauseigenen wissenschaftlichen Nachweis nach dem Goldstandard für das Influenza-Virus oder Schweinegrippe-Virus zu haben - entgegen den Bestimmungen des IfSG, siehe:

https://odysee.com/@impfen-nein-danke:b/barbara_Kahler_ifsg_verletzt:7

und
https://impfen-nein-danke.de/verfassungswidrig/

Wir danken dem RKI, uns darauf aufmerksam gemacht zu haben und somit das alte Flugblatt auf den neuesten Stand der impfkritischen Forschung bringen zu können.

Sie haben ihr Vollzitat, und wir haben eine aktuelle Bestätigung, daß sich am Viruswahn noch nichts geändert hat! Eine Win-Win-Situation also.

Das Flugblatt ist hier zu finden:
https://impfen-nein-danke.de/downloads5/

Weitere klein-klein-Flugblätter und -Infos:
https://impfen-nein-danke.de/downloads3/
https://impfen-nein-danke.de/downloads4/

Die im Flugblatt genannten Adressen und Webseiten sind veraltet. Die aktuelle Webseite ist: www.wissenschafftplus.de.

Anlagen

1. Abmahnbrief des RKI vom 17.03.2022

2. Britische Studie „A Virus Obtained from Influenza Patients“ von Andrewes, Smith und Laidlaw von 1933.
Quelle: https://www.sciencedirect.com/science/article/pii/S0140673600785412

3. Antwort von impfen-nein-danke vom 13.04.2022

4. Brief von Dr. Heinz Rinder, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Leiter des Sachgebiets Infektiologie, an einen ratsuchenden Bürger vom 16.03.2006.
Quelle: https://impfen-nein-danke.de/wissenschaftsbetrug/2/

5. Brief von Prof. Dr. V. Hingst, Präsident des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit, vom 02.08.2006.
Quelle: https://impfen-nein-danke.de/wissenschaftsbetrug/2/

6. klein-klein-newsletter „Regierung belügt Bundestag über Existenzbeweis für Schweinegrippe-Virus“ vom 13.01.2011 (V. 1.1 Aug. 2022).
Quelle: https://impfen-nein-danke.de/downloads3/

7. Dr. Stefan Lanka: Neues Influenza-Supervirus? – WissenschafftPlus-Newsletter vom 25.01.2012. Quelle: https://impfen-nein-danke.de/downloads3/

ROBERT KOCH INSTITUT

Leitungsstab
Grundsatz und Recht

Robert Koch-Institut | Nordufer 20 | 13353 Berlin

An das
Netzwerk Impfentscheid
Herrn Geschäftsführer Daniel Trappitsch
Wetti 41
CH-9470 Buchs SG

Influenza / PDF-Dokument unter https://impfen-nein-danke.de

17.03.2022

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Sehr geehrte Damen und Herren, sehr geehrter Herr Trappitsch,

uns ist kürzlich ein Dokument auf der von Ihnen betriebenen, o. g. Internetseite aufgefallen, in dem unsere Mitarbeiterin Frau Judith Petschelt (Referat Presse- und Öffentlichkeitsarbeit) zitiert wird. Das Zitat, zu finden über die besagte Webseite in einem PDF-Dokument unter https://impfen-nein-danke.de/u/2009-11-07-polizisten-ins-krankenbett.pdf, stammt bereits aus dem Jahr 2009 und ist ganz erheblich aus dem Zusammenhang gerissen:

Die Autoren des Flugblattes zitieren offenbar aus einer Antwort des Robert Koch-Instituts auf Anfragen von Personen, die anscheinend die Existenz des Influenza-Erregers A(H1N1)pdm09 bezweifeln: *„Eine umfassende Erstbeschreibung des Neuen Influenza-Erregers [...] werden Sie allerdings nicht vorfinden"*. Dieser Satz ist aus dem Zusammenhang gerissen, sodass die dort vermittelten, fachlichen Inhalte verzerrt dargestellt werden. Der vollständige Absatz in der genannten Antwort von Frau Petschelt lautete: *„Eine umfassende Erstbeschreibung des Neuen Influenza-Erregers werden Sie allerdings nicht vorfinden. Es handelt sich ja lediglich um einen neuen Stamm des Influenza-Erregers. Das humanpathogene Influenza-Virus als solches wurde schon 1933 durch Andrewes, Smith und Laidlaw vom National Institute for Medical Research, London isoliert und beschrieben. Es war seither Gegenstand unzähliger Forschungsaktivitäten und entsprechender Veröffentlichungen. Aktuelle Forschungsaktivitäten in Bezug auf den neuen Erreger sind vor allem auf dessen Besonderheiten gerichtet."*

Die Autoren des Dokumentes unter https://impfen-nein-danke.de/u/2009-11-07-polizisten-ins-krankenbett.pdf behaupten, ein wissenschaftlicher Nachweis eines Grippevirus sei 1933 noch nicht möglich gewesen. Wie Sie der Veröffentlichung von 1933 im Lancet entnehmen können (siehe Anlage), haben Smith et al. eine vor allem klinisch orientierte Beweisführung zum Nachweis des Grippeerregers

geführt. Dazu gehörte die Übertragung des Krankheitserregers von kranken auf gesunde Tiere, wodurch letztere die gleichen Krankheitssymptome entwickelten, sowie verschiedene diagnostische Methoden wie Hämagglutination und Neutralisation durch Rekonvaleszenten- und andere Immunseren. Dadurch erfolgte zwar kein direkter, aber ein reproduzierbarer klinischer und biochemischer Nachweis des Influenzavirus. Die ersten elektronenmikroskopischen Aufnahmen von Influenzaviren erfolgten unseres Wissens 1943 durch Taylor et al.

Den in dem genannten Dokument unter https://impfen-nein-danke.de/u/2009-11-07-polizisten-ins-krankenbett.pdf erhobenen Vorwurf, das Robert Koch-Institut habe gelogen, können wir daher nicht nachvollziehen; diesen Vorwurf weisen wir hiermit entschieden zurück.

Vor diesem Hintergrund verlangen wir die Löschung des Zitates von Frau Petschelt: *„Eine umfassende Erstbeschreibung des Neuen Influenza-Erregers [...] werden Sie allerdings nicht vorfinden"* aus dem in Rede stehenden Dokument, ebenso die Löschung der beiden darunter stehenden „erläuternden" Sätze der Verfasser des Dokuments; hilfsweise eine Richtigstellung des aus dem Zusammenhang gerissenen Zitats durch Zitierung des vollständigen Absatzes aus der Antwort von Frau Petschelt (siehe oben), in jedem Fall aber Löschung der beiden genannten Sätze, die in der Sache unzutreffend sind und in denen u. a. unsere Mitarbeiterin/bzw. das Robert Koch-Institut fälschlicherweise der Lüge bezichtigt wird.

Für die entsprechende Überarbeitung des Dokuments sowie die Veröffentlichung des insofern korrigierten „Flugblattes" auf Ihrer Internetseite haben wir uns eine Frist bis zum

15.04.2022

notiert. Sollte bis dahin keine Anpassung wie von uns gewünscht erfolgt sein, werden wir die weiteren rechtlichen Schritte prüfen und einleiten.

Mit freundlichen Grüßen

im Auftrag

Dr. Mehlitz

Anlage: Veröffentlichung Smith et al., Lancet 1933

An adequate and well-balanced diet forms an essential part in the treatment of all anæmias—i.e., a diet containing fresh red meat, green vegetables, fresh fruit, milk, and butter.

Transfusion

Hæmopoietic substances are often given to other anæmias than those dependent upon a deficiency of such factors. There is no evidence that they are of any value, and they are often only an unjustified expense. Such anæmias may be benefited by blood transfusions. Transfused blood may apparently act in two ways. It may supply sufficient blood to enable the patient to carry on for a week or fortnight but then has to be repeated as in aplastic anæmia. Transfusion also appears in some instances to stimulate hæmopoiesis. At present it is impossible to predict when this will occur. Provided adequate steps are taken to transfuse compatible blood there are, I believe, no contra-indications to transfusion. I have seen many patients benefited, and only one instance in which transfusion could possibly be said to have shortened life. It is always worthy of trial in anæmias which fail to respond to other treatment. Direct matching of the blood is essential, especially in patients who have been frequently transfused.

Splenectomy

The value of splenectomy in anæmia is as controversial as that of transfusion. It is of undoubted value in acholuric jaundice, and gives at least temporary relief in idiopathic purpura. Its value in early cases of splenic anæmia is less certain. In a certain proportion of patients it appears to cause amelioration of symptoms. Splenectomy should not be attempted until iron therapy has failed. Idiopathic microcytic anæmia may simulate splenic anæmia. The one responds to iron the other does not. This affords an excellent example of the value of carefully controlled treatment in the diagnosis of anæmias.

A VIRUS OBTAINED FROM INFLUENZA PATIENTS

BY WILSON SMITH, M.D. MANCH.
C. H. ANDREWES, M.D. LOND.
AND
P. P. LAIDLAW, B.CHIR. CAMB., F.R.S.

(From the National Institute for Medical Research, Farm Laboratories, Mill Hill)

THE epidemic of influenza at the beginning of 1933 afforded an opportunity of making an experimental study of this disease, the results of which are here embodied in a preliminary communication. Throat-washings were obtained from a number of patients as early as possible after the onset of definite symptoms. On the assumption that the ætiological agent of influenza was probably a filtrable virus the throat-washings were filtered before use through a membrane impermeable to bacteria. The filtrates, proved to be bacteriologically sterile, were used in attempts to infect many different species. All such attempts were entirely unsuccessful until the ferret was used and the first success was only secured towards the close of the epidemic.

The initial successful experiment was made with two ferrets, both of which received a filtrate of human throat-washings, both subcutaneously and by intranasal instillation. Both animals became obviously ill on the third day after infection and exhibited symptoms of the characteristic disease which is described below. It was found that the disease could be transmitted either by contact or by direct transference of nasal washings from a sick to a healthy ferret. At this point therefore the work was transferred to the Institute's farm laboratories at Mill Hill, where it could be carried out under the conditions of rigid isolation of individual experimental animals evolved and used by Dunkin and Laidlaw[1] in their work on dog distemper * (1926).

The Disease in Ferrets

The ferret disease is characterised by a two-day incubation period, a diphasic temperature response, symptoms of nasal catarrh and variable systemic disturbances. In the infected animal the temperature† rises abruptly about 48 hours after infection, often exceeding 105° F. or even 106° F. It subsides on the third or fourth day only to rise again on the fourth or fifth day. In the course of the next day or two the temperature gradually returns to normal, and in most cases remains thereafter within normal limits.

Coincidently with the primary rise of temperature the ferret looks ill, is quiet and lethargic, often refuses food, and may show signs of muscular weakness. The catarrhal symptoms usually begin on the third day. The eyes become watery and there is a variable amount of watery discharge from the nose. This nasal discharge at times becomes sticky and may be mucopurulent, thus causing matting of the fur along the edges and at the corners of the nostrils. The animal sneezes frequently, yawns repeatedly, and in many cases breathes partly through the mouth with wheezy or stertorous sounds which clearly indicate a considerable degree of nasal obstruction. Such obstruction rarely accompanies a copious nasal discharge. The tip of the nose is often very pale. The signs of illness may last for only a few days but sometimes continue for ten days, after which the ferret again becomes perfectly normal. There is considerable variation as regards both the temperature response and the intensity and time of appearance of the local symptoms. In a few ferrets a typical diphasic temperature response has occurred without any nasal symptoms, and in one case well-marked symptoms were noted without any elevation of temperature. These animals when tested later were found to be immune. Very occasionally a ferret, a short time after recovery, has had a relapse in which the temperature curve and the symptoms have been similar to those of the primary illness. The disease has never been fatal in the 64 cases observed throughout the full course of the illness. Fig. 1 illustrates the temperature response of a ferret which had a typical attack of the disease, with a relapse.

In ferrets killed during the first and second febrile periods the mucous membrane of the nasal passages shows acute inflammation. Sections across the turbinate bones show, in the soft parts, acute vascular congestion, dilated lymph channels, numerous leucocytes passing out through the epithelium, and serious

* It is essential, when employing ferrets as experimental animals, that all purchased animals be quarantined for 14 days before being brought into use. If this precaution be omitted it is probable that latent distemper infection will, sooner or later, give rise to serious confusion. There is another acute infectious disorder, the ætiology of which is still obscure, which may also give untold worry to the research worker. Neck abscesses may develop in older animals and examination for these is essential if error is to be avoided.

† The normal temperature of a ferret is somewhat variable but it is unusual in a quiet animal to record a reading of over 103·5° F.

derangement of this structure. There is almost invariably complete disappearance of ciliated cells, and occasionally patchy necrosis of the whole thickness of the epithelium may be observed. No histological feature, such as an inclusion body, has as yet been discovered which can be called characteristic of the disease.

Passage of the Virus

The disease has frequently been transmitted by placing a normal ferret in the same cage as a sick one for 24 hours. The majority of virus passages, however, have been made by the following technique.

The infected animal is killed when showing symptoms, often at the beginning of the second temperature rise. The turbinates are scraped out, ground up with sand, and emulsified in about 20 c.cm. of equal parts of broth and saline. The emulsion is lightly centrifuged, and about 1 c.cm. of the supernatant fluid is dropped into the nostrils of another ferret. In this way 26 serial passages of one strain of virus have been made, and every animal of the series has shown the typical temperature response together with definite symptoms of the disease. A hundredfold dilution of the usual preparation has also been found to be regularly infective.

The method we are forced to employ has the serious disadvantage that it is impossible to make accurate quantitative experiments. The concentration of virus in the emulsion is unknown and it is impossible to determine what proportion of the amount instilled into the nose is retained, but no other route of inoculation has yet proved successful and other tissues tested—e.g., spleen, lymph glands, and blood—have been uniformly non-infective.

Throat-washings from eight human cases diagnosed as influenza have been inoculated into ferrets; five of these produced the ferret disease described above, although four were tested before the importance of utilising temperature records was recognised. From one of these cases throat-washings on the first and second day, and nasal discharge on the third day, were infective for ferrets, but on the sixth day no virus was recoverable from the nasal discharges, and on this day there was considerable improvement in the patient's catarrhal symptoms. A filtrate prepared from an emulsion of lung tissue from a fatal case of influenzal pneumonia likewise produced the ferret disease.

Throat-washings from four human subjects not suffering from influenza were non-infective. Of these, two were taken from men who had recovered from influenza and who at the time of their illness had supplied washings which were the genesis of the transmissible strains of virus with which we have done most work.

The nasal secretions of a man who was suffering from a severe common cold were also non-infective.

Filtrability of the Virus

Most of the human throat-washings were filtered before use through membranes having an average pore size of 0·6 μ. The membranes were Gradocol membranes made and supplied by Mr. W. J. Elford, Ph.D.[2] (1931), to whom we are greatly indebted. The filtrability of the virus after ferret passage was tested repeatedly. Invariably filtrates of an emulsion of the nasal mucosa from a sick ferret through membranes having an average pore size of 0·6 μ were found to produce the typical disease. A tight membrane (a.p.s. 0·25 μ) was used on one occasion only; the resultant filtrate was infective. It is probable therefore that the virus of ferret influenza is no larger than the viruses of vaccinia or herpes febrilis.

The infectivity of the filtrates, coupled with the fact that we failed to grow anything from the filtrate on a variety of media under aerobic or anaerobic conditions, has convinced us that we are dealing with a true virus disease. We have examined a number of bacteria from ferrets and human beings and so far we have failed to discover any micro-organism which will mimic the disease when cultures are instilled into a ferret's nose. *Hæmophilus influenzæ*, *Hæmophilus canis*, and *Hæmophilus influenzæ* (*suis*) administered along with virus produced at most only minor variations in symptoms.

Active Immunity

Ferrets which have recovered from the disease are invariably found to be immune to subsequent infection with the same strain of virus. This holds true whether the immunity test is done a few days after the disappearance of symptoms or five or six weeks later. One of two ferrets tested after the lapse of three months proved to be solidly immune and the other had a very mild attack of the disease with prompt recovery. At the present time it remains uncertain whether viruses from different human sources cross-immunise completely. No means of securing an active immunity apart from giving the disease itself have yet been found.

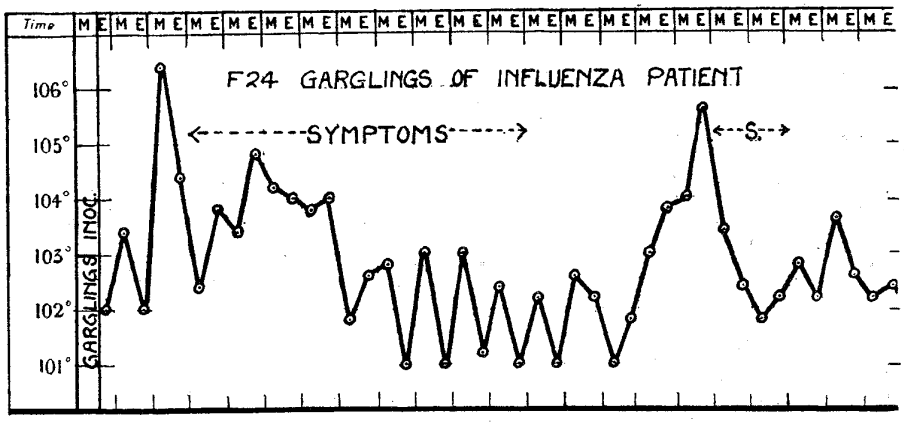

FIG. 1.—Temperature chart of ferret infected with garglings from a patient. The relapse is very unusual.

