Minimum Estimate of Number of Laboratories in the Influenza PPP Research Enterprise

Lynn C. Klotz, Ph.D.
Senior Science Fellow
Center for Arms Control and Non-proliferation
820 1st Street NE, Suite LL-180
Washington, D.C. 20002

Home: 5 Duley Street
Gloucester MA 01930
E-mail: lynnklotz@live.com

Introduction

Research of the Highest Concern (RoHC) is defined as laboratory-creation, laboratory-enhancement, or subsequent research with mammalian-airborne-transmissible (mat) or mammalian-contact transmissible[1] (mct) highly pathogenic influenza viruses. The RoHC categories include highly pathogenic avian influenza viruses that are mat or mct, and past human pandemic influenza viruses for which population immunity (herd immunity) may have been lost. These are among the most worrisome potential pandemic pathogens (PPPs), as they could seed a world-wide pandemic with high fatalities.

The focus here is identifying the number of entities[2] creating, enhancing or researching RoHC, dubbed the "The Influenza PPP Research Enterprise". The greater the number of entities in the Enterprise, the greater the risk of release of a potential pandemic influenza virus into the community; and subsequently, the greater the risk that the release seeds a large outbreak or pandemic.

From the analysis here of Pub Med abstracts from 2012 through November 2018, seventeen RoHC publications from fourteen different Enterprise laboratories were found.

Obtaining a conservative estimate of the number of entities in the Enterprise is important for the calculation of the probability of a laboratory release into the community from an Enterprise lab.

RoHC categories for analysis

The three influenza RoHC categories for this analysis are:

- RoHC 1. Highly-pathogenic-avian-influenza (HPAI) H5N1 and H7N9 viruses that are mammalian-airborne-transmissible (matH5N1 and matH7N9) or are mammalian-contact-transmissible (mctH5N1 and mctH7N9).
- RoHC 2. Other HPAI that are mammalian-airborne-transmissible or are contact transmissible (matHPAI and mctHPAI). The avian viruses looked at here will be restricted to those that have

caused human infections in the past (H5N6, H10N8, H6N1, H1N2, H3N2, H10N7, H7N3, H7N2, H1N2, H7N7, H9N2, H1N2, H1N1, H2N2).

- RoHC 3. Human influenza viruses which have caused past pandemics and for which there is little immunity in the population at present (1918 H1N1, 1957 H2N2), or have been modified (enhanced) to escape immunity and vaccines (2009 H1N1 or 1968 H3N2).

Also briefly discussed are pdm09 H1N1 enhanced viruses that may have higher reproductive number (R_0) than the wild type virus, thus shifting the threshold for herd-immunity that could seed a new outbreak. These would fall into the RoHC 3 category.

Identifying influenza RoHC publications through Pub Med searches is a lengthy, multistep process. A description of the process may be found in Appendix 1.

It is likely that the Pub Med search with over a dozen search words has indeed identified most RoHC publications. What might be missed?

- Using more and wider search terms will download many dozen abstracts where one or two might yield additional RoHC laboratories.
- Unpublished research will be missed.
- Publications in languages other than English may not always be listed in Pub Med. In particular, some Chinese and other Asian research might be missed. Asia is the source of human fatalities from H5N1 and H7N9 avian influenza infections, and much of the recent RoHC seems to be carried out there.

The weeks of extra effort to find a few additional Research Enterprise entities are really not necessary as the risk of a pandemic is already intolerably too high in an Enterprise with fourteen entities. In business market research, unexplored markets are often called “upside market potential.” In analogy, unidentified RoHC entities might be appropriately called “upside RoHC entities,” indicating that it would be nice to have the data but it is not necessary to make the intolerable-risk case.

There appear to be many publications relevant to the RoHC focus from years before 2012; but a limit is needed as to what this project will focus on. The focus here is the recent past and the future, that is, the Enterprise --the collection of entities with ongoing work that creates, enhances or researches potential pandemic influenza viruses. With this focus in mind, the pre-2012 research will not be considered.

