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Cc:	Kawaoka; Vickie Groth
Subject:	Responses to Gryphon"s questions
Date:	Sunday, July 5, 2015 11:42:33 AM
Attachments:	GoF site visit interview goals v2 - With Responses_Final.docx Interview Guide for BioSecurity Task-Gryphon_With Responses_Final.docx

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Dear All,

Please see the attached documents.

I have included a revised version of the response document that everyone has been working on, and also a second document with answers to biosecurity questions.

See you all tomorrow,

Amie

Prepared by:
Gryphon Scientific, LLC
Rocco Casagrande, PI
Corey Meyer, Benefit Assessment Task Lead
Ryan Ritterson, Risk Modeling Task Lead
June 17, 2015

Responses to specific inquiries (in blue text) have been provided by members of the Influenza Research Institute (IRI), UW-Madison officials, and Madison public health officials.
July 3, 2015

Interview Guide for Gain-of-Function Risk and Benefit Assessments, Research Laboratory Site Visits

Background

Gryphon Scientific has been asked on behalf of the NIH Office of Science Policy to conduct formal risk and benefit assessments (RBA) of "gain of function" (GoF) research involving pathogens with pandemic potential. The results of the RBA will inform the NSABB in their development of recommendations to the USG regarding the appropriate level of Federal oversight of GoF research.

As guided by the NSABB's "Framework for Conducting Risk and Benefit Assessments of Gain-of-Function Research," our assessment will focus on evaluation of research involving influenza viruses, SARS-CoV, and MERS-CoV and will include experimental approaches that are reasonably anticipated to confer one or more of the following phenotypic changes to wildtype pathogens:

- Enhanced transmission in mammals, including altered routes of transmission and increased host or tissue tropism;
- Enhanced morbidity and mortality in appropriate animal models;
- Enhanced pathogen production as a result of changes in the replication cycle or growth;
- Resistance to anti-virals or evasion of vaccines and other medical countermeasures (MCMs);
- Evasion of existing natural or induced immunity.

We'll be evaluating all experimental approaches that could produce any of the phenotypic changes listed above, including serial passaging of virus in animals or cells, reverse genetics and reassortment studies, various types of selection, and other approaches.

Although our assessments focus on influenza viruses, SARS, and MERS (hereafter referred to collectively as "PPPs"), we welcome relevant examples featuring other pathogens during the course of our discussion, recognizing that many types of basic research applications are not pathogen-specific.

Interview goals related to the Benefit Assessment (BA)

Our first set of interview questions related to the BA will focus on elucidating gaps in scientific knowledge about PPPs and gaps in public health and medical capabilities related to the prevention and control of PPP outbreaks in humans and animals. This information will provide critical context for our evaluation of the potential benefits of GoF research. Questions will address:

- Critical gaps in knowledge about PPPs;

Note that most of the topics listed here are affected by the current Research Pause

Transmission

We are just beginning to understand the mechanisms of adaptation of avian influenza viruses to humans. Recent studies demonstrated that several features (including HA receptor-binding specificity, HA stability, and

replicative ability) are important for respiratory droplet transmission of avian influenza viruses in ferrets, the best model we have for mammalian adaptation. However, we do not have a ***deep and broad dataset*** that would enable us to use these data reliably to assess the risk of emerging zoonotic threats.

Critical gaps in knowledge include the following topics:

- What are the mechanisms of adaptation of avian influenza viruses to mammals?
  - Role of HA receptor-binding specificity
  - Role of HA stability (note, the significance of HA stability for virus transmission was only discovered through GOF experiments)
  - Role of polymerase complex
  - Potential role of other viral genes/proteins

Pathogenicity

The viral and host factors that determine influenza virus pathogenicity are still poorly understood. A number of (GoF) studies have identified several markers of pathogenicity, but it is almost certain that additional determinants of pathogenicity exist.

Critical gaps in knowledge include the following topics:

- What are the *viral* determinants of pathogenicity (such as HA, NS1, PB1-F2, polymerases)?
  - Which of these determinants are 'global' (such as PB2-627K)?
  - Which of these determinants are subtype- or strain-specific?
  - Which of these determinants 'cross-talk' (compensatory mutations)?
- What are the *host* determinants of pathogenicity?
  - Do they differ among age-groups, gender, ethnicity, etc.?
  - What are the host determinants responsible for severe infections in high-risk groups?

Emergence of pathogens with pandemic potential

Currently, we do not understand the mechanisms and factors that lead to the emergence of pathogens with pandemic potential. Most likely, several viral properties including transmissibility, pathogenicity, antigenicity, and fitness play a role in the emergence of novel influenza viruses.