Virus Neutralisation

The serum of a ferret which has recovered from the disease will neutralise strong emulsions of the virus, provided that the serum and virus are mixed together before being inoculated intranasally into the test animal. Normal ferret serum has no such power of virus neutralisation, even when dilute virus is used in the test. Fig. 2 illustrates a virus neutralisation experiment.

Many human sera are capable of neutralising dilute ferret virus. Sera obtained from ten patients after their recovery from influenza were all found to have neutralising antibodies, but their demonstration was not constant, for two of the sera when retested failed to protect the experimental animals. Such irregularity is probably due to our inability to measure the dosage of virus employed, and it is quite possible,

under the conditions of the only test at present available, that an overwhelming dose of virus is given in one case and not in another. Control ferrets inoculated with virus alone or with virus mixed with normal serum were always included in these neutralisation tests; they invariably developed the disease.

Three human sera from individuals with no history of a recent attack of influenza were also tested: one showed neutralising properties; the other two were inactive. Such a result would be expected in tests on a population shortly after an epidemic of influenza.

Relationship to Swine Influenza Virus

A disease of swine, which arose spontaneously at the time of an influenza epidemic in America, has been described by Shope [3] (1931, 1932). We are indebted to him for samples of the swine influenza virus, and also for cultures of *Hæmophilus influenzæ (suis)*, an organism which plays an important rôle in the serious and fatal cases of the swine disease. The virus when inoculated intranasally into ferrets gave rise to a disease with diphasic temperature response, and all the symptoms described above—in fact a disease indistinguishable from the ferret disease caused by virus of human origin. The swine influenza virus was also readily transmissible serially through ferrets. In striking contrast to swine influenza the ferret disease was not modified in character when cultures of *Hæmophilus influenzæ (suis)* were inoculated together with the virus.

Cross-immunity tests have shown that this swine influenza virus bears a close antigenic relationship to the virus strain of human origin which has been chiefly used in our work. Ferrets after recovery from disease caused by the swine virus proved to be solidly immune to the human strain of virus. Ferrets convalescent from the human virus disease were not completely immune to the pig strain of virus.

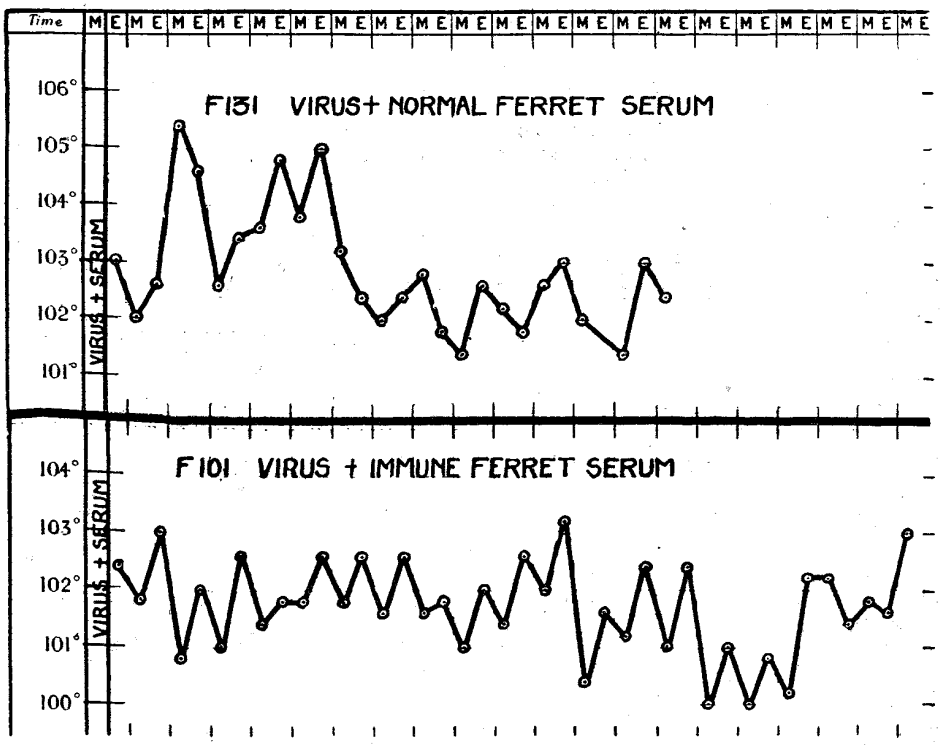

FIG. 2.—Upper temperature chart. Ferret received a mixture of virus with normal ferret serum. Lower temperature chart. Ferret received a mixture of virus with immune ferret serum.

Summary and Discussion

A disease of ferrets, produced by the intranasal instillation of filtrates of throat-washings obtained from influenza patients, is described.

The disease is transmissible serially in ferrets either by contact or by the intranasal instillation of virus-containing material.

The infective agent has, so far, only been recovered from the nasal passages of sick ferrets.

The disease was produced by five of the eight throat-washings obtained from influenza patients in the early stages of the disease.

Throat-washings from healthy persons and influenza convalescents caused no illness in ferrets.

The nasal secretions from a subject with a severe common cold caused no illness in ferrets.

Human sera, particularly those from influenza convalescents, were found to contain antibodies capable of neutralising the virus of the ferret disease.

Swine influenza virus caused a disease in ferrets which was indistinguishable from that produced by virus of human origin, and the pig and human viruses have close antigenic relationships.

We consider that the evidence given above strongly suggests that there is a virus element in epidemic influenza, and we believe that the virus is of great importance in the ætiology of the human disease. This view receives considerable indirect support from the fact that Shope found that the pig virus was the essential factor in swine influenza. The epizoötic disease could only be produced by combining two separate agents: (1) a virus; (2) *Hæmophilus influenzæ (suis)*. The virus *alone* produced a disease so mild that it was difficult to recognise, and the bacillus *alone* appeared to be harmless. Our results with ferrets, so far as they have gone, are consistent with the view that epidemic influenza in man is caused primarily by a virus infection. It is probable that in certain cases this infection facilitates the invasion of the body by visible bacteria giving rise to various complications. Analogous examples of this type of double infection are seen in swine influenza and dog distemper epizoötics. Decisive evidence on this point, and indeed on the importance of the virus we have described, can, we feel, only be secured by intensive study during an influenza epidemic, since direct experiments on man are fraught with difficulties. We are led to the publication of this preliminary note by the hope that our findings may be of assistance to those, wherever they may be situated, whose fate it may be to study the next epidemic of influenza.

We desire to thank the various practitioners through whose kindness we obtained throat-washings from influenza patients.

REFERENCES

1. Dunkin, G. W., and Laidlaw, P. P.: Jour. Comp. Path., 1926, xxxix., 201.
2. Elford, W. J.: Jour. Path. Bact., 1931, xxxiv., 505; Proc. Roy. Soc. B., 1933, cxii., 384.
3. Shope, R.: Jour. Exp. Med., 1931, liv., 349; Lewis, P. A., and Shope, R.: Ibid., p. 361; Shope, R.: Ibid., p. 373; same author: Ibid., 1932, lvi., 575.

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An das
Robert-Koch-Institut
- z. Hd. Frau Dr. Mehlitz, Leitungsstab -
Nordufer 20
13353 Berlin

- nur per Email: zentrale@rki.de -

Buchs, 13.04.2022

Betr.: Ihr Schreiben vom 17.03.2022

Sehr geehrte Frau Dr. Mehlitz,

wir danken Ihnen für Ihr Schreiben vom 17.03.2022, in dem Sie das von uns re-bloggte Flugblatt „Polizisten ins Krankenbett“ vom 07.11.2009 beanstanden, und für die Übersendung der Lancet-Studie von Smith et. al. von 1933.

Aus unserer Sicht sind Ihre Beanstandungen unzutreffend. Das Zitat von Frau Petschelt ist sinnwahrend gekürzt (es handelt sich ja um ein Flugblatt mit wenig Platz und nicht um einen Artikel), und die nachfolgende Paraphrase ist korrekt, denn auch im Jahr 2022 hat das RKI offenbar keine eigene umfassende Erstbeschreibung des angeblichen alten Influenza-Virus, noch eines seiner angeblich neuen Stämme in einzelnen Arbeitsschritten nachvollziehbar dokumentiert (Isolation mit Trennung der viralen Struktur von allen Zellbestandteilen, Aufreinigung, biochemische Charakterisierung, Negativkontrollen usw.).

Ihre Beanstandung: „Die Autoren des Dokumentes unter […] behaupten, ein wissenschaftlicher Nachweis eines Grippevirus sei 1933 noch nicht möglich gewesen“, ist ebenfalls unzutreffend, denn in der Paraphrase wird lediglich der technische Virusexistenznachweis verneint.

Ihre weitere Feststellung, dass die britische Studie (nicht eine des RKI) von Smith, Andrewes und Laidlaw von 1933 „eine vor allem klinisch orientierte Beweisführung“ sei, auf die Sie sich

zum Beweis des Influenza-Virus stützen, bestätigt ja gerade die Feststellung, dass das RKI keinen technischen Beweis hatte und offenbar immer noch nicht hat, sondern nur ein Tiermodell von vor 89 Jahren.

Diese Studie enthält keinen Beweis dafür, dass ein Influenza-Virus existiert, denn die Autoren haben vergebliche Ansteckungsversuche mit den armen Versuchstieren unternommen, bis es endlich bei den Frettchen geklappt haben soll („… attempts to infect many different species… entirely unsuccessful until the ferret was used…"), in vitro-Experimente sind fehlgeschlagen („we failed to grow anything from the filtrate on a variety of media under aerobic or anaerobic conditions…"), Kontrollen werden nur behauptet, nicht dokumentiert, und nicht-virale Kausalitäten ignoriert usw.

Sie schreiben weiter: „Dadurch erfolgte zwar kein direkter, aber ein reproduzierbarer klinischer und biochemischer Nachweis des Influenzavirus."

Es ist erfreulich, dass Sie den Begriff „Direktnachweis" noch in der alten, richtigen Bedeutung benutzen und nicht in der Umdefinition, mit der ein indirekter Nachweis als ein direkter behauptet wird. Aber auch die Behauptung eines indirekten Nachweises geht fehl, weil man indirekt nur etwas nachweisen kann, was es tatsächlich gibt.

Was es nicht gibt, wie offenkundig krankmachende Viren, kann man denklogisch weder direkt noch indirekt nachweisen. Sie begehen damit einen Zirkelschluss. Es ist die Logik wie beim Hexentest vor 500 Jahren während der Inquisition, wo man eine Frau gefesselt in den Fluss warf und ihr Ertrinken als indirekten Beweis („Gottesurteil") behauptete, dass sie eine Hexe sei, ohne vorher geklärt zu haben, ob es überhaupt Hexen gibt.

Die Reproduzierbarkeit ist gerade nicht gegeben, weil das Studiendesign nicht offengelegt ist und es keinen „Materials & Methods"-Abschnitt gibt. Und Korrelation bedeutet bekanntlich nicht Kausalität.

Die EM-Aufnahme von Taylor et. al. von 1943 zeigt keine viralen Strukturen, sondern nur herkömmliche Zellbestandteile. Keine Dichtegradientenzentrifugation, keine Dokumentation der Kontrollen, nur Behauptung „from normal embryos". Es ist auch keine EM-Aufnahme des RKI selbst.

Zu Ihrer Beanstandung des Lügen-Begriffs stellen wir fest, dass uns die diesbezügliche Korrespondenz nicht vorliegt, um feststellen zu können, ob dieses Werturteil zu Recht oder zu Unrecht erfolgt ist oder ob es sich lediglich um eine Selbsttäuschung der Virologen handelt.

Weil dies auch nicht der Hauptpunkt des Flugblattes ist, sondern der Fakt, dass das RKI keinen Virusbeweis hatte und auch im Jahr 2022 nicht hat und Grippesymptome, die herkömmlich erklärbar sind, als viral verursacht behauptet, kommen wir Ihnen dennoch gern entgegen und ändern, ohne Anerkennung einer Rechtspflicht, den beanstandeten Text und übernehmen dabei, bei Wahrunterstellung, Ihr vollständiges Zitat, präzisieren den

Kommentar und fügen die Studie hinzu, damit die Leserinnen und Leser sich selbst informieren können.

Wenn Sie zustimmen, fügen wir auch gerne unsere wechselseitige Korrespondenz hinzu, wie auch eine bestehende oder zukünftige umfassende Virus-Erstbeschreibung des Reinisolats mit allen dokumentierten Arbeitsschritten, wie es guter Wissenschaft entspricht.

Die Neufassung des Textabschnittes lautet:

> „Eine umfassende Erstbeschreibung des Neuen Influenza-Erregers werden Sie allerdings nicht vorfinden. Es handelt sich ja lediglich um einen neuen Stamm des Influenza-Erregers. Das humanpathogene Influenza-Virus als solches wurde schon 1933 durch Andrewes, Smith und Laidlaw vom National Institute for Medical Research, London, isoliert und beschrieben.
>
> Es war seither Gegenstand unzähliger Forschungsaktivitäten und entsprechender Veröffentlichungen. Aktuelle Forschungsaktivitäten in Bezug auf den neuen Erreger sind vor allem auf dessen Besonderheiten gerichtet.“
> (Judith Petschelt, Ref. Presse- und Öffentlichkeitsarbeit des RKI)
>
> Edit 2022: Auch 2022 verweist das RKI noch auf die unzureichenden britischen Tierversuche von 1933 und hat keine umfassende Erstbeschreibung des Influenza-Virus oder seiner angeblichen neuen Stämme veröffentlicht mit Dokumentation der Arbeitsschritte dokumentiert (Isolation mit Trennung der viralen Struktur von allen Zellbestandteilen, Aufreinigung, biochemische Charakterisierung, Negativkontrollen usw.) und behauptet Grippe-Symptome als viral verursacht.
>
> Der Begriff „Isolation“ wird irreführend verwendet und meint gerade nicht die Trennung von allen fremden Bestandteilen.“

Die Neufassung werden wir auf unserer Download-Seite 5 veröffentlichen:
https://impfen-nein-danke.de/downloads5/

Wir gehen davon aus, dass durch unsere Änderung und Präzisierung die Angelegenheit erledigt ist. Sollte dies nicht der Fall sein, bitten wir um weitere Nachricht, der Einfachheit halber auch gern per Email.

Die „Leitlinien zur Sicherung guter wissenschaftlicher Praxis“ der DFG, vom November 2021, Version 1.1 fordern Selbstzweifel und Diskurs:

„Zu den Prinzipien gehört es insbesondere, lege artis zu arbeiten, strikte Ehrlichkeit im Hinblick auf die eigenen und die Beiträge Dritter zu wahren, alle Ergebnisse konsequent selbst anzuzweifeln sowie einen kritischen Diskurs in der wissenschaftlichen Gemeinschaft zuzulassen und zu fördern.“

https://www.dfg.de/download/pdf/foerderung/rechtliche_rahmenbedingungen/gute_wissenschaftliche_praxis/kodex_gwp.pdf

Die Virusbeweisfrage ist auch 2022 noch aktuell, weil 178 Gesundheitsbehörden weltweit auf Bürgeranfragen keine belastbaren Beweise vorlegen können: https://www.fluoridefreepeel.ca/fois-reveal-that-health-science-institutions-around-the-world-have-no-record-of-sars-cov-2-isolation-purification/

Bei uns in der Schweiz läuft gerade ein Gerichtsverfahren, in denen die Behörden aufgefordert werden, Virusnachweise vorzulegen: https://impfen-nein-danke.de/u/2022-01-12-Klagebegehren-EFD-anonymisiert.pdf (Seiten 15-27).

Wir wären Ihnen auch dankbar, wenn Sie uns einen staatlich beschäftigten Virologen benennen könnten, der den mit 1,5 Millionen Euro dotierten Nachweis für das angebliche Corona-Virus vorlegen möchte: https://samueleckert.net/isolate-truth-fund/

Können Sie uns freundlicherweise mitteilen, wann das RKI endlich die im Max von Pettenkofer-Institut in München unter RKI-Beteiligung durchgeführten Kontrollexperimente zum angeblichen Masernvirus veröffentlicht?

Das RKI verweigert bisher ohne erkennbaren Grund eine wissenschaftliche und zivilgesellschaftlich-demokratische Kontrolle, obwohl dafür Steuermittel in Anspruch genommen worden sind, trotz mehrerer Anfragen von Bürgern oder Fachleuten, wie Corona_Fakten dokumentiert hat, und die Überprüfbarkeit massgeblich dazu beitragen könnte, die Frage zu klären, ob das Masernvirus existiert. https://telegra.ph/Das-RKI-best%C3%A4tigt-Wir-f%C3%BChrten-keine-Kontrollexperimente-durch-10-03

Bitte teilen Sie uns auch mit, wie es kommt, dass Frau Prof. Mankertz von einer „complete genome sequence" des angeblichen Masern-Wildvirus berichten kann, wenn niemand bisher eine vollständige Genomsequenzierung durchgeführt hat, und es nur kurze Sequenzen mit der Illumina-Methode gibt, und wie es möglich sein kann, dass in der Studie die vorgeschriebenen Kontrollexperimente nicht dokumentiert sind?