Analysis of the spreadsheet

A spreadsheet with considerable data from the RoHC publications is available upon request. The following three Tables present some of the spreadsheet data. Publication titles from the Pub Med search along with publication year and RoHC class are listed in Table 1. The Tracking Number is simply a device to connect the publications through the three tables.

Tracking Number	Article Title	Pub Year	RoHC class
1	Airborne transmission of influenza A/H5N1 virus between ferrets	2012	RoHC1
2	In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity	2012	RoHC1
3	Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets	2012	RoHC1
4	N-linked glycosylation of the hemagglutinin protein influences virulence and antigenicity of the 1918 pandemic and seasonal H1N1 influenza A viruses	2013	RoHC3
5	Reassortment between Avian H5N1 and human influenza viruses is mainly restricted to the matrix and neuraminidase gene segments	2013	RoHC1, RoHC3
6	H5N1 hybrid viruses bearing 2009/H1N1 virus genes transmit in guinea pigs by respiratory droplet	2013	RoHC1, RoHC3
7	Selection on haemagglutinin imposes a bottleneck during mammalian transmission of reassortant H5N1 influenza viruses	2013	RoHC1
8	Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus	2014	RoHC1
9	Circulating avian influenza viruses closely related to the 1918 virus have pandemic potential	2014	RoHC3
10	Airborne Transmission of Highly Pathogenic H7N1 Influenza Virus in Ferrets	2014	RoHC1
11	Adaptation of H9N2 AIV in guinea pigs enables efficient transmission by direct contact and inefficient transmission by respiratory droplets	2015	RoHC2
12	Testing the Effect of Internal Genes Derived from a Wild-Bird-Origin H9N2 Influenza A Virus on the Pathogenicity of an A/H7N9 Virus	2015	RoHC1
13	Mutations Driving Airborne Transmission of A/H5N1 Virus in Mammals Cause Substantial Attenuation in Chickens only when combined	2017	RoHC1
14	A Single-Amino-Acid Substitution at Position 225 in Hemagglutinin Alters the transmissibility of Eurasian Avian-Like H1N1 Swine Influenza Virus in Guinea Pigs	2017	RoHC2
15	A highly pathogenic avian-derived influenza virus H5N1 with 2009 pandemic H1N1 internal genes demonstrates increased replication and transmission in pigs	2017	RoHC1, RoHC3
16	The T160A hemagglutinin substitution affects not only receptor binding property but also transmissibility of H5N1 clade 2.3.4 avian influenza virus in guinea pigs	2017	RoHC1
17	Comparison of the virulence and transmissibility of canine H3N2 influenza viruses and characterization of their canine adaptation factors	2018	RoHC2

Table 1. List of publications along with publication year and RoHC class from Pub Med for research of highest concern (RoHC) categories 1, 2 and 3, for 2012 through November 2018. The tracking number provides a means of correlating data in other tables with the publications.

Seventeen publications were identified. Almost all Table 1 publications can be downloaded simply by searching only the title on the Internet. The titles are listed in Word format for copying purposes in the End Notes[3]. This is a big change from two years ago, when most publications were only available through a journal subscription or for a fee. Three table entries (publications 5, 6, and 15) are listed as both RoCH1 and RoHC3. The reason is that the research creates reassortants of H5N1 with past pandemic or seasonal influenza viruses.

In Table 2 are data from the spreadsheet that lists the entities (institutions) and nations involved in the research. There are 14 different entities in the Enterprise carrying out RoHC.

Tracking Number	Countries of Research	Institution where BSL Research Performed
1	Netherlands, UK, US	Erasmus Medical Center, Rotterdam Netherlands
2	US	Influenza Division, CDC, Atlanta, GA US
3	US, Japan	University of Wisconsin, Madison WI, US
4	USA	Centers for Disease Control, Atlanta GA US
5	Netherlands	Erasmus Medical Center, Rotterdam Netherlands
6	China	Harbin Veterinary Research Institute, Harbin China
7	US, Japan	University of Wisconsin, Madison WI, US
8	Netherlands	Erasmus Medical Center, Rotterdam Netherlands
9	Japan, US, UK,	Universities of Tokyo, Japan and Wisconsin, Madison US
10	USA, Italy	University of Maryland, College Park MD
11	China	Military Veterinary Research Institute, Changchun China
12	China, US	Research Center for Wildlife Diseases, Beijing, China
13	Netherlands	Erasmus Medical Center, Rotterdam Netherlands
14	China, Japan	Harbin Veterinary Research Institute, Harbin China
15	US	National Animal Disease Center, Ames, Iowa
16	China	Yangzhou University, Yangzhou China
17	Republic of Korea	Chungbuk National University, Cheongju Korea

Table 2. Spreadsheet listing the 14 different entities and the nations participating in the 17 RoHC research studies.