Critical gaps in knowledge include the following topics:

- Transmissibility and Pathogenicity: See above
- Reassortment: What are the factors that determine the outcome of reassortment events?
  - (In)Compatibility at the protein level
  - (In)Compatibility at the nucleotide level
- Antigenic Evolution:
  - Which epitopes are critical for antigenic escape?
  - Can antigenic evolution be predicted?
  - How much antigenic difference is needed to create a pandemic (seasonal H1N1 versus pH1N1)?
- Viral Fitness
  - What is the significance of viral fitness in the emergence of novel pandemic viruses
  - What are the factors that determine viral fitness?

- Critical gaps in existing MCMs for PPPs, including vaccines, therapeutics, and diagnostics;

Current Influenza Vaccines

- Sub-optimal overall effectiveness
- Low effectiveness in certain age groups
- Sometimes low yield / low stability
- Need to be updated frequently
- Lack of vaccines to recently emerged viruses (H7N9, novel H5 viruses)
- In case of a pandemic, the production of a novel vaccine will take too long to protect the public

Influenza Therapeutics

- In the US, only two classes of antivirals are approved: Ion channel and neuraminidase inhibitors
    - Ion channel inhibitors are no longer recommended due to wide-spread resistance to these antivirals
- A polymerase inhibitor has been approved in Japan but only for use in the event of a pandemic
- For current therapeutics, a critical gap is the assessment of combination therapies
- Novel therapeutics (such as siRNAs or broadly cross-reactive antibodies) are in various stages of development

- Challenges in development of new MCMs for PPPs.

- ***The GoF Research Pause is the major challenge in the development of new MCMs for PPPs since many critical studies are on hold or cannot be initiated; among others, the following experiments are on hold:***
    - ***Passages of avian influenza viruses in ferrets to identify mutations that confer transmissibility***
    - ***Generation of virus libraries possessing random mutations in an H5 HA and screening of the resulting mutant H5 viruses for their ability to bind to human-type receptors and transmit among ferrets***
    - ***Identification and characterization of mutations that determine the virulence and pathogenicity of highly-pathogenic influenza viruses***
- Limited research funding (for translational research):
- The GoF Research Pause may negatively affect the recruitment of young scientists

Importantly, our BA will be comparative – that is, we will assess the potential benefits of GoF research *relative* to alternative approaches. Thus, our second set of questions related to the BA will focus on elucidating the potential benefits of GoF research, as well as any barriers that impact the likelihood and timing of the realization of the benefits. Questions will address:

- How GoF research addresses gaps in scientific knowledge;

- GoF research enables researchers to identify the factors that are *necessary **and** sufficient* for a biological trait
    - In contrast, LoF research identifies factors that are necessary for a biological traits; however, these factors may not be sufficient; for example, LoF studies demonstrated that certain mutations in HA abolished transmission; however, these mutations did not convert a non-transmissible virus into a transmissible virus
- GoF research identifies new traits (for example, the role of HA stability in transmissibility was not known until this functional connection was identified through GoF studies)

- GoF research lets us mimic virus evolution and the generation of potential PPPs in a controlled, secure environment
  - Many of the biological processes that may affect the emergence of novel pandemic viruses (mutations, reassortment, antigenic escape, fitness, etc.) can be tested before they may occur in nature
  - GoF research may therefore identify potential pandemic traits before they emerge in nature
  - GoF research may also 'forecast' the (antigenic) evolution of influenza viruses, enabling us to generate vaccine candidate before novel strains emerge (see below)
- GoF research may assist in vaccine virus selection:
  - For *seasonal* influenza viruses, GoF research may lead to the predication of novel antigenic clusters; vaccine candidates could then be prepared *before* the novel cluster becomes widespread in nature
  - For both seasonal and pandemic influenza viruses, GoF research may lead to the prioritization of vaccine candidates: For example, more than 25 vaccine candidates are currently available for H5N1 viruses. Antigenic evolution studies, in combination with surveillance data, could identify strains that are more likely to become pandemic than others

- How GoF research can be applied to gaps in public health and/or medicine;

- See above

- How alternative approaches may address similar gaps in knowledge or public health;

- Studies with *individual viral proteins*: Such studies can assess specific traits (such as replication ability of HA stability), but would not provide reliable information about the significance of a mutation or trait in the context of virus replication (for example, an amino acid change in a polymerase protein may have different effects in minireplicon assays and in virulence studies). In addition, such studies would not reveal the inter-relationships among traits (for example, HA stability has been studied for decades, but its significance for transmissibility was not recognized until transmission studies in ferrets revealed the significance of HA stability for virus transmissibility).