Wie ist die Umgehung der wissenschaftlichen Regeln noch 2014 zu erklären? https://edoc.rki.de/handle/176904/1876

Rechtsrelevante Verstösse können beim ZAAVV gemeldet werden und werden entsprechend aufgearbeitet: https://zaavv.com/de-de/

Behördenmitarbeiter, die ihrem Gewissen folgen und aussteigen wollen, können sich vertraulich an die Mutigmacher wenden: https://mutigmacher.org/

Mit freundlichen Grüssen

Daniel Trappitsch
Netzwerk Impfentscheid
- Geschäftsführer -

Dieses Schreiben wurde maschinell erstellt und ist ohne Unterschrift gültig.

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit
Veterinärstr. 2, 85764 Oberschleißheim

EINGEGANGEN MÄR. 2006

[redacted]

Ihre Nachricht	Unser Aktenzeichen	Ansprechpartner/E-Mail:	Durchwahl und Fax:	Datum
Vom 05.03.06		PD Dr. Dr. Heinz Rinder heinz.rinder@gl.bayern.de	089/31560-214 089/51560-130	16.03.2006

Beweise für die Existenz von Viren

Sehr geehrter [redacted]

vielen Dank für Ihre Anfrage vom 05.03.06 an das Gesundheitsamt Rosenheim, die uns mit der Bitte um Stellungnahme weitergeleitet wurde.

Leider muss ich Ihnen mitteilen, dass der öffentliche Gesundheitsdienst einschließlich der Gesundheitsämter und des Landesamtes für eine Beweisführung für oder gegen die Existenz von Viren nicht zuständig ist. Leider kann ich Ihnen auch keine zuständige Behörde nennen.

Mit freundlichen Grüßen

PD Dr. Dr. Heinz Rinder
Leiter des Sachgebiets Infektiologie

cc: GA Rosenheim

Dienstsitz:
LGL

Diese Dienststelle schreibt Ihnen:
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Präsident

Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit
Eggenreuther Weg 43, 91058 Erlangen

Ihre Nachricht	Unser Aktenzeichen	Ansprechpartner/E-Mail:	Durchwahl und Fax:	Datum
1.7.2006		Prof. V. Hingst volker.hingst@lgl.bayern.de	09131/764-100 09131/764-102	02.08.2006

4.8.06

Vogelgrippe– bzw. Geflügelpestvirus
Ihre Anfrage vom 01.07.2006

Sehr geehrter Herr

ich bestätige Ihnen den Eingang Ihres als Petition bezeichneten Schreibens am 05.07.2006.

Ihren Ausführungen entnehme ich, dass Ihnen der einschlägige Schriftverkehr zwischen mir und Herrn Birner, Deining vollständig bekannt ist. Ich darf Sie daher bitten, die darin aufgezeigten Möglichkeiten wahrzunehmen, über öffentlich zugängliche Bibliotheken (z.B. Stadtbibliothek Neumarkt, Weiherstr. 7, 92318 Neumarkt in der Oberpfalz per Fernleihe) die aufgezeigten Informationsträger zu beschaffen, falls Sie von Ihrem Internet-Anschluss keinen Gebrauch machen wollen.

Dies gilt beispielsweise für die nach Literaturangaben als erstmalige Beschreibung einzustufende Publikation der Autoren **W. Smith, C. Andrewes und P. Laidlaw: A virus obtained from influenza patients**, publiziert in der angesehenen Zeitschrift **Lancet, Band 225 (1933) Seite 66 – 68**. Falls Sie neben dieser Herrn Birner bereits mitgeteilten medizinhistorisch interessanten Arbeit auch an neueren Forschungsergebnissen interessiert sind, möchte ich Ihnen mit der beiliegenden Übersichtsarbeit den Zugang zu dieser Literatur erleichtern.

Die von Ihnen zitierte Aussage meines Mitarbeiters, Herrn Dr. Dr. Heinz Rinder wurde so mitgeteilt und ist selbstverständlich zutreffend. Die Aufgabenfelder des öffentlichen Gesundheitsdienstes wie auch des Landesamtes sind in gesetzlichem und rechtlichem Rahmen festgelegt. Die Erfüllung dieser Aufgaben vollzieht sich vor dem jeweiligen aktuellen Stand des einschlägigen Wissens unter möglichst umgehender Wahrnehmung neuester Erkenntnisse der Wissenschaft und ihrer Einrichtungen.

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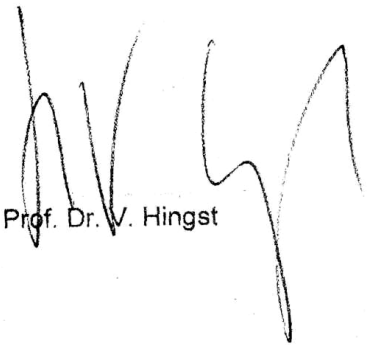
Bezogen auf die infektiologische Bedeutung von Influenzaviren für die Ausbildung von Erkrankungen bei Tieren und Menschen, für die vom Grundsatz her unter den einschlägigen wissenschaftlichen Einrichtungen und Organisationen ein weltweiter Konsens besteht, ergibt sich für uns für eine nochmalige Beweisführung in der Sache oder Infragestellung dieses Konsens kein Handlungsbedarf.

Mit freundlichen Grüßen



Prof. Dr. V. Hingst

P.S.: Nach dem einleitenden Satz Ihres obigen Schreibens hätte ich mit Ihnen schon korrespondiert. Dies ist mir ebenso wenig erinnerlich wie „Anfragen ohne jede Antwort".

newsletter klein-klein-verlag vom 13.01.2011

Regierung belügt Bundestag über den Existenzbeweis für das Schweinegrippe-Virus

Schweinegrippe: Lüge der Bundesregierung über existierende Beweise der biologischen Virusexistenz vor dem Deutschen Bundestag.

Erst jetzt wurde uns die dreiste Antwort der Bundesregierung (Bundesgesundheitsministerium, Staatssekretär Dr. Klaus Theo Schröder) vom 22. Juli 2009 auf die „Kleine Anfrage" des fraktionslosen Bundestagsabgeordneten Henry Nitsche bekannt.

Drucksache des Bundestages zur Schweinegrippe:

im Ergebnis finanziert hat. Der Ausgang dieses Verfahrens bleibt abzuwarten.

34. Abgeordneter **Henry Nitzsche** (fraktionslos)

Inwiefern ist der H1N1-Virus in einem direkten Nachweisverfahren (exakte Isolierung aus menschlichem Serum/Plasma, biochemische Charakterisierung und elektronenmikroskopische Aufnahme; sog. Direktnachweis) nachgewiesen worden, und ist nach Auffassung der Bundesregierung die Anordnung von Schutzimpfungen nach § 20 Absatz 6 und 7 des Infektionsschutzgesetzes (IfSG) auch ohne einen Direktnachweis des H1N1-Virus zulässig?

Antwort des Staatssekretärs Dr. Klaus Theo Schröder vom 22. Juli 2009

Das Virus Influenza A/H1N1 ist mit anerkannten wissenschaftlichen Methoden nachgewiesen worden; dies wurde in renommierten Fachzeitschriften publiziert (N Engl J Med. 2009 Jun 18; 360(25): 2605-15. Epub 2009 May 7).

Darüber hinaus wurde bei vielen Patientinnen und Patienten aus deren Probenmaterial das neue A/H1N1-Virus isoliert und angezüchtet, im Elektronenmikroskop nachgewiesen und mit verschiedenen Methoden (Sequenzanalyse, Untersuchungen mittels Immunseren) umfassend charakterisiert.

Schutzimpfungen sind in Deutschland freiwillig. Eine Impfpflicht besteht nicht. Auch die derzeitige Ausbreitung der Influenza A/H1N1 gibt keinen Anlass, dies zu ändern. Um einen Impfstoff entwickeln zu können, muss der Krankheitserreger bekannt sein. Der Nachweis des Krankheitserregers ist somit sachnotwendig vor einer auf § 20 Absatz 6 oder Absatz 7 des Infektionsschutzgesetzes gestützten Rechtsverordnung vorhanden.

[Transkription:

Drucksache 16/**13831** - 28 - Deutscher Bundestag - 16. Wahlperiode

34. Abgeordneter **Henry Nitzsche** (fraktionslos)

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In einem demokratischen Rechtsstaat hat das Volk die Regierung, die es verdient.

In einem demokratischen Rechtsstaat, wie ihn das geduldige Papier, auf dem das Grundgesetz und das Infektionsschutzgesetz gedruckt sind, von der BRD verlangt, dieser zu sein, ist die Regierung so schlecht, wie die Staatsbürger dulden, dass die Regierung schlecht sein darf.

Die Bundesregierung behauptet gegenüber dem Bundestag, dass das Schweinegrippevirus oft isoliert, elektronenmikroskopisch fotografiert und mit mehreren Methoden charakterisiert (beschrieben) wurde. Das Robert-Koch-Institut (RKI) gesteht aber zwei Monate später ein, dass eine solche Erstbeschreibung nicht existiert, nachdem ich das RKI nach einer Publikation fragte, in der die Isolation, das Foto und die Charakterisierung des Schweinegrippe-Virus zu finden ist.

Die Behauptung der Bundesregierung gegenüber dem Deutschen Bundestag vom

22.07.2009 ist unvereinbar mit der Aussage des RKI mit Datum vom 21.09.2009, die infolge o.g. Anfrage an den damaligen Vizepräsidenten des RKI und heutigen Präsidenten Prof. Burger erfolgte.

Diese zwei unvereinbaren Aussagen sind auf [dem anliegenden] Blatt zusammengefasst.

Dieses Blatt kann selbstverständlich weitergereicht werden, insbesondere als Instrument, zu dem Zwecke, dass wir in Deutschland eine Bundesregierung bekommen, wie wir sie brauchen und wollen, die im Gesundheitsbereich weder die Bevölkerung noch den Deutschen Bundestag dreist belügen darf.

Wer ein berechtigtes Interesse daran hat, dass wir eine Bundesregierung bekommen, wie wir sie wollen und brauchen, auch zur Erhaltung der natürlichen Lebensgrundlagen in Verantwortung für künftige Generationen, (Grundgesetz (GG) Art. 20 a), der kann dieses Instrument einsetzen und beim Bundestag, beim Bundesgesundheitsministerium, bei seinen Bundestagsabgeordneten, bei den Spitzen der Parteien usw. durch konkrete Fragen vor dem Hintergrund dieser Aussage der Bundesregierung gegenüber dem Deutschen Bundestag auf eine Klärung drängen.

Hierzu ein Formulierungsvorschlag:

Absender
Adresse

Ort, Datum

Lässt sich der Deutsche Bundestag durch das Bundegesundheitsministerium belügen?
Schweinegrippe (H1N1-Virus).
Sind die Voraussetzungen für die Impfstoffzulassungen erfüllt?

Sehr geehrte(r) … …

In der Anlage sende Ich Ihnen ein Blatt vom klein-klein-verlag vom 10.01.2011.

Die beiden Aussagen sind unvereinbar.

Das Robert-Koch-Institut (RKI) gesteht ein, dass man eine Erstbeschreibung des A/H1N1-Virus nicht vorfinden wird, und behauptet dann, dass das Influenzavirus aber schon 1933 isoliert und beschrieben wurde, also zu einer Zeit, als ein elektronenmikroskopischer Nachweis eines Virus und seine biochemische Charakterisierung technisch noch nicht möglich war. [1]

Die Bundesregierung (Staatssekretär Dr. Klaus Theo Schröder) behauptet aufgrund einer „Kleinen Anfrage“ eines Abgeordneten gegenüber dem Deutschen Bundestag, dass das neue A/H1N1 Virus isoliert und angezüchtet, im Elektronenmikroskop nachgewiesen und mit verschiedenen Methoden umfassend charakterisiert worden ist.

Wie das wissenschaftlich-technisch möglich sein soll, ohne dass eine umfassende Erstbeschreibung vorliegt, verschweigt die Bundesregierung gegenüber dem demokratisch legitimierten Deutschen Bundestag, dessen Aufgabe u.a. die Kontrolle der Bundesregierung ist, die u.a. durch Kleine Anfragen von einzelnen Abgeordneten wahrgenommen wird.

Die Bundesregierung behauptet gleichzeitig, dass Voraussetzung für die Entwicklung eines Impfstoffes ist, dass der Krankheitserreger bekannt ist und der Nachweis des Krankheitserregers sachnotwendig ist.

Fragen:

1. Wie kann der Existenzbeweis eines viralen Krankheitserregers erfolgt sein, wenn keine Erstbeschreibung (Direktnachweis) existiert? Ohne Erstbeschreibung kann der Krankheitserreger wissenschaftlich nicht bekannt sein.

2. Die Regierung setzt als logische und sachnotwendige Voraussetzung für eine Impfstoffentwicklung, dass der Krankheitserreger bekannt sein muss. Hat das für die Impfstoffzulassung in der BRD zuständige Paul-Ehrlich-Institut (PEI) in den vergangenen Jahrzehnten jemals einen Impfstoff als Schutz vor einer behaupteten viralen Erkrankung, z.B. Grippe, zugelassen, bei dem die von der Bundesregierung vor dem Deutschen Bundestag erhobene Forderung erfüllt war und ist?

Zufolge der unvereinbaren zwei Aussagen kann diese Voraussetzung zumindest bei einem Grippeimpfstoff nicht erfüllt sein, da es zufolge des RKI an einer Erstbeschreibung (Direktnachweis) mangelt.

Mit freundlichem Gruß

Unterschrift
Name

Das auf geduldigem Papier gedruckte Konzept des demokratischen Rechtsstaates ist keine Gabe irgendwelcher gottähnlichen Kräfte an den Staatsbürger, sondern eine Aufgabe für den Staatsbürger. Für wen denn sonst wohl?

Staatsbürger in einem formell beschriebenen demokratischen Rechtsstaat haben nicht die Regierung, die sie verdienen, sondern haben die Regierung, die sie sich selbst verdient haben.

Für die klein-klein-Bewegung

Ihr Christoph Hubert Hannemann, Karl Krafeld, Stefan Lanka

PS.

Die Weiterleitung an Staatsbürger in der BRD, die meinen, sie selbst, aber auch ihre Kinder

und Enkelkinder, hätten unter dem Gesichtspunkt der Erhaltung der natürlichen Lebensgrundlagen für künftige Generationen (GG Art. 20 a) nicht die Regierung verdient, die wir in der BRD haben, ist ausdrücklich erwünscht.

Bis heute verweigert die Bundesregierung der BRD, in der Verantwortung der Elite-Katholikin, Bundesforschungsministerin Schavan (Cusanerin), die Finanzierung der Erforschung der Wirkung der Impfstoffe, insbesondere zur Klärung der Frage, welche vererbbaren Schäden, die die natürlichen Lebensgrundlagen zerstören, durch die Impfstoffe erwirkt werden.

Bei der Weiterleitung als Rundmail bitte die anderen Adressen von privaten Empfängern aus Gründen des Daten- und Persönlichkeitsschutzes unkenntlich machen.

Fußnote
[1] Vgl. das Flugblatt "2009-11-07 Polizisten ins Krankenbett (Edit 2022)" unter Downloads 5, wo das RKI sich auch 2022 noch auf die unwissenschaftliche Tierstudie von 1933 beruft, die das Influenza-Virus und ebenso das Schweinegrippe-Virus beweisen soll.

Quelle
klein-klein-verlag (offline), s. Waybackmachine. Rekonstruiert, zuletzt August 2022, externe Texte integriert, Korrektur einiger Tippfehler, Fußnote und Transkription hinzugefügt.

Weitere ehemalige klein-klein-newsletter unter Downloads 3. Haben Sie noch unveröffentlichte Newsletter? Dann bitten wir um Zusendung.

Version 1.1 (Update August 2022)

Schweinegrippe-Virus-Existenz

Ist die Existenz des „Schweinegrippevirus" (A/H1N1) bewiesen?

zwei Anfragen an staatliche Stellen

Ja, ist sie! **www.klein-klein-verlag.de** Nein, ist sie nicht!

Henry Nitzsche
Abgeordneter (fraktionslos)

Christoph H. Hannemann
Staatsbürger der BRD

Anfrage des Bundestages

Inwiefern ist der H1N1-Virus in einem direkten Nachweisverfahren (exakte Isolierung aus menschlichem Serum/Plasma, biochemische Charakterisierung und elektronenmikroskopische Aufnahme; sog. Direktnachweis) nachgewiesen worden,

und ist nach Auffassung der Bundesregierung die Anordnung von Schutzimpfungen nach § 20 Absatz 6 und 7 des Infektionsschutzgesetzes (IfSG) auch ohne einen Direktnachweis des H1N1-Virus zulässig?

Anfrage ans Robert Koch-Institut

Bevor jemand darüber reden kann, was das Virus tut, wie es funktioniert, wie man es bekämpfen kann usw., muss das Virus ja erst einmal analysiert worden sein. Und zu dem Zweck der Analyse ist eine vorherige Isolation des Virus nötig.

Die Frage beim Bürgerforum zielte lediglich darauf ab, von Ihnen, Herr Prof. Burger, eine einzige Publikation genannt zu bekommen, in der der Direktnachweis (Isolation, elektronenmikroskopisches Foto, biochemische Untersuchung) des A/H1N1-Virus überprüf- und nachvollziehbar dokumentiert ist.

Antworten

22. Juli 2009
Dr. Klaus Theo Schröder
Staatssekretär

Darüber hinaus wurde [...] das neue A/H1N1-Virus isoliert und angezüchtet, im Elektronenmikroskop nachgewiesen und mit verschiedenen Methoden [...] umfassend charakterisiert.

Um einen Impfstoff entwickeln zu können, muss der Krankheitserreger bekannt sein.