Table 3 presents brief quotes from the 17 RoHC publications, which provide some indication of their RoHC status. For a better understanding, their abstracts and full publications must be read.

	Tracking Number		Selected Article Quotes
	1		"Using a combination of targeted mutagenesis followed by serial virus passage in ferrets, we investigated whether A/H5N1 virus can acquire mutations that would increase the risk of mammalian transmission" "Our experimental rationale to obtain transmissible A/H5N1 viruses was to select a mutant…A/H5N1 virus with receptor specificity for a-2,6–linked SA…" "[W]e investigated whether airborne-transmissible viruses were present in the heterogeneous virus population generated during virus passaging in ferrets"
	2		" By in vitro selection for binding α2-6 sialosides, we identified…a mutant virus combining Q196R with mutations from previous pandemic viruses (Q226L and G228S) revealed predominantly α2-6 binding. Unlike the wild type H5N1, this mutant virus was transmitted by direct contact in the ferret model although not by airborne respiratory droplets. However, a reassortant virus with the mutant hemagglutinin, a human N2 neuraminidase and internal genes from an H5N1 virus was partially transmitted via respiratory droplets."
	3		"We identified a reassortant virus with H5 HA possessing four mutations in a 2009 pandemic H1N1 virus backbone capable of droplet transmission in a ferret model…" "The mutant H5 HA reassortant (H5HA-mutant/pdm09) was capable of respiratory droplet transmission in ferrets. "The mutant H5 HA reassortant (H5HA-mutant/pdm09) was capable of respiratory droplet transmission in ferrets… "
	4		"[R]ecombinant viruses containing WT and mutant HA genes of SI/06 or 1918 virus [were] generated on a PR8 backbone…"[M]ice [were] infected with the 1918-HA-WT virus or glycan addition mutant viruses…" Further characterization of 1918 recombinant viruses in mice and human airway cells revealed that the 1918 HA gene is essential for virulence and maximum virus replication of this pandemic strain."
	5		"[W]e investigated the ability of HPAI H5N1 and contemporary human H3N2, H1N1 and p[andemic]H1N1 influenza viruses to reassort. "The HA gene of these human viruses was omitted to make sure that only viruses with the HA of H5N1 virus were generated" ""[N]o aerosol or respiratory droplet transmission was detected…" [*LK note: A no transmission result is beside the point. The experiments could have produced mat viruses, Thus, these experiments are RoHC*]
	6		"Using reverse genetics, we systematically created 127 reassortant viruses between a duck isolate of H5N1, specifically retaining its hemagglutinin (HA) gene throughout, and a highly transmissible, human-infective H1N1 virus. "Transmission studies showed that the H1N1 virus genes encoding acidic polymerase and nonstructural protein made the H5N1 virus transmissible by respiratory droplet between guinea pigs without killing them.
	7		""Here we evaluate the impact of within-host viral genetic diversity on the replication and transmission of H5N1 reassortant viruses described in our previous study" "To evaluate transmissibility in ferrets, we …used a virus isolate called VN1203-HA(3)-CA04). [E]ach infected ferret was paired with an uninfected 'contact' ferret placed in an adjacent cage that prevented direct contact between the animals but permitted airborne droplet transmission of the influenza virus. VN1203-HA(3)-CA04 was transmitted between animals." Note that no new virus transmission experiments were conducted for this study." [*LK note: Transmission studies only involved mat viruses from previous study, so this study further researched mat ferrets.*