- Studies using *replication-incompetent viruses*: We have developed viruses which lack the coding sequence for an essential viral protein such as PB2 or HA, but can be propagated in cell lines expressing the respective protein. While these 'biologically-contained' viruses allow studies in cultured cells, many biologically critical traits (for example, transmissibility) need to be tested in infected animal models.

- Studies in the *genetic background of lab-adapted viruses* (such as A/Puerto Rico/8/34 (PR8), which is commonly used for human vaccine production): Certain mutations or traits can be tested by introducing a viral segment or mutation into the background of PR8. This approach has now been proposed in a number of studies.

- Studies with *viruses with restricted tropism*: Garcia-Sastre et al. generated a virus with a target site for an miRNA expressed in human cells and mice, but not in ferrets. The resulting virus is therefore replication-competent in ferrets, but attenuated in mice. While this is an attractive approach, the effects of the mutations and the genetic stability of the mutant virus should be tested more thoroughly.

- *Loss-of-function (LoF) studies*: LoF and GoF studies are typically carried out side-by-side. As stated earlier, LoF studies alone would identify mutations or traits that may be necessary, but are not sufficient.

- How GoF research may provide unique benefits to knowledge/public health;

The emergence of H7N9 viruses in China, the continued spread of MERS, the recent Ebola virus outbreak, worldwide outbreaks of highly pathogenic avian influenza in poultry (including the introduction of highly pathogenic H5 viruses of several neuraminidase subtypes into North America) have created high levels of concern about pandemic preparations. More and better scientific information is important to developing the critical MCMs needed to effectively prepare for viral threats. We need to learn as much as possible about viral threats as soon as we can.

In summary, GoF studies have been critical to the following achievements:

- Demonstration that H5 viruses can acquire transmissibility in mammals
- Identification of previously unknown traits that confer ferret-transmissibility to avian influenza viruses
- Identification of genes and mutations important for efficient replication of avian influenza viruses in mammals
- Identification of antigenic variants that may cause epidemics in humans

- Concerns related to the likelihood that benefits to public health would be achieved, as well as the timescale for realization of the benefits, including both scientific and non-scientific 'co-factors'
    - An example of the former is uncertainty regarding whether mutations will confer the same phenotypic changes in different genetic contexts; an example of the latter is insufficient surveillance data.

- Benefits may not be realized until a new epidemic or pandemic occurs
- However, *no benefits* will be achieved if the respective experiments cannot be carried out (***with appropriate reviews, containment, training, and approvals in place***)

Interview goals related to the Biosafety Risk Assessment (RA)

In the context of research involving PPPs, our biosafety risk assessment will consider:

- The probability that laboratory accidents lead to loss-of-containment;
- The probability that loss-of-containment leads to human infections and subsequent secondary transmission in the community;
- The consequences of the outbreak should it spread beyond local control.

Questions for Laboratory Personnel

To inform the laboratory accident and initial outbreak modeling components of the RA, we are interested in collecting the following data about the types of experiments you conduct involving influenza viruses, SARS, and MERS:

1) What types of experiments involving influenza/SARS/MERS do you conduct in your lab, including both GoF and alternative approaches?

Note: The list of GoF research provided below includes activities that are currently suspended due to the pause in GoF research. No GoF research is currently being performed at the Influenza Research Institute (IRI) at the UW-Madison.

GoF Research

- Passage of wild-type viruses in ferrets to identify mutations that promote transmissibility
- Generation of virus libraries possessing random mutations in an H5 HA protein and screening of the resulting mutant H5 viruses for their ability to bind to human-type receptors and transmit among ferrets
- Generation and passaging of reassortant viruses to identify non-HA dependent contributors to transmissibility
- Generation and characterization of 1918-like avian influenza viruses
- Introduction of mutations expected to increase pathogenicity or transmissibility of influenza viruses
- Passaging wild-type and 'mutant library viruses' (possessing random mutations in PB2 and/or NP) in MxA- or Mx1-expressing cell lines to obtain Mx1/MxA resistant viruses (Note: MxA/Mx1 is a potent host restriction factor for influenza virus)

Alternative Approaches

Studies not involving viruses

- *In vitro* assays are used to help define whether mutations in specific viral proteins affect known activities. These include minireplicon assays (to assess polymerase activity), budding assays, protein-protein interaction assays, and assays that assess the ability of NS1 to counteract innate immune responses. It is important to note that, while *in vitro* assays can be useful, the *in vitro* activities of viral proteins in isolation do not always reflect the behavior of the same virus proteins in the context of an authentic virus backbone.
- We also use computational analysis of the sequences of natural influenza virus isolates to try to identify sequence determinants of host specificity, pathogenicity and/or transmissibility. While such analyses are important for furthering understanding of these processes, they are limited to assessment of available virus genomic sequences, which are unlikely to fully represent variability in natural influenza virus reservoirs. In addition, computational predictions remain theoretical until they have been experimentally tested by generating virus mutants, at which time, these studies cross over into GoF research.