Der Nachweis des Krankheitserregers ist somit sachnotwendig vor einer auf § 20 Absatz 6 oder Absatz 7 des Infektionsschutzgesetzes gestützten Rechtsverordnung vorhanden.

21. September 2009
Judith Petschelt
Robert Koch-Institut

Eine umfassende Erstbeschreibung des Neuen Influenza-Erregers, wie Sie sie in ihrem Schreiben skizzieren, werden Sie allerdings nicht vorfinden. Es handelt sich ja lediglich um einen neuen Stamm des Influenza-Erregers. Das Influenza-Virus als solches wurde schon 1933 isoliert und beschrieben.

Anmerkung: 1933 gab es noch keine Elektronenmikroskope. 1933 gab es auch noch keine ausreichend ausgereifte Biochemie, um es charakterisieren zu können. 1933 war ein Existenznachweis für ein Virus technisch noch nicht möglich. Ohne Erstbeschreibung kann der Krankheitserreger nicht bekannt sein.

Berlin, 10. Januar 2011, Christoph Hubert Hannemann, Hindenburgdamm 96a, 12203 Berlin

Ergänzender Newsletter zum Zwei-Monats-Magazin "WISSENSCHAFFTPLUS"

Neues Influenza-Supervirus?

Newsletter | **WISSEN**SCHAFFT**PLUS** | 25.01.2012

Sehr geehrte Damen und Herren!

In den Massenmedien wird behauptet, dass es Forschern gelungen sei, aus dem Vogelgrippe-Virus und dem Schweinegrippe-Virus ein neues, hochinfektiöses und tödliches Influenza-Virus zu züchten. Damit islamistische Terroristen dieses Virus nicht nachbauen und den Westen infizieren, hat die US-Amerikanische Regierung die Forscher angewiesen, ihre Ergebnisse nicht zu veröffentlichen.

Weil hier eine riesen Chance liegt, wenn die Wirklichkeit hinter diesen Behauptungen öffentlich wird, aber gleichzeitig auch eine riesen Gefahr liegt, wenn es den Betreibern gelingt, mit ihren Virus-Ideen eine reale Panik und massenhaftes Ersticken durch den Blutverdicker Tamiflu auszulösen, möchte ich Ihnen erklären, was exakt die Forscher tun, um ihre Aussagen mit Experimenten scheinbar zu belegen.

Ich demonstriere das anhand der aktuellsten Publikation zu diesem Thema (PLoS Pathog 7(12): e1002443), in der Dr. Seema S. Lakdawala und seine Kollegen vom US-Amerikanischen Institut für Allergien und Infektionskrankheiten in Bethesda behaupten, dass sie herausgefunden hätten, welche Gen-Ausstattung es sei, die H1N1 zum Pandemie-Virus macht.

Als Modell für den Menschen benutzen sie junge Frettchen, die, mit implantieren Meßelektroden versehen, in einer Unterdruckkabine festgeschraubt sind. Ihnen wird die Kehle aufgeschnitten und in die Luftröhre ein Schlauch eingebracht, durch den langsam Flüssigkeit in die Lunge tropft. Die Flüssigkeit entstammt aus Zellkulturen, die einmal mit Flüssigkeit von einem Tier in Kontakt gebracht wurden, von der behauptet wurde, dass sie mit H1N1 infiziert sei.

Tierversuche

Je nach Tropfgeschwindigkeit und Zusammensetzung der verwendeten Tropflösung entzünden sich Luftröhre und Lunge und sterben die Tiere mehr oder weniger schnell. Je nachdem, wie sich die Luftröhren und die Lungen entzünden, und welches Organ zuerst, und mit welchen weiteren Symptomen die Tiere sterben, werden unterschiedliche Viren-Typen behauptet.

Obwohl noch niemals ein Influenza-Virus in einem Menschen oder Tier fotografiert oder isoliert werden konnte, sondern die Viren nur als existent gelten, da viele Forscher jeweils eine indirekte Entdeckung als einen Bestandteil eines Virus behaupten und alle indirekten Behauptungen zusammen ein Modell eines Virus ergeben sollen, ist zentraler Beweis für die Existenz UND die Gefährlichkeit der Viren das Leiden und Sterben der Versuchstiere.

Es gibt weltweit nicht einen wissenschaftlichen Beweis, dass jemals ein Virus, wie das Influenzavirus, in einem Menschen oder Tier gesehen, geschweige denn isoliert und fotografiert

und untersucht wurde. Die Viren gelten lediglich als existent, weil viele Forscher ihre Laborarbeiten als indirekte Entdeckungen von einzelnen Bestandteilen eines Virus behaupten, ohne dass jemals ein komplettes Virus gesehen wurde, dem man die Bestandteile wissenschaftlich zuordnen könnte.

Viele Forscher tätigen viele solcher Behauptungen, und die Summe dieser Behauptungen soll dann in ihrer Gesamtheit das Modell des ganzen Virus ergeben. Obwohl noch niemals ein Influenza-Virus in einem Menschen oder Tier fotografiert oder isoliert werden konnte, sondern die Viren nur als existent gelten, gilt das Leiden und Sterben der Versuchstiere als der zentrale Beweis für die Existenz UND die Gefährlichkeit der Viren.

Kontroll-Experimente

Um Ergebnisse als „wissenschaftlich“ publizieren zu dürfen, fordern der Wissenschaftliche Kodex und die Bestimmungen der Fachmagazine, dass Kontroll-Experimente stattgefunden haben und dokumentiert werden müssen, die einen Irrtum ausschließen sollen. Solche Kontrollexperimente finden im gesamten Bereich der Infektionshypothesen nicht statt, was immer ein Hinweis auf Betrugstaten ist. In allen anderen Bereichen würden Publikationen nicht angenommen, wenn Kontrollexperimente nicht durchgeführt und veröffentlicht worden sind.

In einem solchen Kontroll-Experiment müssten die gleichen Flüssigkeiten verwendet werden, die aber als nicht infiziert gelten, um zu beweisen, dass die erzielten Effekte nichts mit dem Luftröhrenschnitt und der Tropfengabe in die Lunge zu tun haben. Ich kann versichern, dass die gleichen „Influenza“-Effekte ausgelöst werden, wenn destilliertes Wasser in die Lunge getropft wird. Sie können das ja mal an sich testen oder einen Forscher bitten, er möge den Gegenbeweis antreten.

Im Frettchen entzünden sich nun Luftröhre und Lunge. Das Tier versucht die Flüssigkeit auszuhusten, was es ihm aber ab einer gewissen Dauer des Eintropfens bzw. bei einer zu großen Menge an Flüssigkeit nicht mehr gelingt. Im Todeskrampf hustet das Tier besonders große Mengen an Flüssigkeit und Blut aus, von denen behauptet wird, dass sich darin die Viren in großer Zahl befinden. Das Husten selbst wird natürlich auch als ein durch das Virus ausgelöstes Symptom behauptet.

Anstatt die Viren in der ausgehusteten Flüssigkeit zu isolieren, fotografieren und biochemisch zu charakterisieren, werden aus dem ausgehusteten Schaum nur Eiweiße und deren RNA-Vorlagen entnommen, von denen – ohne jegliche Beweisführung – behauptet wird, dass sie den Viren entstammen würden und deswegen Viren anwesend seien. Für Presse-Fotos und in Filmen werden deswegen Schutzkleidung und Masken getragen, was im Labor, wenn die Forscher diese Versuche ohne Anwesenheit einer Fernsehkamera durchführen, nicht der Fall ist.

Zwei ganz normale Eiweiße

Als Bestandteile der Influenza-Viren werden zwei Eiweiße ausgegeben, die in jedem menschlichen und tierischen Organismus eine zentrale Rolle spielen. Das eine ist ein Enzym, die Neuraminidase, die durch Spaltung der Sialinsäure unsere Zellen mit elektrischer Ladung versorgt. Da sich die negativ geladenen Blutkörperchen untereinander abstoßen und nicht zusammenkleben, bleibt das Blut flüssig. Tamiflu hemmt spezifisch dieses Enzym, was zum Verdicken des Blutes und zum Ersticken führt.

Das andere Enzym, was wider besseres Wissen als Bestandteil eines Influenza-Virus ausgegeben wird, ist ein Matrix-Eiweiß, welches beim Auf- und Abbau unserer Zellen und Gewebe benötigt wird. Es ist klar, dass durch Entzündung und Absterben von Zellen und Gewebe diese Eiweiße vermehrt gebildet werden.

Der Beweis, dass die beteiligten Wissenschaftler das ganz genau wissen ist, dass sie auf Nachfrage niemals eine konkrete Publikation benennen, in der ein Virus behauptet wird, obwohl Anzahl und Beteiligung an solchen Publikationen Voraussetzung für die staatliche Anstellung und die Höhe der Einkünfte ist.

Fragen Sie Ihren Influenza-Forscher.
Wer nicht fragt, bleibt... ahnungslos.

In diesem Sinne!
Ihr Dr. Stefan Lanka

PS: Die Basis-Informationen zur Grippe, Influenza, der Grippe-/Influenza-Virus-Idee und dem Blutverdicker Tamiflu gibt es **HIER**. [sowie Buchwerbung von 2009 **HIER** und aktueller Shopeintrag **HIER**]

PPS: Regelmäßige kostenlose Informationen auf höchstem Niveau? Unseren kostenlosen E-Mail-Newsletter gibt es **HIER**.

PPPS: Die aktuelle Ausgabe unseres Zweimonat-Magazins WISSENSCHAFFTPLUS gibt es einmalig kostenlos & unverbindlich ~~HIER~~. [Angebot ungültig. Leseproben als pdf **HIER.**]

Veränderungen von impfen-nein-danke: Titel aus Email-Newsletter übernommen, Links z. T. aktualisiert, Studie in pdf-Datei hinzugefügt, einige wenige Tippfehler korrigiert.

Quelle: Newsletter-Email und https://web.archive.org/web/20120618075110/http://www.klein-klein-verlag.de/Viren-%7C-Erschienen-in-2012/25012012-influenza-neues-supervirus.html

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PLoS PATHOGENS

Eurasian-Origin Gene Segments Contribute to the Transmissibility, Aerosol Release, and Morphology of the 2009 Pandemic H1N1 Influenza Virus

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Abstract

The epidemiological success of pandemic and epidemic influenza A viruses relies on the ability to transmit efficiently from person-to-person via respiratory droplets. Respiratory droplet (RD) transmission of influenza viruses requires efficient replication and release of infectious influenza particles into the air. The 2009 pandemic H1N1 (pH1N1) virus originated by reassortment of a North American triple reassortant swine (TRS) virus with a Eurasian swine virus that contributed the neuraminidase (NA) and M gene segments. Both the TRS and Eurasian swine viruses caused sporadic infections in humans, but failed to spread from person-to-person, unlike the pH1N1 virus. We evaluated the pH1N1 and its precursor viruses in a ferret model to determine the contribution of different viral gene segments on the release of influenza virus particles into the air and on the transmissibility of the pH1N1 virus. We found that the Eurasian-origin gene segments contributed to efficient RD transmission of the pH1N1 virus likely by modulating the release of influenza viral RNA-containing particles into the air. All viruses replicated well in the upper respiratory tract of infected ferrets, suggesting that factors other than viral replication are important for the release of influenza virus particles and transmission. Our studies demonstrate that the release of influenza viral RNA-containing particles into the air correlates with increased NA activity. Additionally, the pleomorphic phenotype of the pH1N1 virus is dependent upon the Eurasian-origin gene segments, suggesting a link between transmission and virus morphology. We have demonstrated that the viruses are released into exhaled air to varying degrees and a constellation of genes influences the transmissibility of the pH1N1 virus.

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Introduction

Influenza A viruses pose a global threat to human health. They circulate in animal hosts and can reassort to generate a virus to which the human population is naïve, creating a potential pandemic threat. Efficient person-to-person transmission of influenza A viruses via RDs is a feature of seasonal epidemics and of pandemics. Influenza viruses have caused several pandemics in the past, including one in 1918 caused by an avian-origin virus that killed 50 million people, and the most recent pandemic occurred in the spring of 2009 [1,2]. The 2009 pandemic of swine-origin H1N1 influenza virus spread to over 215 countries from April 2009 to August 2010 and was responsible for at least 18,000 laboratory-confirmed deaths [3]. Determination of the molecular requirements for influenza viruses to transmit efficiently from person-to-person is an essential contribution to our understanding of potential pandemic threats. For example, the animal influenza viruses, avian H5N1, swine H1N1, and swine H1N2 viruses, have sporadically infected humans [4–8] but have not caused an influenza pandemic, presumably because they were unable to transmit efficiently throughout the human population.

The influenza A virus genome consists of 8 negative strand RNA gene segments that encode at least 11 proteins. The viral envelope is predominantly composed of the hemagglutinin (HA), neuraminidase (NA), and matrix (M1 and M2) proteins. HA is responsible for receptor binding and viral entry into a cell, while NA aids in release from the infected cell by cleaving sialic acids on the cell surface. The M1 protein lines the inside of the plasma membrane enveloping the viral RNA and gives structure to the virion, while M2 is an ion channel important for uncoating of the virus in the endosome and for virus release [9,10]. The segmented genome allows reassortment to occur in nature, enhancing the genetic diversity of the virus. The 2009 pandemic H1N1 (pH1N1) virus arose from a reassortment event between a North American triple reassortant swine virus (TRS) and a Eurasian swine virus. The Eurasian swine viruses contributed the NA and M gene segments to the pH1N1 strain, while the remaining 6 gene

Author Summary

Influenza A viruses spread rapidly from person-to-person via respiratory droplets (RDs). In this study we used a ferret model to explore viral functions involved in RD transmission of influenza viruses. The 2009 pandemic H1N1 (pH1N1) virus originated by reassortment of a North American triple reassortant swine (TRS) virus with a Eurasian swine virus. Both TRS and Eurasian swine viruses had previously caused sporadic infections in humans, but failed to spread from person-to-person, unlike the pH1N1 virus. We evaluated the release of influenza virus-containing aerosols and the transmissibility of the pH1N1, TRS, and Eurasian viruses in ferrets and found that the Eurasian-origin gene segments contributed to efficient RD transmission of the pH1N1 virus by modulating the release of influenza viral RNA-containing particles into the air. The increased release of viral RNA-containing particles correlated with increased viral neuraminidase activity and production of filamentous viral particles. These observations enhance what we currently know about the viral requirements for influenza virus RD transmission and have implications for assessing the potential of novel influenza viruses to spread.

segments came from the TRS virus [7,11]. The pH1N1 precursor viruses, TRS and Eurasian swine, have transmitted from pigs to humans sporadically but secondary human cases did not occur [5,8]. Recent studies have attempted to identify the genetic requirements for transmission of the pH1N1 virus [12,13]. However, they did not identify the biological mechanisms by which these gene segments confer efficient transmission. Therefore, the biological determinants responsible for transmission of the pH1N1 virus that are lacking in the TRS and Eurasian swine viruses are still unknown.

Transmission of influenza virus has been studied extensively in animal models such as guinea pigs and ferrets [14], yet the precise mechanism or requirements for transmission are still unclear. Previous studies have suggested that host-range determinants such as receptor binding specificity and human-specific PB2 amino acid residues are important for transmission [15–19]. However, recent studies have demonstrated that these host-range determinants are not sufficient for transmission [20,21]. Additionally, the HA protein from both the pH1N1 and TRS viruses is from the classical swine lineage that binds α2,6-linked sialic acids and both of these viruses contain avian-specific amino acids 627 and 701 in the PB2 gene, suggesting that those characteristics alone do not determine the transmissibility of these viruses. These observations suggest a role for other gene products in the transmissibility of the pH1N1 virus.

Three modes of influenza virus transmission have been defined: contact transmission, droplet spray transmission, and aerosol transmission. Contact transmission includes direct or indirect contact with a contaminated surface. Droplet spray transmission refers to person-to-person transmission via larger droplets that are deposited onto mucous membranes of the upper respiratory tract. Aerosol transmission is person-to-person transmission via aerosols composed of small, respirable particles that can be inhaled into the lower respiratory tract. The relative contribution of these different modes of transmission to person-to-person spread of influenza viruses is not known. In our study, the term respiratory droplet (RD) transmission includes both droplet spray and aerosol transmission. Studies attempting to distinguish between large and small aerosols have used aerosol samplers to measure the size of influenza virus-containing particles released by humans. Bioaerosol sampling has been performed in various environmental settings such as hospitals, airplanes, and daycare centers [22–25]. These studies suggest that humans predominantly release small respirable particles that contain influenza virus, although larger particles containing influenza virus were also detected.

There are three components to consider when studying RD transmission of influenza virus: the donor, the environment, and the recipient. The donor must be infected with a virus that replicates efficiently in the upper respiratory tract and infectious virus must be released into the surrounding air. Environmental factors can alter the size, morphology, and amount of influenza virus-containing particles present in the air that is shared by the donor and recipient [26]. Recipients must be susceptible to viral infection and exposed to enough infectious virus to establish a productive infection. Modulation of any of these parameters, including viral host-range determinants, severity of disease symptoms, environmental temperature, humidity, and susceptibility of the recipient can alter the transmissibility of a virus [27,28].