	8		"Here, five substitutions proved to be sufficient to determine this [A/H5N1 influenza virus] airborne transmission phenotype.
	9		"[W]e generated and characterized a virus composed of avian influenza viral segments with high homology to the 1918 [human pandemic] virus. This virus exhibited pathogenicity in mice and ferrets higher than that in an authentic avian influenza virus. Further, acquisition of seven amino acid substitutions in the viral polymerases and the hemagglutinin surface glycoprotein conferred respiratory droplet transmission to the 1918-like avian virus in ferrets." "The 1918-like avian influenza virus showed…pathogenicity [in mouse and ferret models] higher than that of an authentic avian influenza virus."
	10		"[A] fully avian H7N1 virus was adapted to become capable of airborne transmission in ferrets. The virus also showed transmission to all cohoused animals""
	11		"[W]e have adapted an avian H9N2 virus that initially displayed an ability to recognize both α 2,3 and α 2,6-linked SA residues via serial passage in guinea pigs. Three amino acid substitutions introduced into the H9N2 AIV during serial were sufficient to increase contact transmission efficiency between guinea pigs." "These results suggest that the adapted H9N2 virus is less efficiently transmitted by respiratory. droplets in guinea pigs and ferrets as compared to transmission by direct contact."
	12		"[We] generated 63 H7N9 reassortants derived from an avian H7N9 and a wild-bird-origin H9N2 virus. Compared with the wild-type parent, 25/63 reassortants had increased pathogenicity in mice. [T]hree substitutions associated with adaptation to mammals conferred airborne transmission to the virus." "These results imply that these H7N9 mutants are transmitted between guinea pigs by airborne respiratory droplets."
	13		"A fully avian A/H5N1 influenza virus was previously shown to acquire airborne transmissibility between ferrets upon accumulation of five or six substitutions…" "The virus containing all substitutions associated with transmission in mammals was highly attenuated in chickens." "This data indicate that an A/H5N1 virus that is airborne-transmissible between mammals is unlikely to emerge in chickens…"
	14		"[W]e explored the molecular basis for transmission of Eurasian avian-like H1N1 (EAH1N1) swine influenza viruses by comparing two viruses that are genetically similar but differ in their transmissibility in guinea pigs… We used reverse genetics to generate a series of reassortants and mutants in the GX/18 background and tested their transmissibility in guinea pigs. [T]he substitution of E for G at the same position (G225E) in HA1 enabled HLJ/27 to transmit in guinea pigs."
	15		"We observed a relative increase in infectivity and transmission in swine of an HPAI-derived H5N1 following incorporation of H1N1pdm09 internal genes" "Direct-contact pigs were introduced…Virus was isolated from all contact pigs exposed to NY/09- challenged pigs…
	16		"We generated and characterized site-directed HA mutants…of H5N1 in order to identify the key determinants in hemagglutinin rendering the dual affinity to both α-2,3 (avian-type) and α-2,6 (human-type)linked sialic acid receptors…" "Here, we verified that the T160A mutation enabled H5N1 clade 2.3.4 virus to not only acquire binding affinity for human-type glycans but also transmit among guinea pigs."
	17		"[A]mong the reassortant virus infected dogs, the LPM91-Ca-NA/NP/M virus showed the highest viral titer in nasal washes and was readily transmitted into [respiratory droplet]-contact animals"