Studies with viruses

- To test the receptor-binding properties of HA proteins of highly pathogenic avian influenza virus isolates, we are using modified HA genes/proteins that no longer encode a multibasic sequence at the HA cleavage site, in the genetic background of the mouse-adapted A/Puerto Rico/8/34 (PR8, H1N1) virus. Note: The multibasic cleavage site of the HA molecule is a critical pathogenicity determinant, which allows for highly pathogenic avian virus dissemination to non-respiratory sites in the host.
- Mutations expected to increase pathogenicity will be introduced into viruses with modified HA genes/proteins that no longer encode a multibasic sequence at the HA cleavage site.
- We will carry out cell line passaging experiments (e.g., for identification of mutations that confer resistance to MxA/Mx1) with a replication-incompetent virus. Such a virus does not replicate in wild-type cells but can be propagated in a cell line expressing HA, which is available in our group.
- We will perform loss-of-function (LoF) studies, in which mutations that are expected to reduce pathogenicity are introduced into highly pathogenic viruses. It is important to note that, while LoF studies provide an important counterpoint to GoF studies, the results can also be misleading. For example, introducing changes to highly conserved amino acids may lead to virus attenuation, but this attenuation may be unrelated to virulence determinants.
- We study the host response (transcriptome, proteome, metabolome, lipidome) to natural influenza virus isolates in target cells and tissues, and use computational analyses to predict host factors that are critical for the regulation of influenza virus replication and/or pathogenicity. These predictions are then tested (also with natural virus isolates) in cells using RNA interference (RNAi) or protein overexpression, or in knock-out or knock-in mice.

- We are performing a host genetic screen in mice to identify host factors that regulate susceptibility to influenza virus pathogenicity.

2) Please describe the logistical aspects of conducting each type of experiment:
   a. Please walk through the basic protocol, including the containment level in which each step is conducted.
   b. What type of PPE are you wearing during each step?
   c. How much pathogen do you have on hand during the experiment? Please describe volumes and pathogen concentrations. What types of containers are you using (e.g. what type of microcentrifuge tubes, what type of caps, etc.)? [Want units in PFU or TCID50s]
   d. How often do you conduct this experiment?
   i. Note – recognize that experimental activity waxes and wanes throughout the lifetime of a research project. If experiment is performed regularly, then elicit average experiment frequency. If not, then elicit experiment frequently when experiment is being actively performed, and length of time period during which experiment is regularly performed.
   e. If the experiment involves animals, which type, and how many are used at one time, and over the course of a particular experimental series? How long are animals infected during the course of an experiment?
   f. What types of waste does this experiment generate? How is liquid waste disposed of? How is solid waste disposed of? How are animal carcasses handled?

General Considerations for All Types of GoF and non-GoF Experiments: PPE and Waste Disposal

PPE

For work performed in BSL-2 containment at the IRI, researchers use the following PPE:

- Lab coats
- Gloves
- Eye protection

The following PPE is always used in all of our BSL-3 facilities when viruses are in use:

- Dedicated scrubs
- Shoe covers over feet
- Dedicated shoes (garden clogs)
- Shoe covers over shoes
- Tyvek booties or sleeve covers over feet/ankles
- Tyvek suit
- Tyvek shroud
- Powered air purifying respirator (PAPR)
- One pair of nitrile gloves under Tyvek suit sleeve (sleeve hooked over thumb)
- One pair of nitrile gloves over the Tykek suit sleeve

When working with (potentially) mammalian-transmissible influenza viruses, Tyvek sleeve covers are required in addition to all the PPE listed above.

When handling non-anesthetized ferrets at any stage of an experiment (i.e., either before or after infection), researchers wear leather or thin welding gloves to prevent breaks in PPE and/or skin due to ferret bites.