In this study, we used viruses generated by reverse genetics and biological isolates from human infections to explore the impact of the Eurasian-origin NA and M gene segments on transmissibility of the 2009 pH1N1 virus in a ferret model. We included the pH1N1 virus, representative Eurasian and TRS viruses that are putative precursor viruses, and a reassortant pH1N1 virus in which the NA and M gene segments were replaced with corresponding gene segments from a TRS virus. We found that the Eurasian NA and M gene segments contribute to efficient transmission of the pH1N1 virus. We used cyclone-based aerosol samplers to assess the amount and size distribution of influenza viral RNA-containing particles released by infected ferrets and determined the susceptibility of ferrets to the pH1N1 and its precursor viruses. Ferrets infected with viruses containing the Eurasian-origin NA and M gene segments efficiently released influenza viral RNA-containing particles into the air; this release correlated with higher NA activity of the pH1N1 and Eurasian viruses. Eurasian gene segments also contribute to the pleomorphic phenotype of the pH1N1 virus and this correlated with efficient RD transmission, suggesting a constellation of genes was responsible for the release of influenza virus-containing aerosols and transmissibility of the pH1N1 virus.

Results

Eurasian-Origin Gene Segments Confer Increased Transmission of pH1N1 Virus

RD transmission of pH1N1 virus has been shown to be highly efficient in the ferret model, with transmission efficiency ranging from 66% to 100% [29–31]. To assess whether the Eurasian-origin gene segments contribute to this phenotype, we used reverse genetics to create a recombinant pH1N1 virus and a 6:2 reassortant pH1N1 virus in which the Eurasian-origin NA and M gene segments were replaced with the North American TRS NA and M gene segments (Table 1). We confirmed that the recombinant wild-type (wt) 2009 pH1N1 virus rescued by reverse genetics behaved similarly to the biological wt virus *in vitro* and *in vivo* (Figure S1). The titer of biological pH1N1 and recombinant pH1N1 viruses differed in the lungs of ferrets on day 1 (Figure S1B); however, by day 5 post-infection, viral replication in the lungs was equivalent. Therefore, we used the recombinant 2009 pandemic virus (rec A/California/07/2009), hereafter referred to as Rec pH1N1, as a surrogate for the biological virus in further studies.

Table 1. Genotype of the viruses.

			Origin								
Virus	**Derived**	**Abbr Name**	**PB2**	**PB1**	**PA**	**HA**	**NP**	**NA**	**M**	**NS**	**Reference**
rec A/CA/07/2009 V4 (H1N1)	Reverse genetics	Rec pH1N1	N. Am avian	Human H3N2	N. Am avian	CS	CS	ERAS	ERAS	CS	Chen Z et al 2010 [52]
rec A/CA/07/2009+A/OH/02/07 NA and M (H1N1)	Reverse genetics	6:2 Reassort	N. Am avian	Human H3N2	N. Am avian	CS	CS	CS	CS	CS	This Study
A/OH/02/2007 (H1N1)	Biological isolate	TRS	N. Am avian	Human H3N2	N. Am avian	CS	CS	CS	CS	CS	Shinde et al 2009 [8]
A/Thailand/271/2005 (H1N1)	Biological isolate	Eurasian	ERAS	ERAS	ERAS	CS	ERAS	ERAS	ERAS	ERAS	Komadina et al 2007 [5]

Adapted from Garten et al 2009 Science [11].
Key: CS (Classical Swine); ERAS (Eurasian avian-like swine); N. Am (North American).
doi:10.1371/journal.ppat.1002443.t001

RD transmission studies were carried out in four independent transmission cages with the Rec pH1N1 virus and the recombinant A/California/07/2009+A/Ohio/02/2007 NA and M (6:2 reassortant) virus. We measured viral titers in the nasal secretions of ferrets on alternate days for 14 days and determined levels of influenza-specific serum antibodies on day 14. A ferret was considered infected if it shed virus in the nasal secretions or seroconverted. We found that both the Rec pH1N1 and 6:2 reassortant virus replicated to high titers in the nasal secretions of the infected ferrets (Figure 1A and B, left panels). Infected ferrets shed virus for 6 days, with a mean peak titer between $10^{4.2}$–$10^{5.2}$ $TCID_{50}$/mL on days 2 or 4 post-infection. All four of the naïve ferrets exposed to Rec pH1N1 virus shed infectious virus in the nasal secretions. Three of the naïve exposed ferrets shed virus from days 3 to 7 post-exposure, with a peak titer of $10^{3.2}$–$10^{3.7}$ $TCID_{50}$/mL of virus on day 5 for two of the ferrets (Figure 1A); this pattern of viral shedding is similar to data observed by us and others on the transmission of the biological pH1N1 virus (Figure S1D and [32]). The fourth ferret (naïve 4) shed virus much later than the other three (day 9 post-exposure). Presence of influenza-specific antibodies was found in all ferrets that shed virus in the nasal secretions (Figure 1C). Antibody titers for naïve ferret 4 were lower compared to the other ferrets, most likely because of the late onset of viral shedding. Since virus was detected in all 4 naïve ferrets and they all seroconverted by HAI, we concluded that RD transmission efficiency for the Rec pH1N1 virus was 100% in our system.

RD transmission of the 6:2 reassortant virus was less efficient; virus was detected in the nasal secretions of two out of four naïve ferrets (Figure 1B), with peak shedding of $10^{3.2}$–$10^{3.7}$ $TCID_{50}$/mL on day 7 post-exposure. Influenza-specific antibodies were detected in the ferrets that shed virus in their nasal secretions (Figure 1C). These data demonstrate that replacement of the Eurasian-origin gene segments in the 6:2 reassortant virus resulted in reduced transmission efficiency.

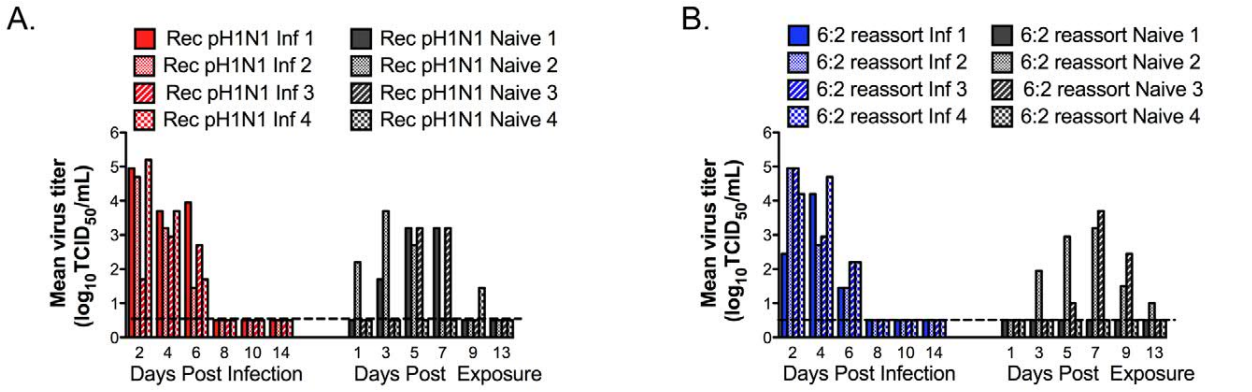
Additionally, we observed severe weight loss in two out of four ferrets infected with Rec pH1N1 virus and three out of four ferrets infected with the 6:2 reassortant virus (Table S1), indicating that disease severity, as measured by weight loss, does not correlate with efficiency of RD transmission. Sneezing was observed in one of four ferrets for both viruses. Interestingly, in each case the naïve partner became infected, suggesting that generation of aerosols by sneezing may enhance transmission.

Transmission Efficiency of the Pandemic Precursor Viruses

To determine whether the reduced transmission efficiency between the Rec pH1N1 and the 6:2 reassortant virus was due to the Eurasian-origin gene segments, we evaluated the transmission efficiency of the pandemic precursor viruses. These experiments were conducted with swine-origin viruses that were isolated from humans (Table 1): for the North American TRS, an isolate obtained from an adult male in Ohio in 2007 (A/Ohio/02/2007) [8] and for the Eurasian swine virus, a virus isolated from a child in Thailand in 2005 (A/Thailand/271/2005) [5]. Both of these viruses had transmitted from pigs to humans, but did not spread from person-to-person [5,6,8].

Ferrets infected with the A/Ohio/02/2007 (TRS) virus had much lower titers of virus in their nasal secretions (Figure 2A) than those infected with the Rec pH1N1 or 6:2 reassortant viruses. The peak titer was $10^{2.2}$ $TCID_{50}$/mL for most ferrets on day 2-post infection. However, in other experiments performed in our lab and by others, this virus replicated to higher levels in the upper respiratory tract of ferrets (Table 2, Figure S1A and [32]). Ferrets infected with the A/Thailand/271/2005 (Eurasian) virus shed high titers of the virus (Figure 2B). Peak viral shedding was observed on days 2 or 4 post-infection, with peak titers of $10^{2.95}$–$10^{4.95}$ $TCID_{50}$/mL. A matched non-parametric two-way ANOVA of the nasal wash titers in animals infected with Eurasian, pH1N1, or 6:2 reassortant virus showed no statistical difference among these groups of viruses.

One of four ferrets each infected with either the TRS or Eurasian viruses developed a distinctive cough, similar to croup (Table S1). The naïve ferrets paired with the croupy ferret became infected with influenza and shed virus in their nasal secretions, suggesting that the aerosols released by coughing enhanced RD transmission. These were the only ferrets that shed virus in the nasal secretions after exposure to ferrets infected with the TRS or Eurasian viruses. As seen with the Rec pH1N1 and 6:2 reassortant viruses, all of the ferrets with detectable virus in the nasal secretions also produced anti-influenza antibodies (Figure 2C). However, with both the TRS and Eurasian viruses, one naive ferret (TRS naïve 3 and Eurasian naïve 1) that did not shed virus in the nasal secretions seroconverted. Others have also found serologic evidence of infection in the absence of virologic evidence in a ferret transmission model [33]. Thus, we conclude that the two pandemic precursor viruses transmitted with 50% efficiency in ferrets. Our data demonstrate that the Eurasian-origin NA and M gene segments are necessary, but not sufficient, for RD transmission in our ferret model. To



C.

	Virus	Infected 1	2	3	4	% Sero-positive (Infected)	Naïve 1	2	3	4	% Sero-positive (Naive)
HAI assay	Rec pH1N1	640	640	320	640	100%	1280	160	1280	80	100%
	6:2 reassort	640	1280	640	1280	100%	<10	640	1280	<10	50%
Neutralization assay	Rec pH1N1	160	320	101	640	100%	127	40	127	<20	75%
	6:2 reassort	127	226	80	493	100%	<20	226	226	<20	50%

Figure 1. Eurasian-origin NA and M gene segments contribute to RD transmission of the pH1N1 virus. Four ferrets were inoculated IN to test the RD transmission of Rec pH1N1 (A) or the 6:2 reassortant (B) viruses. Nasal washes were collected on the indicated days. Each bar represents the titer of virus from an individual ferret. Inf stands for infected ferret. The limit of detection is represented as the dashed line and is $10^{0.5}$ $TCID_{50}$ per mL. Serum was collected on day 0 and day 14. Anti-influenza antibodies were measured by HAI and neutralization assay (C). The limit of detection is 1:10 for HAI and 1:20 for the neutralization assay. Antibody titers in the day 0 sera were below the limit of detection.
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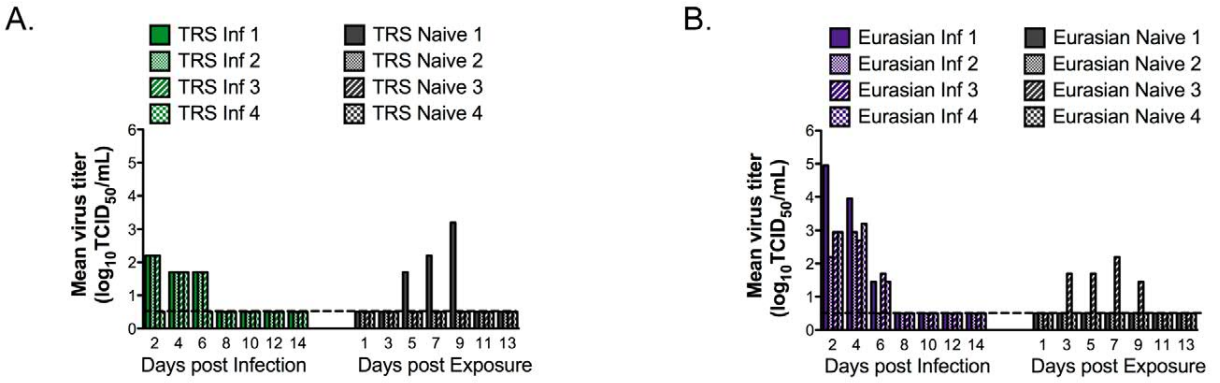
confirm that the reduced RD transmission of the TRS (A/Ohio/02/2007) was not due to the lower viral replication in the experimentally infected ferrets, we re-evaluated the replication and transmissibility of this virus with a larger number of animals. We confirmed the earlier finding of reduced transmissibility, even in the face of higher titers of virus in the experimentally infected ferrets. The TRS virus replicated to variable titers in the nasal secretions of experimentally infected ferrets; some infected ferrets had low titers ($10^{1.7}$–$10^{2.95}$ $TCID_{50}$/mL), consistent with the titers we had observed in the first study (Figure 2A) and others had higher titers ($10^{3.7}$–$10^{3.95}$ $TCID_{50}$/mL) of virus (Figure S2A). The peak of viral shedding was on day 2, as previously observed. In the new transmission study, only 2 out of 6 naïve animals became infected, as defined by isolation of virus in their nasal secretions and/or seroconversion (Figure S2B). The reduced transmission efficiency of the TRS virus has also been reported by others [13,32]. Additionally, since ferrets infected with the pH1N1, 6:2 reassortant, or Eurasian virus all shed virus to similar levels, we believe that RD transmission is not dependent upon efficient virus replication in the nasal secretions of animals. Therefore, efficient RD transmission is likely due to other factors such as infectivity of the virus for the naïve host or release of viral particles into the air.

Infectivity of the Pandemic and Precursor Viruses for Ferrets

To determine whether the infectivity of the viruses for ferrets varied, we determined the dose of virus at which 50% of ferrets were infected (FID_{50}). Ferrets were inoculated with 10,000, 100, or 10 $TCID_{50}$ of virus, and infectivity was measured by the presence of infectious virus in nasal secretions or by seroconversion. Table 2 lists the number of ferrets at each dose that were infected among the ferrets that were inoculated with each dose. Peak virus titers obtained from the nasal secretions are also presented in Table 2. In this experiment, ferrets infected with the TRS virus shed virus in the nasal wash at titers equivalent to the other viruses, confirming that this virus has variable replication in the upper respiratory tract of ferrets. Interestingly, administration of doses of virus as low as 10 $TCID_{50}$ resulted in peak viral titers similar to that of 1000-fold higher doses. Based on the data presented in Table 2, Rec pH1N1 and TRS viruses have a similar FID_{50}, and the 6:2 reassortant and Eurasian viruses are more infectious. Surprisingly, all 3 animals infected with 10 $TCID_{50}$ of the Eurasian virus shed virus in nasal washes and seroconverted (Table 2). These data demonstrate that while the pandemic virus and its precursors may differ in their infectivity in ferrets, this does not correlate with transmissibility of these viruses via RD transmission.

Release of Influenza Viral RNA-containing Particles into the Air Depends on the Presence of the Eurasian-Origin Gene Segments

Influenza virus particles must be released into the air for RD transmission to occur. Much work has been done recently exploring the size distribution of particles containing influenza



C.

	Virus	Infected 1	Infected 2	Infected 3	Infected 4	% Sero-positive (Infected)	Naïve 1	Naïve 2	Naïve 3	Naïve 4	% Sero-positive (Naive)
HAI assay	TRS	2560	5120	5120	5120	100%	5120	<10	2560	<10	50%
	Eurasian	640	640	2560	640	100%	160	<10	640	<10	50%
Neutralization assay	TRS	2560	5120	5120	2560	100%	1280	<20	5120	<20	50%
	Eurasian	320	640	1280	640	100%	40	<20	640	<20	50%

Figure 2. Pandemic precursor viruses transmit to 50% of exposed ferrets by RD. Four ferrets were inoculated IN to test the RD transmission of TRS (A) or the Eurasian (B) viruses. Nasal washes were collected on the indicated days. Each bar represents the titer of virus from an individual ferret. Inf stands for infected ferret. The limit of detection is represented as the dashed line and is $10^{0.5}$ $TCID_{50}$ per mL. Serum was collected on day 0 and day 14. Anti-influenza antibodies were measured by HAI and neutralization assay (C). The limit of detection is 1:10 for HAI and 1:20 for the neutralization assay. Antibody titers in the day 0 sera were below the limit of detection.
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virus that are released by humans [22–24,34,35]. However, few studies have been done in animal models to correlate the amount of particles released with influenza virus transmission [36,37]. To determine the size of influenza virus particles in the air exhaled by infected ferrets, we used cyclone-based aerosol samplers that separate particles based on size; these samplers have previously

Table 2. Infectivity of pH1N1 influenza and precursor viruses for ferrets.