Table 3. Brief quotes from the publications listed in Table 1.

Lynn Klotz
February 2019

Laboratory-enhanced herd-immunity-threshold increase

The recent 2009 influenza pandemic (pdm09 H1N1) infected 20-27% of the world's population[4] before it ended. The acquired herd immunity to the virus plus vaccination may not provide protection against new infections from an accidental release into the community of a lab-enhanced, more virulent, and more transmissible strain of pdm09 H1N1.

If enhanced virulence is accompanied by enhanced airborne or contact transmission, then the basic reproductive number, R_0, could increase, creating a higher herd-immunity threshold. A release into the community would then cause the number of people infected to increase substantially until the new threshold is reached. Nearly a billion new world-wide infections with potentially higher case-fatality rates could result since the new strains are more virulent.

The arguments and calculations leading to this conclusion may be found in the publication, Herd Immunity: A Cautionary Note. The research identified in this article should be classified as research of concern as well.

Other research of concern

There are other kinds of research that should receive oversight. In particular, the so-called Fink report, *Biotechnology Research in an Age of Terrorism*, broadly defines research of concern (RoC). The report lists seven categories of (RoC):

> "1. **Would demonstrate how to render a vaccine ineffective**. This would apply to both human and animal vaccines. Creation of vaccine resistant smallpox virus would fall into this class of experiments.
> 2. **Would confer resistance to therapeutically useful antibiotics or antiviral agents.** This would apply to therapeutic agents that are used to control disease agents in humans, animals or crops. Introduction of ciprofloxacin resistance in Bacillus anthracis would fall into this class.
> 3.**Would enhance the virulence of a pathogen or render a nonpathogen virulent.** This would apply to plant, animal, and human pathogens. Introduction of cereolysin toxin gene into Bacillus anthracis would fall into this class.
> 4. **Would increase transmissibility of a pathogen.** This would include enhancing transmission within or between species. Altering vector competence to enhance disease transmission would also fall into this class.
> 5. **Would alter the host range of a pathogen.** This would include making non zoonotics into zoonotic agents. Altering the tropism of viruses would fit into this class.
> 6.**Would enable the evasion of diagnostic/detection modalities.** This could include microencapsulation to avoid antibody-based detection and/or the alteration of gene sequences to avoid detection by established molecular methods.
> 7. **Would enable the weaponization of a biological agent or toxin**. This would include the environmental stabilization of pathogens. Synthesis of smallpox virus would fall into this class of experiments."

The focus of this analysis is categories 4 and 5. For all seven categories, the Fink report recommends institutional or outside review:

> "All the experiments that fall within the seven areas of concern should currently require review by an Institutional Biosafety Committee (IBC)… In most cases…it would either designate the project as acceptable to move forward or as one raising concerns that need further consideration…"

From a perusal of the downloaded Pub Med abstracts, several dozen avian influenza research publications since 2012 would fit the Fink report definition of research of concern for further review. Many publications before 2012 would fit the Fink definition as well.

Identifying and cataloging all the publications in all the categories of the Fink report is much too broad a focus for the analysis here, which is the Enterprise that creates or researches mammalian airborne or contact transmissible highly-pathogenic influenza viruses.

The Enterprise research risk in the context of other pandemic risks

Besides a release from a laboratory, there are at least two other routes to an influenza pandemic: (1) A pandemic seeded from reassortants of two influenza viruses in an animal infected simultaneously with both. (2) Evolution in nature of an HPAI to mammalian airborne transmission with high virulence in humans.

Based on occurrence of pandemics in the last hundred years, the likelihood is lower of a pandemic from a release from an Enterprise laboratory but within an order of magnitude of the likelihood of a natural pandemic, likely closer. Therefore, concern over an Enterprise laboratory release should rival our grave concern over a natural pandemic. The difference being that a pandemic from a lab release might be prevented using alternative research approaches not employing live virus; whereas, it is difficult to nearly impossible to prevent a natural pandemic.

In a detailed analysis, *The Potential for Respiratory Droplet–Transmissible A/H5N1 Influenza Virus to Evolve in a Mammalian Host*, several scientists, working with Fouchier and Yamamoto, have attempted to calculate the probability of evolution of avian HPAI H5N1 virus to human airborne transmission. They note that "As few as five amino-acid substitutions, or four with reassortment, might be sufficient for mammal-to-mammal transmission through respiratory droplets." The goal of the analysis was to calculate the probability of this occurring in nature. After their considerable work, they concluded there was not enough data available to calculate a probability.