When performing dissecting procedures with razor blades, researchers wear cut-resistant surgical gloves to prevent breaks in PPE and/or skin due to razor cuts. ***Here, it is important to note that the use of razor blades is minimized or eliminated whenever possible, and that all researchers are trained in and utilize additional safety measures whenever sharps are in use. Moreover, the use of any sharp instrument requires the presence of at least two researchers at all times.*

Waste Disposal

For all experiments described below, both solid and liquid wastes contaminated with viruses would be generated, as well as PPE waste and other waste items. Additionally, all experiments with animals will generate contaminated carcasses. Methods for dealing with each waste type are described below.

Solid, virus-contaminated waste is decontaminated with a solution of fresh 5% MicroChem Plus for 1-16 hours inside of a biosafety cabinet (BSC). Waste items are completely submerged in the 5% MicroChem Plus solution, and importantly, the insides of pipette tips and serological pipettes are rinsed with 5% MicroChem Plus prior to submersion. If an item cannot be completely submerged (e.g., the upper ends of serological pipettes), then it is soaked with 70% ethanol. At the end of the decontamination period, solid waste is strained into the sink, and the 5% MicroChem Plus solution is poured down the drain. In the BSL-2 lab, the liquid waste goes directly into the city sewer system. In the BSL-3 labs, the liquid waste goes into the effluent decontamination system (EDS), where it undergoes autoclaving and cooling before release into the city sewer system. Drained, decontaminated solid waste materials are discarded in biohazard trash bags – with the exception of serological pipettes, which are transferred to designated hard plastic containers – and all solid waste materials are autoclaved prior to incineration.

Solid sharps waste is rinsed with 5% MicroChem Plus or 70% ethanol and collected in designated, hard plastic sharps containers inside of BSCs. Syringes are filled with 5% MicroChem Plus prior to discarding into sharps containers. When sharps containers are full, they are soaked with 70% ethanol, closed and taped shut, removed from the BSC, and autoclaved prior to incineration.

Liquid wastes (e.g., virus-contaminated cell culture supernatants) are decontaminated with 5% MicroChem plus (if collected inside of a BSC) or with 10% Vesphene (if collected in a vacuum aspirator device). After 1-16 hours of incubation, liquids are discarded into the sink as just described.

PPE is decontaminated with 70% ethanol prior to removal during facility exit procedures. All PPE are collected in biohazard bags and autoclaved prior to disposal by incineration.

All other paper and plastic waste that are not contaminated with virus are soaked with 70% ethanol prior to disposal in a biohazard bag. This waste is then autoclaved prior to disposal by incineration.

Contaminated mouse cages and water bottles are placed in biohazard bags for autoclaving at the conclusion of an experiment. After autoclaving, bedding and leftover food are discarded and the cages are washed in a cage washer. Cage washing rooms are located outside of BSL-3 containment, but inside the SA-registered space in the IRI facility.

Ferret feces and cage pan liners are discarded in biohazard bags and autoclaved throughout any ferret experiment. At the conclusion of a ferret experiment, cages are disassembled and decontaminated with 1% Virkon, and then rinsed with water.

Animal remains (including eggs) are double-bagged inside of the BSC, and the bags are labeled with the contents (i.e., the type of animal remains and the virus that was used for infection). Bags are then sprayed completely with 70% ethanol and allowed to decontaminate for 10 minutes inside of the BSC. After removal from the BSC, the remains are transferred to a designated animal remains freezer. At the conclusion of an experiment, animal remains are thawed and autoclaved prior to incineration.

Specific Experimental Details – GoF Research

A. ***Passage of wild-type viruses in ferrets*** **–** The purpose of these experiments is to develop novel mammalian transmissible viruses from wild-type, highly pathogenic avian influenza viruses that do not exhibit naturally efficient transmission in mammals (including H5N1 and H7N9 avian influenza viruses). The overarching goals are to promote a deeper understanding of the molecular determinants that promote avian influenza virus transmission, and to determine whether mechanisms that promote transmission are conserved

across different influenza virus strains and subtypes. This information is essential for accurately identifying influenza viruses of concern that already exist in nature, and for preparing countermeasures against natural influenza viruses that pose the greatest threats to humans. To develop novel, mammalian-transmissible viruses, we plan to serially passage wild-type, non-transmissible viruses multiple times in the ferret model, selecting for viruses that exhibit transmissible properties. This work is described in more detail below.