Virus	Dose ($TCID_{50}$) of virus administered[a]	No. seroconverted/total[b]	No. shedding virus (Culture pos/total)	50% ferret infectious dose (FID_{50})[c]	Mean peak titer in nasal wash (log_{10} $TCID_{50}$/mL)
	10	1/3	1/3		3.2
Rec pH1N1	100	3/3	3/3	18	2.4
	10,000	3/3	3/3		4.2
	10	1/3	2/3		3.45
6:2 Reassort	100	3/3	3/3	18	4.95
	10,000	3/3	3/3		3.7
	10	1/3	1/3		4.45
TRS	100	3/3	3/3	18	3.7
	10,000	3/3	3/3		4.45
	10	3/3	3/3		4.3
Eurasian	100	3/3	3/3	<10	3.7
	10,000	3/3	3/3		3.6

[a] Virus dose delivered in 500 μL volume.
[b] Seroconversion was determined by HAI assay.
[c] If the endpoint was not reached at a dose of 10 $TCID_{50}$, we assumed that at a dose of 1 $TCID_{50}$ no ferrets would be infected; therefore, the FID_{50} value is shown as <10.
doi:10.1371/journal.ppat.1002443.t002

been used in clinical settings to assess exposure of health care workers to influenza [22,23]. The samplers have three collection surfaces: a 15 mL conical tube captures particles greater than 4 μm, a 1.5 mL tube captures particles between 1 and 4 μm, and a filter traps all submicron (<1 μm) particles. The samplers were secured to the outside of the cage, between the inner and outer doors (Figure S3A), and the air on the infected ferret's side of the cage was sampled for one hour on alternate days at a rate of 3.5 liters per minute. The ferrets were undisturbed during air sampling. The distribution of influenza viral RNA-containing particles released by infected ferrets was determined for each virus in the study at all 3 sizes: >4 μm (Figure 3), 1 to 4 μm (Figure 4), and <1 μm (Figure 5). This system does not allow for the measurement of the total count of particles released by each ferret nor the isolation of infectious virus because the collection tubes are dry. However, it does allow for the quantification of particles containing influenza viral RNA. We used this measurement as a surrogate for the amount of viral particles present in aerosols of various sizes. We found that ferrets predominantly released influenza virus into the air in large particles (>4 μm) (Compare Figures 3, 4, and 5). The duration for which the large particles containing influenza viral RNA was detected correlated with the length of time that virus was detected in the nasal washes of the ferrets. In the case of the Rec pH1N1 virus, all four infected ferrets consistently released large particles containing influenza viral RNA for 6 days post-infection, with a peak on day 2 (Figure 3A). Although virus was not detected in the nasal wash on day 8 post-infection, a low level of aerosol particles containing influenza viral RNA was observed. Ferrets infected with the Eurasian swine virus also consistently released influenza viral RNA-containing particles into the air, and in a larger quantity than the Rec pH1N1 infected ferrets (Figure 3B). Ferrets infected with the 6:2 reassortant and TRS viruses sporadically released large (>4 μm size) influenza viral RNA-containing particles (Figure 3C and D). To compare the trend of influenza viral RNA-containing particles released by animals infected with these viruses, we calculated the average area under the curve (AUC) for each virus per collection tube. We found that AUC of the Rec pH1N1 and Eurasian viruses for the 15 mL collection tube are 5540 and 29,384 respectively. These values are higher than those for the 6:2 reassortant and TRS viruses, which are 1338 and 2333, respectively. These data demonstrate that while ferrets predominantly released large influenza viral RNA-containing particles, the ferrets infected with Rec pH1N1 and Eurasian viruses released more than those infected with either the TRS or 6:2 reassortant virus. A similar phenomenon was found with the release of 1 to 4 μm-sized particles (Figure 4). Viruses containing the Eurasian-origin gene segments (Rec pH1N1 and Eurasian) also had a more consistent release of influenza viral RNA-containing particles at the 1–4 μm size (Figure 4A and B), while the TRS and 6:2 reassortant virus had a more sporadic release of influenza viral RNA-containing particles (Figure 4 C and D). This phenomenon was confirmed by analysis of the average AUCs for each respective graph; the Rec pH1N1 and Eurasian viruses had AUC values (636.5 and 5464, respectively) higher than the TRS and 6:2 reassortant viruses (124.8 and 59.3, respectively). Ferrets infected with pH1N1 virus released 1–4 μm particles containing influenza viral RNA from day 2 to 4 with a peak at day 2, while some animals infected with the Eurasian virus released these particles consistently on days 2, 4, and 6. Very few influenza viral RNA-containing particles were detected 6 days post-infection (Figure 4). Although it is possible that the sporadic release of influenza viral RNA-containing particles from ferrets infected with the TRS virus may be linked to the low viral titers in the nasal secretions (Figure 2A), a similar pattern of sporadic release was also seen in the repeat experiment of ferrets infected with the TRS virus (Figure S2C). Additionally, ferrets infected with the 6:2 reassortant virus shed virus to high titers in the nasal secretions and also displayed a sporadic release of particles containing influenza viral RNA. Therefore, we conclude that the Eurasian-origin gene segments contribute to the release of influenza viral RNA-containing particles greater than 1 μm.

Interestingly, the pattern of release of submicron particles containing influenza viral RNA by the Rec pH1N1 virus was different from the other viruses (Figure 5 A and B). Ferrets infected with the Rec pH1N1 virus consistently released submicron particles containing influenza viral RNA into the air, and this release was detected at every time point tested, with a similar amount on days 2 and 6 post-infection. Interestingly, infected ferret number 4 released a considerable amount of submicron particles containing influenza viral RNA on days 8 and 10 post-infection, which correlates with the late infection of its naïve pair (refer to Figure 1A). In contrast to the Rec pH1N1-infected ferrets, those infected with the Eurasian virus released submicron influenza viral RNA-containing particles only sporadically. Infection with the TRS and 6:2 reassortant virus did not result in release of submicron influenza viral RNA-containing particles into the air that were detectable by our sampling system (Figure 5 C and D). There was a higher background observed in the Rec pH1N1 infected ferrets on day 0 compared to the other viruses that may be due to an environmental contaminant. Despite this, the release of influenza viral RNA-containing particles from ferrets infected with the Rec pH1N1 virus was found to be distinct from the other viruses. A comparison of the average AUC values from days 2 to 10 confirms this observation; the pH1N1 virus had an AUC value of 1043 and all of the other viruses had AUC values ranging from 56 to 59. Additionally, a two-way ANOVA found that the difference in the amount of submicron particles that contained influenza viral RNA released by ferrets infected with Rec pH1N1 virus compared with all other viruses was significant. The release of submicron influenza viral RNA-containing particles correlates with transmission efficiency and it is tempting to speculate that RD transmission is associated with these submicron particles.

Overall, our air sampling studies have found that ferrets infected with viruses that lacked the Eurasian-origin NA and M gene segments, the TRS and 6:2 reassortant viruses, only sporadically released influenza viral RNA-containing particles of all sizes into the air (Figures 3, 4, and 5). This finding suggests that the Eurasian-origin gene segments contribute to the transmissibility of the pH1N1 virus by influencing the release of influenza viral RNA-containing particles into the air.

Release of Influenza Viral RNA-containing Particles Correlates with NA Activity and Virus Morphology

The neuraminidase activity of the influenza NA protein cleaves sialic acids from the proteins on the cell surface and on the viral surface [9]. The cleavage of sialic acids by the viral neuraminidase aids in viral release and the prevention of viral agglutination after release. Therefore, it is plausible that infection with a virus with a more active NA could result in the release of more virus particles into the air. To determine whether the activity of the Eurasian-origin NA differs from that of the classical swine NA, we used an enzyme-linked lectin assay to determine the neuraminidase activity of viruses that had been normalized for infectivity using fetuin as a substrate (Figure 6A). Viruses that contain the Eurasian NA (the biological and recombinant pH1N1 and the Eurasian viruses) had higher NA activity than the TRS and 6:2 reassortant viruses,

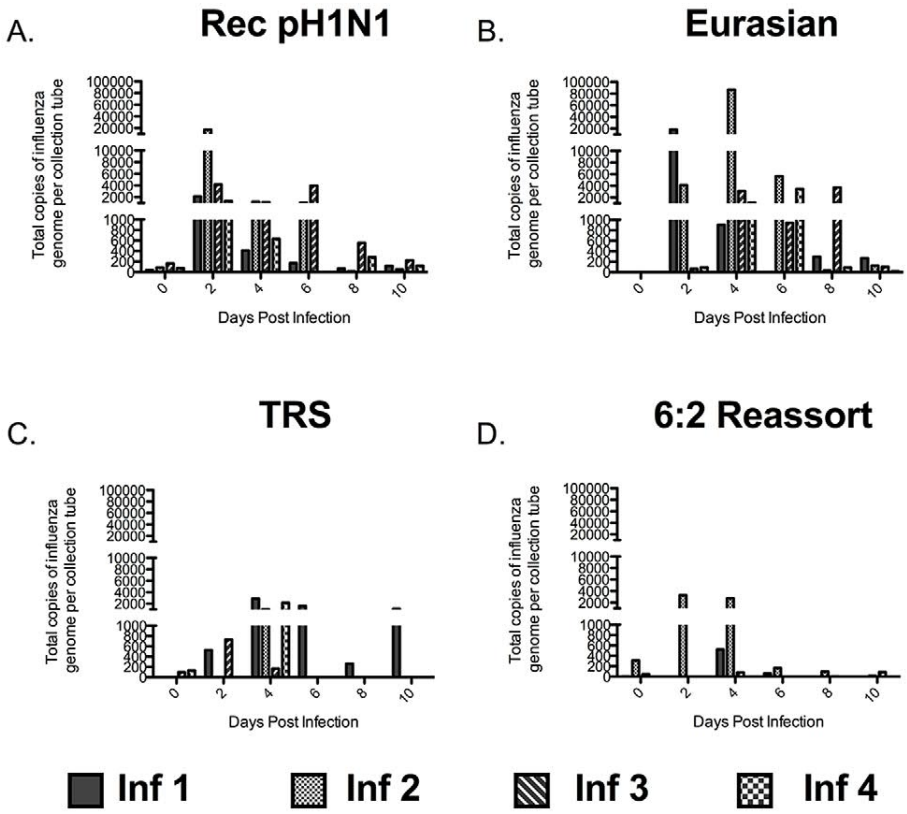

Figure 3. The Eurasian-origin NA and M gene segments contribute to abundant release of large (>4 μm) particles containing influenza virus. Quantitative (Q)-PCR for influenza A M gene in RNA extracted from the 15 mL collection tube of the cyclone-based air samplers. Air was collected for one hour on the outside of the infected ferret cage. Each bar represents the amount of genome copies of influenza in particles released by a single ferret infected with Rec pH1N1 (A), Eurasian (B), TRS (C), or 6:2 reassortant (D). Absolute amount of RNA was quantitated using a standard curve of in vitro transcribed influenza M gene RNA. Inf stands for infected ferret.
doi:10.1371/journal.ppat.1002443.g003

which have a classical swine NA protein. A similar observation has been made previously using MUNANA as a substrate [13]. To confirm these results, we performed a neuraminidase assay using MUNANA as a substrate (Figure 6B). MUNANA and fetuin differ in size; MUNANA is a short α2,6-linked sialic acid substrate while fetuin is much larger and contains both α2,3- and α2,6-linked sialic acids [38,39]. Since little is known about the biological substrates cleaved by NA in vivo, it is difficult to determine which substrates are biologically most relevant. We found that the Rec pH1N1 virus had a lower NA activity than the biological pH1N1 virus in both assays. The consensus sequence for the NA gene was identical for these viruses, suggesting that differences in the minor quasispecies composition of the respective virus populations may be the factor. Interestingly, with MUNANA, the Eurasian virus had lower NA activity than the pH1N1 virus, suggesting that NA proteins may have variable activity on different substrates. Our data indicate that the pH1N1 virus has a higher neuraminidase activity than the TRS and 6:2 reassortant viruses with both long and short substrates, and higher neuraminidase activity than the Eurasian virus with short substrates. These observations suggest that NA activity correlates with the release of virus particles and increased viral release is important for efficient RD transmission of the pH1N1 virus.

The Eurasian swine virus contributed both the NA and M gene segments to the pH1N1 virus and the M protein has been implicated in determining the filamentous or spherical morphology of influenza viruses [40–42]. Therefore, we compared the morphology of the Rec pH1N1, 6:2 reassortant, Eurasian, and TRS viruses by electron microscopy (Figure 7). The pH1N1 virus has previously been reported to be pleomorphic [29] and similar morphology was observed for the Rec pH1N1 virus (Figure 7A). We counted 20 or more particles and found that 60% of the Rec pH1N1 virus particles were filamentous, while the 6:2 reassortant virus was predominantly spherical with only 4% filamentous particles (Figure 7B). These data suggest that the Eurasian-origin gene segments specify the pleomorphic phenotype of the pH1N1 virus. The pH1N1 precursor viruses (Eurasian and TRS) were both predominantly spherical (Figure 7C and D), with only 9.5% or 0% filamentous particles, respectively. Taken together, these observations indicate that the Eurasian-origin gene segments alone are not sufficient to specify the pleomorphic morphology of the pH1N1 virus. The cytoplasmic tails of both HA and NA have previously been shown to contribute to influenza viral morphology [43]. However, the viruses used in this manuscript all contain the classical swine HA. Therefore, it is likely that specific adaptations in the pH1N1 viral gene segments that are distinct from the Eurasian swine gene segments have arisen and these changes may have contributed to the pleomorphic nature of the pH1N1 virus. Additionally, the complete passage history of the Eurasian virus is not known but may be relevant to its morphology.

Previous studies have suggested that receptor specificity correlates with RD transmission [17,44]. However, all of the viruses tested in this study have HA proteins that are evolutionarily similar to the classical swine virus (Table 1) and are antigenically

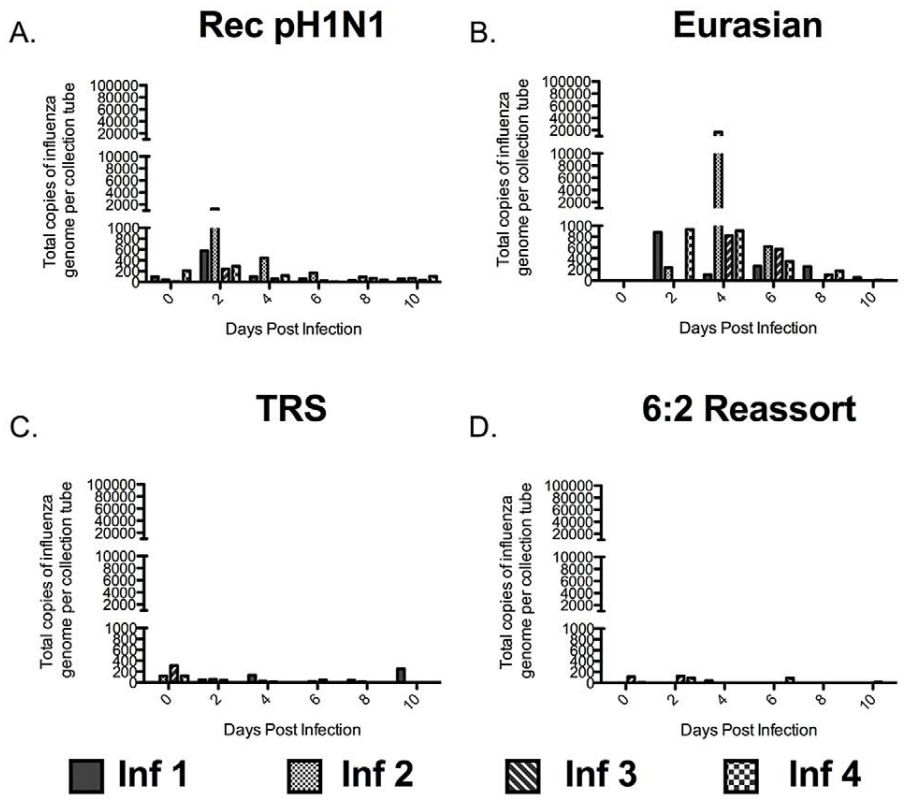

Figure 4. The Eurasian-origin NA and M gene segments contribute to the abundant release of 1 to 4 μm particles containing influenza virus. Q-PCR for influenza A M gene on RNA extracted from the 1.5 mL collection tube of the cyclone-based air samplers. Air was collected for one hour on the outside of the infected ferret cage, each bar represents the amount of particles released by a single ferret infected with Rec pH1N1 (A), Eurasian (B), TRS (C), or 6:2 reassortant (D). Absolute RNA was quantitated using a standard curve of in vitro transcribed influenza M gene RNA. Inf stands for infected ferret.
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similar to each other (data not shown). We evaluated receptor binding specificity using an in vitro assay with chicken RBCs specifically sialylated with α2,3 or α2,6 sialyltransferases (Figure S4A) and demonstrated that all of the viruses predominantly associate with α2,6-linked sialic acids.

Since virus-receptor affinity may be altered during viral evolution [45], we tested whether the viruses used in this study differed in their affinity for the α2,6 receptor by measuring their ability to agglutinate chicken red blood cells (RBCs) that had been treated with varying amounts of neuraminidase (Figure S4B). We found that all of the viruses bound to RBCs that were desialylated with similar concentrations of bacterial neuraminidase; therefore, we conclude that neither receptor specificity nor receptor affinity are responsible for the particle release observed in this study.

Taken together, our data suggest a role for the Eurasian-origin segments in the morphology and NA activity of the pH1N1 virus, one or both of which contribute to its efficient transmission.