Lynn Klotz
February 2019

APPENDIX 1

(Methodology for identifying RoHC)

Pub Med abstracts from 2012 through mid-November 2017 were downloaded into a single Word document, and Pub Med abstracts for 2018 were downloaded separately. A number of search terms were employed to identify Pub Med abstracts, which yielded a few hundred-page Word document. The employed search terms along with the number of publications found, in parentheses, were:
For the period 2012 through mid- November 2017:

- mammalian transmissible HPAI (9 items)
- mammalian transmissible H5N1 (95 items)
- mammalian transmissible H7N9 (63 items)
- mutagenesis 1918 H1N1 (24 items)
- mutagenesis 1957 H2N2 (4 items)
- mammalian transmissible H5N6 (3 items)
- mammalian transmissible H10N8 (2 items)
- reassortant HPAI (68 items)
- recombination HPAI mammals (33 items)
- reassortant HPAI mammals (43 items)
- mammal transmissible avian influenza (items 123)
- reverse genetics H5N1 mammalian transmissible (items 5)
- reverse genetics H7N9 mammalian transmissible (items 3)

Many of the abstracts appeared more than once, so extra copies were deleted from the Word document, leaving a 100-page Word document. The remaining abstracts were quickly scanned to delete the obviously irrelevant ones, leaving an over twenty-page Word document. The remaining Abstracts were read; and for each, the publications were downloaded and relevant-text read to verify that the research was indeed RoHC.

For the first eleven months of 2018:

- mammalian transmissible HPAI (0 items)
- mammalian transmissible H5N1 (1 item)
- mammalian transmissible H7N9 (1 item)
- mutagenesis 1918 H1N1 (1 items)
- mutagenesis 1957 H2N2 (0 items)
- mammalian transmissible H5N6 (0 items)
- mammalian transmissible H10N8 (0 items)
- reassortant HPAI (15 items)
- recombination HPAI mammals (1 item)
- reassortant HPAI mammals (6 items)
- mammal transmissible avian influenza (4 items)
- reverse genetics H5N1 mammalian transmissible (0 items)
- reverse genetics H5N1 (used only for 2018 search) (9 items)

Lynn Klotz
February 2019

An Excel spreadsheet of important data was created for statistical study.

---

[1] Contact transmissibility is determined by placing infected mammals in the same cage with uninfected mammals, then observing if the uninfected mammals become infected. Airborne transmissibility is determined by placing infected mammals in cages separated by a short distance from cages with uninfected mammals.

[2] Here, the words "entity" and facility have the same meaning. Entity or facility are the terms used by the Federal Select Agent Program. Each entity may have a number of high-containment laboratories. It is usually an entity official, not laboratory Principal Investigators, who reports accidents and incidents to the FSAP and NIH

[3] Airborne transmission of influenza A/H5N1 virus between ferrets
In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity
Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets
N-linked glycosylation of the hemagglutinin protein influences virulence and antigenicity of the 1918 pandemic and seasonal H1N1 influenza A viruses
Reassortment between Avian H5N1 and human influenza viruses is mainly restricted to the matrix and neuraminidase gene segments
H5N1 hybrid viruses bearing 2009/H1N1 virus genes transmit in guinea pigs by respiratory droplet
Selection on haemagglutinin imposes a bottleneck during mammalian transmission of reassortant H5N1 influenza viruses
Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus
Circulating avian influenza viruses closely related to the 1918 virus have pandemic potential
Airborne Transmission of Highly Pathogenic H7N1 Influenza Virus in Ferrets
Adaptation of H9N2 AIV in guinea pigs enables efficient transmission by direct contact and inefficient transmission by respiratory droplets
Testing the Effect of Internal Genes Derived from a Wild-Bird-Origin H9N2 Influenza A Virus on the Pathogenicity of an A/H7N9 Virus
Mutations Driving Airborne Transmission of A/H5N1 Virus in Mammals Cause Substantial Attenuation in Chickens only when combined
A Single-Amino-Acid Substitution at Position 225 in Hemagglutinin Alters the transmissibility of Eurasian Avian-Like H1N1 Swine Influenza Virus in Guinea Pigs
A highly pathogenic avian-derived influenza virus H5N1 with 2009 pandemic H1N1 internal genes demonstrates increased replication and transmission in pigs
The T160A hemagglutinin substitution affects not only receptor binding property but also transmissibility of H5N1 clade 2.3.4 avian influenza virus in guinea pigs
Comparison of the virulence and transmissibility of canine H3N2 influenza viruses and characterization of their canine adaptation factors

[4] *One-fifth of the world had swine flu due to 2009 pandemic – WHO report*, https://www.rt.com/news/swine-flu-pandemic-who-815/