Basic protocol:

- For wild-type virus passaging experiments in ferrets, a stock virus may be prepared (as described in the answer to question 3 below) if such a stock is not already available.
- Passaging experiments are performed with pairs of inoculated and naïve (i.e., "contact") ferrets housed in adjacent, wire-framed cages inside an isolator unit; inoculated and contact ferrets are not able to physically touch each other. The development of infection in a contact ferret indicates virus transmission by the aerosol route, and viruses derived from these ferrets would be preferentially selected for additional passaging experiments. If viruses do not transmit to contact ferrets, then viruses derived from inoculated ferrets may be used for additional passaging experiments.
- Ferrets are inoculated by the intranasal route (in a BSC) after anesthetization and are kept in isolator cages for the duration of the experiment. After inoculation, ferrets are transferred between the BSC and isolator cages in empty, filter-topped rat cages. It should be noted that all ferret experiments are performed in the BSL-3Ag suite, and in this suite, each room is considered primary containment by itself.
- To collect virus for passaging purposes, ferrets are anesthetized and transferred to the BSC at multiple time points after inoculation or co-housing with an inoculated animal, and then 1 ml of PBS (containing antibiotics) is passed through the choanae (via the mouth) and into the nasal cavity by using an animal feeding needle with a bent tip connected to a 3 ml syringe. PBS that is secreted through the nostrils (i.e., the 'nasal wash') is collected in 2 ml screw-cap tubes (with gaskets in the caps) for downstream use.
- The presence of virus in a nasal wash sample is assessed by using standard plaque assays, and only nasal washes containing quantifiable amounts of virus are used for additional ferret inoculation/passaging experiments.
- Virus "Passaging" experiments – Nasal washes from the initial ferret inoculation may be used to directly inoculate additional ferrets; nasal wash virus may be amplified in MDCK cells to create virus stocks that will be used to inoculate additional ferrets; or virus stocks may be prepared from plaque-picks of ferret nasal wash samples and used to inoculate additional ferrets.
- A portion of nasal wash samples or virus stocks may be used to extract viral RNA for sequencing purposes. RNA is extracted using the Qiagen RNeasy kit. Briefly, samples that have been lysed in RLT buffer (provided in the kit) containing β-mercaptoethanol and mixed with 70% ethanol are transferred out of the BSL-3Ag suite (using an established standard operating procedure), and the RNA extraction procedure is completed in BSL-2 containment. The mixture of RLT buffer/β-mercaptoethanol/70% ethanol is a well-established method for inactivating influenza viruses.
- Infected and contact ferrets are bled at the conclusion of each experiment to determine whether the animals sero-converted to the inoculating virus (as determined by hemagglutination inhibition [HI] assays).

Containment: Generation of wild-type virus stocks may be performed in BSL-3Ag or ABSL-3+ containment. Viruses that must be transferred between suites or between rooms in the BSL-3Ag suite are transported using locked plastic containers (whose outer surfaces have been soaked with 70% ethanol) according to approved protocols to prevent spillage or loss of any material. All ferret passaging experiments, nasal wash plaque assays, plaque picking and generation of virus stocks from nasal washes are conducted in BSL-3Ag containment. As described above, RNA extractions are initiated in BSL-3Ag containment and inactivated samples are transferred to BSL-2 containment for the completion of the procedure. Virus-containing samples that need to be centrifuged are placed in gasket-sealed centrifuge containers or rotors, and centrifuges are fitted with HEPA filters. All procedures using viruses are performed inside of a BSC.

Number of animals: Typically, 3 pairs of inoculated and contact ferrets (6 ferrets in total) are used for each round of passaging for each virus that will be passaged. Up to 18 pairs (36 ferrets in total) can be housed simultaneously in isolator units in the IRI BSL-3Ag suite.

Amount of pathogen: Influenza virus stocks typically range in concentration from 10^5 PFU to 10^9 PFU per ml, and stock virus preparations are usually prepared in a 25 ml volume (distributed into 500 µl aliquots). Stock viruses are stored in 2 ml screw-cap tubes with rubber gaskets in the caps. A standard dosage for ferret inoculation is 1×10^6 plaque forming units (PFU) in 0.5 ml of PBS; inocula are prepared in 15 conical tubes. Virus titers in ferret nasal washes range in concentration from 0 PFU to 10^7 PFU per ml.

Duration: Generation and titration of virus stocks takes about 1 week (the majority of this being incubation time). Influenza viruses are typically detected in the upper respiratory tract of ferrets for 3-6 days, and transmission between inoculated and contact ferrets may (theoretically) occur at any point in this time-frame. To accommodate all potential timelines for transmission to contact animals and allow for a full analysis of virus replication in contact animals (particularly for viruses with unknown replication and transmission properties), we usually collect nasal washes for 13 days after inoculation or after housing adjacent to an inoculated animal. Ferrets are bled at 17-21 days post-inoculation for sero-conversion studies prior to euthanasia. In short, each nasal wash sample collection experiment takes about 3 weeks to perform, and 4 weeks are required if a virus stock needs to be generated before starting. Follow-up experiments (i.e., plaque assays, plaque picks, virus stock generation, RNA extractions, HI assays) are often performed in parallel to nasal wash sample collection, and thus, can be completed in 3-4 weeks (Note: Different teams of researchers are responsible for performing ferret procedures and follow-up analyses if these activities are performed in parallel). If follow-up analyses are performed after sample collections have been completed, a 1-4 week time-frame is required depending on the number of samples that need to be analyzed.