Discussion

This study was designed to identify the molecular determinants that confer transmissibility of the pH1N1 virus and the mechanism by which they promote transmission. RD transmission can be modulated at the level of the infected donor, the environment, and the recipient. We established an RD transmission caging system that allowed for aerosol sampling of infected ferrets. In our system, the Rec pH1N1 virus transmitted to 100% of the naïve animals and replacement of the NA and M gene segments with the corresponding gene segments from TRS resulted in reduced transmission efficiency. These findings indicate that the Eurasian-origin NA and M gene segments contribute to the efficient transmission of the Rec pH1N1 virus. The fact that the Eurasian virus only transmitted to 50% of the naïve animals demonstrates that gene constellation may influence this phenotype as it does other properties such as virulence [46]. Yen et al. have recently suggested that a balance between HA and the Eurasian-origin NA contribute to the transmissibility of the pH1N1 virus [13]. Unlike our study, they used swine isolates that had not infected humans; therefore, any compensatory mutations that promote the initial transmission from an animal host to human were not taken into account. Based on our results, we believe that the biological properties of both Eurasian-origin gene segments influence particle release and thus efficient RD transmission. In our study, we found that susceptibility of the recipient ferrets to the specific virus, measured as the FID_{50} of the virus, did not correlate with transmission efficiency. Since environmental factors such as temperature and relative humidity were unaltered during the study, they did not contribute to the transmission phenotype. Therefore, we focused our attention on the release of influenza viruses by the infected donor ferrets. The viruses used in this study shared similar receptor specificity and replicated efficiently in the upper respiratory tract of ferrets. These two factors have been implicated in the transmissibility of other influenza viruses but they did not contribute to the enhanced transmission phenotype of the

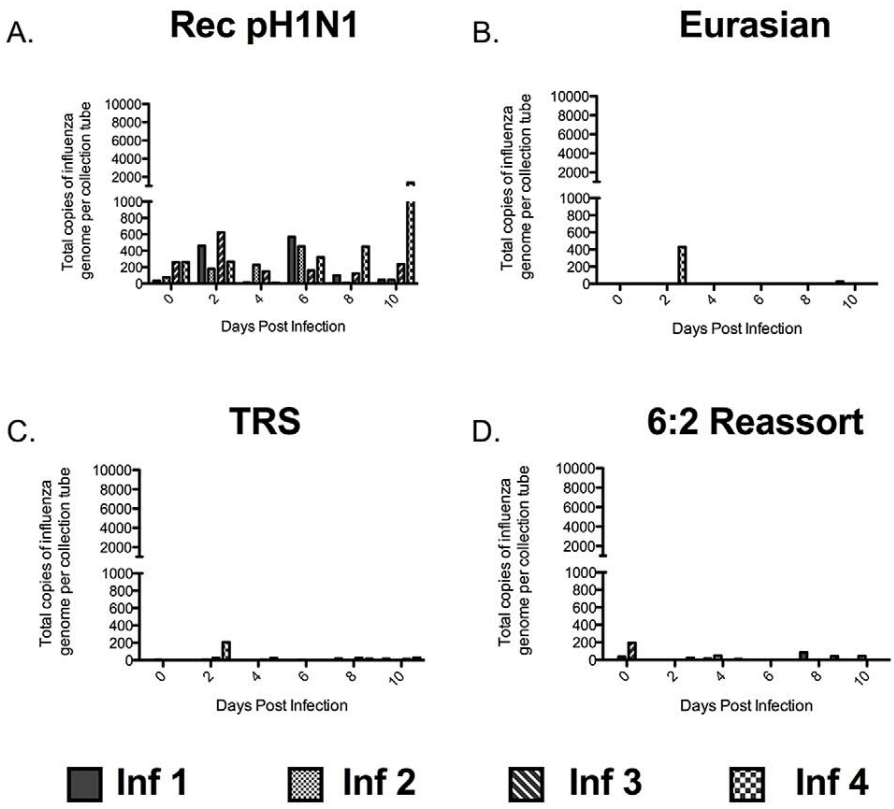

Figure 5. Ferrets infected with the recombinant pH1N1 virus release submicron particles containing influenza virus. Q-PCR for influenza A M gene on RNA extracted from the filter of the cyclone-based air samplers. Air was collected for one hour on the outside of the infected ferret cage, each bar represents the amount of particles released by a single ferret infected with Rec pH1N1 (A), Eurasian (B), TRS (C), or 6:2 reassortant (D). Absolute RNA was quantitated using a standard curve of in vitro transcribed influenza M gene RNA. Inf stands for infected ferret.
doi:10.1371/journal.ppat.1002443.g005

pH1N1 virus in our study. Using aerosol biosamplers to measure the release of virus into the air, we found that viruses containing the Eurasian-origin NA and M gene segments released influenza viral RNA-containing particles into the air consistently and this correlated with increased NA activity of these viruses. The Eurasian-origin gene segments also conferred the pleomorphic phenotype of the pH1N1 virus. Our observations extend our knowledge of the molecular determinants of RD transmission and provide an explanation for the epidemiological success of the pH1N1 virus.

An infected donor can generate aerosols during normal breathing or upon sneezing and coughing [47]. In our study, we used ferrets as donors because they are highly susceptible to influenza viruses and can both transmit the virus to humans and acquire infection from humans [48]. Ferrets infected with influenza viruses develop clinical symptoms such as weight loss,

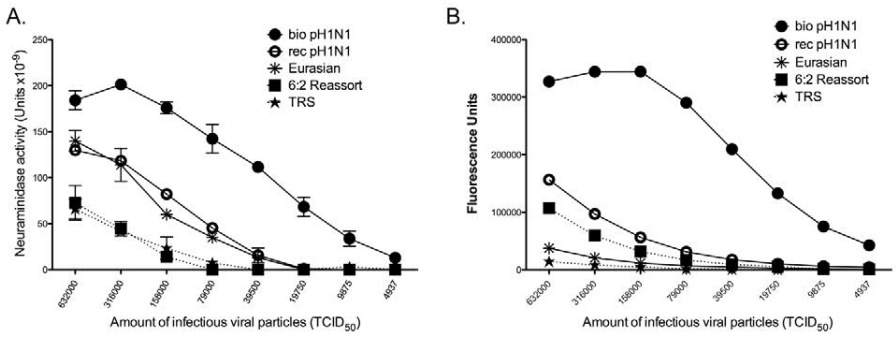

Figure 6. Viruses with Eurasian-origin NA have greater neuraminidase activity than viruses with a classical swine NA. An ELLA assay using fetuin as a substrate was used to determine the NA activity for the biological pH1N1 (●), rec pH1N1 (○), 6:2 reassortant (■), TRS (★), and Eurasian (*) viruses (A). Neuraminidase activity of these viruses was also measured using MUNANA as a substrate (B). Viruses were normalized for equal infectivity in all assays. The data are displayed as an average of 2 independent assays performed in duplicate. Error bars represent the standard error.
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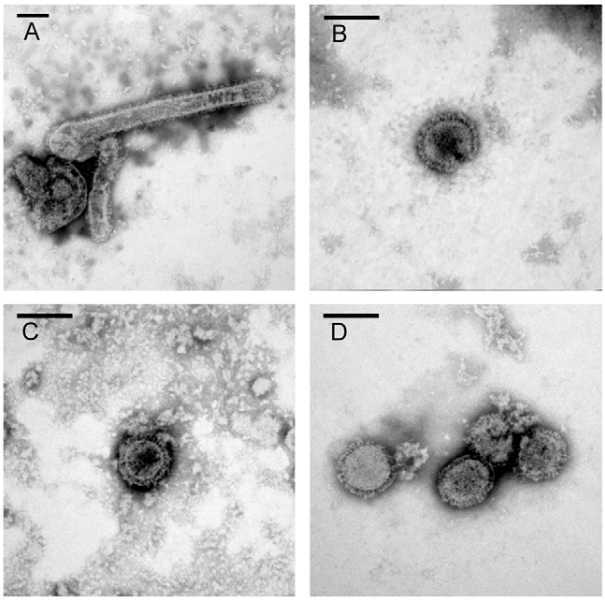

Figure 7. Eurasian-origin gene segments confer filamentous morphology of pH1N1 virus. Electron micrographs of negatively stained virus preparations are shown for Rec pH1N1 (A), 6:2 reassortant (B), TRS (C), and Eurasian (D) viruses. Representative images are shown for each virus. Bar; 100 nm.
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sneezing, and lethargy [49]. Disease severity in ferrets and humans varies by strain, with highly pathogenic strains such as H5N1 avian influenza viruses causing more severe disease than seasonal influenza strains [29–31]. We found that the 2009 pH1N1 virus and its precursor viruses caused similar disease severity in ferrets, defined by >10% weight loss and presence of clinical symptoms like sneezing and runny nose (Table S1). However, we also found that one out of four ferrets infected with TRS or Eurasian viruses developed croup and were able to efficiently transmit the virus to their naïve partners. Upon further analysis, we found a correlation between infected ferrets that were observed sneezing or coughing and infection of their naïve neighbors, indicating that generation of aerosols by sneezing and coughing enhances RD transmission.

In this study, we examined the size of influenza viral RNA-containing particles released from ferrets infected intranasally (IN) with influenza viruses and found that the ferrets primarily released influenza viral RNA-containing particles greater than 4 μm in size into the air (Figure 3). Consistent with our observations, Gustin et al. reported that anesthetized ferrets infected IN predominantly released large (>4.7 μm) infectious particles during normal breathing. However, they found that ferrets infected by aerosol released much smaller (0.65 to 4.7 μm) particles containing infectious virus into the air [37]. We found that ferrets inoculated IN with pH1N1 and Eurasian viruses released large (>4 μm) and small (1 to 4 μm) influenza viral RNA-containing particles more consistently than ferrets infected with the TRS and 6:2 reassortant viruses (Figure 3 and 4). The viruses with more consistent release of virus had a higher NA activity than viruses that were associated with sporadic release of influenza viral RNA-containing particles (Figure 6). Thus, NA activity correlates with the release of both large and small influenza viral RNA-containing particles. However, these particles are not sufficient for efficient RD transmission since the Eurasian virus, which consistently released large and small influenza viral RNA-containing particles, transmitted to only 50% of the naïve animals (Figure 2B). Additionally, in animals infected with the TRS virus, we only detected the presence of large particles containing influenza viral RNA in the air, yet this virus transmitted to 50% of the naïve animals. These data suggest that the large particles (>4 μm) may contribute to RD transmission of viruses in the ferret model system. Release of large particles containing influenza has been observed in human clinical studies [23]. However, the relative importance of these particles in human transmission is unclear.

Interestingly, release of submicron influenza viral RNA-containing particles differed between pH1N1 and the Eurasian viruses (Figure 5). The Rec pH1N1 infected ferrets consistently released submicron influenza viral RNA-containing particles while ferrets infected with the Eurasian virus did not. Given that the animal cages have a continuous air flow rate of 40 cubic feet per minute, it is also possible that we were unable to thoroughly capture the submicron particles released by the ferrets by sampling on the outside of the cage. Aerosol sampling in different environments suggests that humans predominantly release small, respirable particles that likely result in the respiratory or aerosol transmission of influenza viruses [22,23,34]. Since the pH1N1 infected ferrets released more submicron particles than ferrets infected with any of the other viruses, it is possible that the submicron particles are responsible for the efficient aerosol transmission of the pH1N1 virus.

Previous studies have demonstrated a role for HA receptor binding specificity and specific amino acid residues in the PB2 protein on RD transmission of influenza A viruses [17–19,50]. The emergence and transmissibility of the 2009 pH1N1 virus cannot be explained by these molecular determinants of transmissibility of the virus via RDs. Instead, our study illustrates the importance of the NA and M proteins in the transmissibility of the pH1N1 virus. We found that NA activity correlates with the release of particles greater than 1 μm in size and this may be necessary, but not sufficient, for RD transmission. Additionally, we found that viral morphology correlated with transmissibility of swine-origin viruses in the ferret model. The pleomorphic Rec pH1N1 virus was more efficiently transmitted than the spherical 6:2 reassortant, TRS, and Eurasian viruses, suggesting that this phenotype may be important for RD transmission of swine-origin viruses. While there are many examples of α2,6-specific receptor binding influenza viruses that do not transmit in animal models or in the human population [14,51], there are no reports of RD transmission of α2,3-specific receptor binding influenza viruses. Therefore, virus receptor binding specificity is also necessary, but not sufficient, for transmission.

Our data indicate that in order to more accurately assess pandemic threat potential, phenotypes that are important for transmission such as viral replication in the upper respiratory tract of ferrets, release of respirable influenza virus-containing particles, and receptor specificity of novel influenza viruses should be characterized.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The National Institutes of Health and MedImmune Animal Care and Use Committee (ACUC) approved the animal experiments that were conducted at the respective facilities. All efforts were made to minimize suffering.

Cells and Viruses

Madin-Darby canine kidney (MDCK) cells, obtained from the ATCC, were maintained in minimum essential media (MEM) and 10% fetal bovine serum (FBS). 293T cells, obtained from ATCC, were maintained in Dulbecco's MEM with 10% FBS.

The reverse genetics system for generating the 2009 pandemic H1N1 virus (A/California/07/2009) were previously described [52]. The NA and M gene segments for the North American TRS virus (A/Ohio/02/2007) were constructed as previously described [52]. The recombinant viruses generated from the reverse genetics plasmids were rescued from MDCK/293T cell co-culture and propagated in specific pathogen free (SPF) embryonated eggs as described [53] for 2 passages. Viruses generated by reverse genetics were confirmed by genomic sequencing. The A/Ohio/02/2007 (H1N1) and A/Thailand/271/2005 (H1N1) viruses were obtained from the Centers for Disease Control and Prevention (CDC) and were subsequently propagated in MDCK cells. The passage histories for the biological isolates are C5 and CX,C3/C2/C2 for A/Ohio/02/2007 and A/Thailand/271/2005, respectively where X indicates an unknown number of passages.

Ferret Infections and Nasal Wash Collection

All transmission studies consisted of four RD transmission cages, 3 male cages and 1 female cage. Each transmission cage contained two ferrets – 1 naïve and 1 infected, per cage (Figure S3). For each study 6 male and 2 female, 5–8 month old adult ferrets obtained from Triple F farms (Sayre, PA) that were seronegative for seasonal H3 and H1 viruses, and all of the viruses used in this study. As in other RD transmission studies [27,33,51] the sample size is small. Ferrets were inoculated intranasally (IN) with $10^{6.5}$ $TCID_{50}$ of virus in 500 µL of Leibovitz-15 medium. All ferrets were monitored for clinical signs including sneezing, coughing, lethargy, weight loss, and body temperature changes. In accordance with NIAID Animal Care and Use Committee (ACUC) guidelines, ferrets were euthanized if they lost more than 20% of their initial body weight.

Ferret infectivity studies were performed at MedImmune (Mountain View, CA). Two male and one female adult ferrets (5–6 month old) were inoculated IN with each dose (10, 100, or 10,000 $TCID_{50}$ per 500 µL) of virus. Ferrets were considered infected if one of the following criteria was met: detection of virus in nasal secretions or by the presence of >40 influenza-specific antibody titer in the sera. Ferret infectious dose 50 (FID_{50}) values were calculated using the method described by Reed and Muench [54].

Nasal secretions were collected by washing the right nostril of an anesthetized ferret with sterile PBS and 500 µL of liquid that was expelled from the left nostril was collected. These nasal secretions were analyzed for the presence and titer of infectious viruses and expressed as 50% tissue culture infectious doses ($TCID_{50}$) per mL.

Transmission Studies

We designed the caging system for transmission studies based on earlier reports [33]. Briefly, large stainless steel ventilated ferret cages from Allentown (Allentown, New Jersey) were modified for the RD transmission studies (Figure S3). Two perforated stainless panels were welded together, 0.5 inches apart, and placed into the cage with a floor and ceiling guide to stabilize the panel. A door, with separate feeder and water bottles on each side of the dividing panel, was manufactured for each cage. Infected ferrets were placed into the section of the cage closest to the air inlet one day prior to infection. One day post-infection, a naïve ferret was placed into the cage on the other side of the divider. Environmental conditions inside the laboratory were monitored daily and were consistently 19±1°C and 56±2% relative humidity. The transmission experiments were conducted in the same room, to minimize any effects of caging and airflow differences on aerobiology. Nasal washes were collected and clinical signs were recorded on alternate days for 14 days. Air samples were collected between 9 am to 12 pm on alternate days for 10 days. On day 14 post-infection, blood was collected from each animal for serology. The naïve ferret was always handled before the infected ferret. Great care was taken during nasal wash collections and husbandry to ensure no direct contact occurred between the ferrets.

Serology

Ferret sera were tested for the presence of anti-influenza antibodies by hemagglutination inhibition (HAI) assay using turkey red blood cells (RBC) and neutralization assay using MDCK cells as described previously [53,55]. Ferrets were considered to have seroconverted if the antibody titer was higher than the limit of detection. The limit of detection is 1:10 for the HAI assay and 1:20 for the neutralization assay.

Aerosol Particle Sampling

Aerosol sampling of the ferret cages was performed between 9 am and 12 pm on alternate days for 10 days, prior to nasal wash collection. The air samples were collected by placing cyclone-based air samplers (BC251) developed by the National Institute for Occupational Safety and Health (Morgantown, WV) [22] on the outside of the infected side of the ferret transmission cage. A designated air sampler was used for each ferret to reduce cross-contamination between animals. A baseline or day 0 reading was obtained on the infected ferrets prior to inoculation and 24 hrs after the animal was placed into the transmission cage. Air was sampled for one hour at a flow rate of 3.5 liters per minute. The aerosol sampler flow rate was calibrated before each use using a flow meter (TSI 4100 series). The NIOSH BC251 samplers separate particles based upon size. Each sampler contained an empty 15 mL conical that collected particles greater than 4 µm, a 1.5 mL conical that collected particles between 1–4 µm, and a 3 µm pore Fluoropore membrane filter (Millipore) to collect submicron particles.