Frequency: Each ferret passaging experiment takes about one month to complete, so no more than 12 experiments may be performed in one year. In the absence of any pause in GoF research, it is standard for 6 ferret experiments to be performed at the IRI in one year.

Alternative approaches: No alternative approaches have been identified or approved by the NIAID for the experiments described in this section.

B. Generation of virus libraries possessing random mutations in an H5 HA protein and screening of the resulting mutant H5 viruses for their ability to bind to human-type receptors and transmit among ferrets – The purpose of these experiments is to use a forward genetics approach to identify the spectrum of mutations that promote transmission of avian influenza viruses containing H5 HA proteins (among all influenza virus proteins, HA is the strongest determinant of transmissibility). The overarching goals of these experiments are similar to that described for passaging of wild-type influenza viruses in ferrets (Section A, above). However, importantly, these experiments will further expand knowledge about the molecular determinants of HA-mediated transmission by increasing the total pool of HA mutations that will be analyzed. In brief, we will generate a library of viruses possessing mutations across the entire HA open reading frame and screen this library in ferrets, selecting for mutants that facilitate transmission. Receptor binding properties of selected transmissible viruses will be examined in-depth. This work is described in more detail below.

Basic protocol:

- To generate virus libraries containing random mutations in H5 HA proteins, a plasmid carrying an H5 gene will be subjected to PCR-based random mutagenesis. Then, a virus library stock will be generated by reverse genetics using H5 HA library plasmids in place of an individual H5 HA gene plasmid.
- Original virus library stocks may be used directly to perform ferret transmission experiments, as described above. Alternatively, the virus library may be subjected to one or more rounds of selection – for example, by passaging the library in cells that preferentially express human-like influenza virus receptors – prior to use for ferret inoculation.

- Ferret nasal wash sample collection will be performed as described above. Contact ferret nasal washes exhibiting detectable levels of virus replication (as determined by plaque assays) will be selected for further analyses.
- Viruses will be plaque-purified from contact ferret nasal wash samples and virus stocks will be prepared and titrated by using plaque assays. RNA will be extracted from the virus stock for sequencing analysis.
- To confirm that plaque-purified virus(es) maintain the ability to transmit by the aerosol droplet route, virus stocks from plaque-purified viruses will be used for an additional round of ferret transmission studies. Viruses that maintain transmission capabilities after plaque purification will be selected for in-depth characterization of receptor binding properties.
- Mutant, transmissible viruses will be subjected to one or more receptor binding assays, as follows:
  - *Binding to human respiratory tissue sections:* Virus stocks are incubated with human respiratory tissue sections and virus binding is detected by an immunofluorescence-based method. Stained slides are fixed with 10% formalin (a well-established method for inactivating influenza virus) and are transferred to BSL-2 containment (according to approved protocols) for confocal microscopy analysis.
  - *Solid phase binding assay:* Virus stocks are incubated with plates pre-loaded with α-2,3 or α-2,6 carbohydrate moieties (specific for avian-like and human-like receptor binding properties, respectively), and virus binding is detected by reading absorbance of an immunohistochemical signal. All procedures are performed in the BSL-3Ag suite.
  - *Glycan binding array:* Array assays are performed by a collaborating laboratory on viruses that have been concentrated by ultra-centrifugation and inactivated with betapropiolactone (BPL). BPL-mediated inactivation is a standard procedure used at the IRI, and for each BPL inactivation experiment, inactivation is verified prior to removing samples from BSL-3 containment and shipment to the collaborating laboratory.

Containment: PCR-based mutagenesis for H5 HA library generation is conducted in BSL-2 containment. All experiments involving generation or manipulation of viruses (in cell cultures or ferrets) are performed in BSL-3Ag containment. Viruses that must be transferred between rooms in the BSL-3Ag suite are transported using locked plastic containers (whose outer surfaces have been soaked with 70% ethanol) according to approved protocols. Nasal wash RNA extractions are initiated in BSL-3Ag containment and inactivated samples are transferred to BSL-2 containment for the remainder of the RNA isolation procedure. Virus-containing samples that need to be centrifuged are placed in gasket-sealed centrifuge containers or rotors, and centrifuges are fitted with HEPA filters. All procedures using virus are performed inside of a BSC. BPL-inactivated specimens are removed from BSL-3Ag containment for additional analyses in BSL-2 containment following verification of successful inactivation.