Processing of the samplers was performed in a bio-safety cabinet; 500 µL of Ambion RNA lysis binding buffer was placed into each collection tube and vortexed vigorously. RNA was extracted from each collection tube on a QIAGEN EZ Robot using the QIAGEN EZ1 virus mini kit, per the manufacturer's recommendations. The total amount of influenza RNA was quantified using Applied Biosystems Taqman one-step RT-PCR kit with primers (F – 5′AGATGAGTCTTCTAACCGAGGTCG3′, and R - 5′GCAAAGACATCTTCAAGTCTCTG3′) and a probe (FAM-TCA GGC CCC CTC AAA GCC GA–[NFQ]) specific for the influenza A M gene segment. In vitro transcribed RNA corresponding to this region of the M gene segment was used as a standard for absolute quantification. The RNA standard was created by, linearizing a pCDNA3.1 plasmid containing the T7 promoter and M gene sequences of influenza virus strain A/Beijing/262/95. This DNA was used as templates with the MEGAScript In Vitro Transcript Kit (Ambion) to generate Flu A M gene transcript. Transcripts were purified by extraction with Phase Lock Gel (PLG) (Heavy) tubes (Eppendorf Scientific, Inc.) two times, followed by phenol/chloroform, chloroform extraction and ethanol precipitation. The dried RNA pellet was resuspended in RNase-free RNA storage buffer (1 mM sodium citrate, pH 6.4; Ambion). The concentration of the purified transcript was determined by measuring absorbance at

260 nm. 10-fold serial dilution of the FluAM transcript in RNA storage buffer was performed to generate transcript at the level of 5×10^6 down to 5 copies/μL.

The limit of detection of the NIOSH BC251 samplers is unknown. After each use, the BC251 samplers were decontaminated by first rinsing each sampler with distilled water, making sure to wash the air inlet and other holes, then washing the samplers with isopropanol, again running the alcohol through the air inlet and all other holes.

Neuraminidase Activity

Activity of the NA protein of each virus was determined using a peanut-agglutinin based enzyme-linked lectin assay (ELLA). The ELLA assay was slightly modified from a previously described assay [56]. The ELLA assay uses fetuin as a substrate for the viral neuraminidase. Viruses were normalized for infectivity, $10^{6.5}$ $TCID_{50}$ per 500 μL, prior to performing the assay. Neuraminidase activity using MUNANA as a substrate was preformed using the NA star kit obtained from Applied Biosystems, and following the manufacturers instructions.

Electron Microscopy for Virus Morphology

A 2 mL aliquot of each stock virus, described in the 'Viruses' section, was concentrated by ultra-centrifugation using a Beckman Coulter L-100 XP ultracentrifuge with a SW-55i rotor. Viruses were pelleted at 24K rpm for 2 hrs; the pellet was resuspended in 20 μL of 1x Karnovsky's fixative solution. Concentrated viruses were sent to the Electron Microscopy unit at Rocky Mountain Laboratory (Hamilton, MT) for negative stain and analysis. Freshly glow discharged Formvar-carbon coated copper grids (Ted Pella, Inc., Redding, CA) were submerged in droplets of each sample and incubated overnight at 4 degrees C in a humid chamber. The grids were washed three times for 5 min each in deionized water, and negatively stained for 15 sec with methylamine tungstate (Nanoprobes, Inc., Brookhaven, NY). The grids were examined at 80 kV on a Hitachi H7500 transmission electron microscope. Digital images were captured on an HR-100 CCD camera (Advanced Microscopy Techniques, Danvers, MA), and rendered using Adobe PhotoShop (Adobe Systems, Inc., San Jose, CA). The percent filamentous particles were calculated by counting over 20 particles for each virus from blind pictures taken randomly on the grid.

Supplemental Methods

In vitro replication kinetics. MDCK cells were infected with each virus at an MOI of 0.1 and supernatant from infected cells in triplicate was collected at 8, 24, 30, and 48 hours post-infection. Supernatants were titrated on MDCK cells by serial dilution as previously described [57].

In vivo replication kinetics. Replication of viruses in the upper and lower respiratory tract of 8–12 wk old ferrets was determined as previously described [53]. Each ferret was inoculated IN with 10^6 $TCID_{50}$ of virus in 500 μL. Nasal turbinates and lung sections were harvested on 1 and 5 days post infection. Viral titers in each organ were determined as previously described [57].

Influenza receptor binding assay. Receptor specificity was determined using an in vitro receptor binding assay as described previously [58]. Chicken RBCs (Lampire Biological Laboratories Inc) were desialylated with *Clostridium perfringens* neuraminidase (SIGMA). The desialylated RBC were resialylated using specific α2,3 (SIGMA) or α2,6 (Calbiochem) sialyltransferases. Viruses known to bind specifically to α2,3- and α2,6-linked sialic acids were used as controls for each experiment.

Receptor affinity assay. The affinity of a virus was determined as previously described [45]. Briefly, chicken RBCs (Lampire Biological Laboratories Inc) were treated with serial dilutions of *Clostridium perfringens* neuraminidase (SIGMA) to remove sialic acids. Agglutination of RBCs treated with the different neuraminidase concentrations was determined using a standard amount of each virus (4 HAU).

Supporting Information

Figure S1 The Rec pH1N1 virus behaves like the biological pH1N1. Ferrets, 6–8 weeks old, were infected with either Rec pH1N1 or biological pH1N1. Virus titers were measured on days 1 and 5 post infection in the nasal turbinates (A) or lung (B). MDCK cells were infected with biological or Rec pH1N1 and virus titers were determined at the time indicated (C). Transmission efficiency of the biological pH1N1 virus was determined using 3 transmission cages with 6 adult ferrets (D).
(TIFF)

Figure S2 Reduced transmission and release of particles containing influenza viral RNA from ferrets infected with TRS virus. Six ferrets were inoculated IN to test the RD transmission of TRS. Nasal washes were collected on the indicated days (A). Each bar represents the titer of virus from an individual ferret. Inf stands for infected ferret. The limit of detection is represented as the dashed line and is $10^{0.5}$ $TCID_{50}$ per mL. Serum was collected on day 0 and day 14. Anti-influenza antibodies were measured by HAI and neutralization assay (B). The limit of detection is 1:10 for HAI and 1:20 for the neutralization assay. Antibody titers in the day 0 sera were below the limit of detection. Aerosol sampling was performed on four of the infected animals (Inf 1–4) to determine the presence of particles containing influenza viral RNA (C). Each bar represents an individual animal. Absolute RNA was quantified using a standard curve of in vitro transcribed influenza M gene RNA.
(TIFF)

Figure S3 Schematic of respiratory droplet transmission cage setup. Commercially available cages from Allentown were modified to prevent direct contact between the two ferrets. A top-down view of the modified cage illustrates the location of the infected and naïve ferret in relation to the airflow (A). A door containing separate water and feeding tray for each ferret (B) and a perforated stainless steel panel (C) prevented any contact between the ferrets.
(TIFF)

Figure S4 The 2009 pandemic H1N1 virus and precursors share receptor specificity and affinity. An in vitro receptor-binding assay using desialylated chicken RBCs was used to determine the receptor binding of the Rec pH1N1, 6:2 reassortant, TRS, and Eurasian swine viruses (A). Viruses with differential receptor specificity, previously identified by MedImmune, were used as controls in the receptor-binding assay. The α2,3 standard is A/Japan/305/1957 (H2N2) Q226, G228 and the α2,6 standard is A/Japan/305/1957 (H2N2) L226, S228. Receptor affinity was assessed by agglutination of partially desialylated RBCs (B). Viruses defined previously to have differential receptor affinity [59] were used as standards.
(TIFF)

Table S1 Summary of clinical signs in infected and naïve ferrets.
(DOC)

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Author Contributions

Conceived and designed the experiments: SSL EWL ALS YM KS. Performed the experiments: SSL EWL ALS WW CPS LV. Analyzed the data: SSL ALS. Contributed reagents/materials/analysis tools: ALS WGL HJ. Wrote the paper: SSL.

References

1. Morens DM, Taubenberger JK (2011) Pandemic influenza: certain uncertainties. Rev Med Virol 21: 262–284.
2. Centers for Disease Control and Prevention (CDC) (2009) Outbreak of swine-origin influenza A (H1N1) virus infection - Mexico, March-April 2009. Morb Mortal Wkly Rep 58: 467–470.
3. WHO (2010) WHO Global Alert and Response: Pandemic (H1N1) 2009 - update 101, weekly update.
4. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, et al. (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279: 393–396.
5. Komadina N, Roque V, Thawatsupha P, Rimando-Magalong J, Waicharoen S, et al. (2007) Genetic analysis of two influenza A (H1) swine viruses isolated from humans in Thailand and the Philippines. Virus Genes 35: 161–165.
6. Myers KP, Olsen CW, Gray GC (2007) Cases of swine influenza in humans: a review of the literature. Clin Infect Dis 44: 1084–1088.
7. Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931–939.
8. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, et al. (2009) Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. N Engl J Med 360: 2616–2625.
9. Palase P, Shaw ML (2007) Orthomyxoviridae: The viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, et al. (2007) Fields Virology. 5th Edition. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. pp 1647–1689.
10. Rossman JS, Jing X, Leser GP, Lamb RA (2010) Influenza virus M2 protein mediates ESCRT-independent membrane scission. Cell 142: 902–913.
11. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, et al. (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325: 197–201.
12. Chou YY, Albrecht RA, Pica N, Lowen AC, Richt JA, et al. (2011) The M segment of the 2009 new pandemic H1N1 influenza virus is critical for its high transmission efficiency in the guinea pig model. J Virol 85: 11235–11241.
13. Yen HL, Liang CH, Wu CY, Forrest HL, Ferguson A, et al. (2011) Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proc Natl Acad Sci U S A 108: 14264–14269.
14. Belser JA, Maines TR, Tumpey TM, Katz JM (2011) Influenza A virus transmission: contributing factors and clinical implications. Expert Rev Mol Med 12: e39.
15. Subbarao EK, London W, Murphy BR (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67: 1761–1764.
16. Lowen AC, Steel J, Mubareka S, Carnero E, Garcia-Sastre A, et al. (2009) Blocking interhost transmission of influenza virus by vaccination in the guinea pig model. J Virol 83: 2803–2818.
17. Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, et al. (2007) A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 315: 655–659.
18. Gao Y, Zhang Y, Shinya K, Deng G, Jiang Y, et al. (2009) Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog 5: e1000709.
19. Van Hoeven N, Pappas C, Belser JA, Maines TR, Zeng H, et al. (2009) Human HA and polymerase subunit PB2 proteins confer transmission of an avian influenza virus through the air. Proc Natl Acad Sci U S A 106: 3366–3371.
20. Herfst S, Chutinimitkul S, Ye J, de Wit E, Munster VJ, et al. (2011) Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. J Virol 84: 3752–3758.
21. Maines TR, Chen LM, Van Hoeven N, Tumpey TM, Blixt O, et al. (2011) Effect of receptor binding domain mutations on receptor binding and transmissibility of avian influenza H5N1 viruses. Virology 413: 139–147.
22. Blachere FM, Lindsley WG, Pearce TA, Anderson SE, Fisher M, et al. (2009) Measurement of airborne influenza virus in a hospital emergency department. Clin Infect Dis 48: 438–440.
23. Lindsley WG, Blachere FM, Davis KA, Pearce TA, Fisher MA, et al. (2010) Distribution of airborne influenza virus and respiratory syncytial virus in an urgent care medical clinic. Clin Infect Dis 50: 693–698.
24. Yang W, Elankumaran S, Marr LC (2011) Concentrations and size distributions of airborne influenza A viruses measured indoors at a health centre, a day-care centre and on aeroplanes. J R Soc Interface 8: 1176–1184.
25. Troko J, Myles P, Gibson J, Hashim A, Enstone J, et al. (2011) Is public transport a risk factor for acute respiratory infection? BMC Infect Dis 11: 16.
26. Shaman J, Pitzer VE, Viboud C, Grenfell BT, Lipsitch M (2010) Absolute humidity and the seasonal onset of influenza in the continental United States. PLoS Biol 8: e1000316.
27. Lowen AC, Mubareka S, Steel J, Palese P (2007) Influenza virus transmission is dependent on relative humidity and temperature. PLoS Pathog 3: 1470–1476.
28. Yang W, Marr LC (2011) Dynamics of airborne influenza a viruses indoors and dependence on humidity. PLoS One 6: e21481.
29. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, et al. (2009) In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460: 1021–1025.
30. Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, et al. (2009) Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science 325: 484–487.
31. Munster VJ, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, et al. (2009) Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science 325: 481–483.
32. Belser JA, Gustin KM, Maines TR, Blau DM, Zaki SR, et al. (2011) Pathogenesis and transmission of triple-reassortant swine H1N1 influenza viruses isolated before the 2009 H1N1 pandemic. J Virol 85: 1563–1572.
33. Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, et al. (2006) Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A 103: 12121–12126.
34. Fabian P, McDevitt JJ, DeHaan WH, Fung RO, Cowling BJ, et al. (2008) Influenza virus in human exhaled breath: an observational study. PLoS One 3: e2691.
35. Lindsley WG, Blachere FM, Thewlis RE, Vishnu A, Davis KA, et al. (2010) Measurements of airborne influenza virus in aerosol particles from human coughs. PLoS One 5: e15100.
36. Mubareka S, Lowen AC, Steel J, Coates AL, Garcia-Sastre A, et al. (2009) Transmission of influenza virus via aerosols and fomites in the guinea pig model. J Infect Dis 199: 858–865.
37. Gustin KM, Belser JA, Wadford DA, Pearce MB, Katz JM, et al. (2011) Influenza virus aerosol exposure and analytical system for ferrets. Proc Natl Acad Sci U S A 108: 8432–8437.
38. Shtyrya YA, Mochalova LV, Bovin NV (2009) Influenza Virus Neuraminidase: Structure and Function. Acta Naturae 2: 26–32.
39. Baenziger JU, Fiete D (1979) Structure of the complex oligosaccharides of fetuin. J Biol Chem 254: 789–795.
40. Bourmakina SV, Garcia-Sastre A (2003) Reverse genetics studies on the filamentous morphology of influenza A virus. J Gen Virol 84: 517–527.
41. Hughey PG, Roberts PC, Holsinger LJ, Zebedee SL, Lamb RA, et al. (1995) Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology 212: 411–421.
42. Smirnov Yu A, Kuznetsova MA, Kaverin NV (1991) The genetic aspects of influenza virus filamentous particle formation. Arch Virol 118: 279–284.
43. Jin H, Leser GP, Zhang J, Lamb RA (1997) Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. Embo J 16: 1236–1247.
44. Jayaraman A, Pappas C, Raman R, Belser JA, Viswanathan K, et al. (2011) A single base-pair change in 2009 H1N1 hemagglutinin increases human receptor affinity and leads to efficient airborne viral transmission in ferrets. PLoS One 6: e17616.
45. Hensley SE, Das SR, Bailey AL, Schmidt LM, Hickman HD, et al. (2009) Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift. Science 326: 734–736.
46. Suguitan AL, Jr., Marino MP, Desai PD, Chen LM, Matsuoka Y, et al. (2009) The influence of the multi-basic cleavage site of the H5 hemagglutinin on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus. Virology 395: 280–288.
47. Verreault D, Moineau S, Duchaine C (2008) Methods for sampling of airborne viruses. Microbiol Mol Biol Rev 72: 413–444.
48. O'Donnell CD, Subbarao K (2011) The contribution of animal models to the understanding of the host range and virulence of influenza A viruses. Microbes Infect 13: 502–515.
49. Reuman PD, Keely S, Schiff GM (1989) Assessment of signs of influenza illness in the ferret model. J Virol Methods 24: 27–34.

50. Steel J, Lowen AC, Mubareka S, Palese P (2009) Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog 5: e1000252.
51. Sorrell EM, Wan H, Araya Y, Song H, Perez DR (2009) Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc Natl Acad Sci U S A 106: 7565–7570.
52. Chen Z, Wang W, Zhou H, Suguitan AL, Jr., Shambaugh C, et al. (2010) Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. J Virol 84: 44–51.
53. Min JY, Chen GL, Santos C, Lamirande EW, Matsuoka Y, et al. (2010) Classical swine H1N1 influenza viruses confer cross protection from swine-origin 2009 pandemic H1N1 influenza virus infection in mice and ferrets. Virology 408: 128–133.
54. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. Am J Hyg 27: 493–497.
55. Chen GL, Min JY, Lamirande EW, Santos C, Jin H, et al. (2011) Comparison of a live attenuated 2009 H1N1 vaccine with seasonal influenza vaccines against 2009 pandemic H1N1 virus infection in mice and ferrets. J Infect Dis 203: 930–936.
56. Lambre CR, Terzidis H, Greffard A, Webster RG (1990) Measurement of anti-influenza neuraminidase antibody using a peroxidase-linked lectin and microtitre plates coated with natural substrates. J Immunol Methods 135: 49–57.
57. Joseph T, McAuliffe J, Lu B, Vogel L, Swayne D, et al. (2008) A live attenuated cold-adapted influenza A H7N3 virus vaccine provides protection against homologous and heterologous H7 viruses in mice and ferrets. Virology 378: 123–132.
58. Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, et al. (2005) A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 79: 11533–11536.
59. Hensley SE, Das SR, Gibbs JS, Bailey AL, Schmidt LM, et al. (2011) Influenza A virus hemagglutinin antibody escape promotes neuraminidase antigenic variation and drug resistance. PLoS One 6: e15190.