Number of animals: At least 3 pairs of inoculated and contact ferrets (6 animals in total) will be used for the initial library screening experiment. Additional animals would be used for follow-up studies as needed.

Amount of pathogen: Influenza virus stocks typically range in concentration from 10^5 PFU to 10^9 PFU per ml. For all virus stocks needed for this work (i.e., HA library stocks and stocks of individual virus clones), we expect to prepare a 25 ml volume and distribute into 500 µl aliquots. Stock viruses are stored in 2 ml screw-cap tubes with rubber gaskets in the caps. Ferret inoculations will be performed with a dosage of 1 x 10^6 PFU per animal in 0.5 ml of PBS, and inocula will be prepared in 15 ml conical tubes. Virus titers in ferret nasal washes range in concentration from 0 PFU to 10^7 PFU per ml. For human respiratory tissue binding and solid phase binding assays, we will use virus amounts that are standardized by activity in hemagglutination (HA) assays (e.g., 128 HA units). For glycan array assays, virus stocks (at least 25 ml) will be concentrated by ultra-centrifugation prior to inactivation.

Duration: Generation and titration of the mutant virus library stock (not including mutagenesis) is expected to take 1 week. If a selection method is used, an additional 1-2 months may be needed. Each ferret transmission study takes 3 weeks, and follow-up analyses of collected samples (including virus titration, plaque-picking, and

virus stock preparation and sequence analysis) may take additional weeks to months, depending on the number of plaques that are picked.

Frequency: The frequency of this experiment (i.e., whether the entire set of procedures or parts of the procedures are performed more than once) is dependent on the results.

Alternative approaches: No alternative approaches have been identified or approved by the NIAID for assessing transmission of mutant H5 HA virus libraries in ferrets. Receptor-binding properties of HA proteins of highly pathogenic avian influenza virus isolates will be tested by using modified HA genes/proteins that no longer encode a multibasic sequence at the HA cleavage site, in the genetic background of the mouse-adapted A/Puerto Rico/8/34 (PR8, H1N1) virus. Note: The multibasic cleavage site of the HA molecule is a critical pathogenicity determinant, which allows for highly pathogenic avian virus dissemination to non-respiratory sites in the host.

C. *Generation and passaging of reassortant viruses to identify non-HA dependent contributors to transmissibility –* The purpose of these experiments is to identify mutations in virus proteins other than HA that promote transmission of avian influenza viruses. While HA is the most dominant factor in promoting influenza virus transmission, non-HA proteins also contribute to this process, and yet very little is known about these contributions. The overarching goals of these experiments are similar to those described in Sections A and B, above, but these experiments will also further expand knowledge of molecular determinants of transmission by increasing the total pool of mutations in individual non-HA proteins that will be analyzed. This work is described in more detail below.

Basic protocol:

- In these experiments, reassortant influenza viruses possessing different combinations of genome segments derived from transmissible and non-transmissible viruses will be rescued by using reverse genetics and virus stocks will be prepared.
- The ability of reassortant viruses to transmit via the respiratory droplet route will be assessed in ferrets as described above.
- Nasal wash samples from contact ferrets that contain influenza viruses will be serially passaged in ferrets, using the methods described in Section B above, to promote the accumulation of mutations that promote efficient transmission.

Containment: All experiments involving generation or manipulation of viruses will be performed in BSL-3Ag containment. Viruses that must be transferred between rooms in the BSL-3Ag suite are transported using locked plastic containers (whose outer surfaces have been soaked with 70% ethanol) according to approved protocols. Nasal wash RNA extractions are initiated in BSL-3Ag containment and inactivated samples are transferred to BSL-2 containment for the remainder of the RNA isolation procedure. Virus-containing samples that need to be centrifuged are placed in gasket-sealed centrifuge containers or rotors, and centrifuges are fitted with HEPA filters. All procedures using virus are performed inside of a BSC.

Number of animals: At least 3 pairs of inoculated and contact ferrets (6 ferrets in total) will be used for each round of passaging for each virus that will be passaged.

Amount of pathogen: Influenza virus stocks typically range in concentration from 10^5 PFU to 10^9 PFU per ml. For all virus stocks needed for this work, we expect to prepare a 25 ml volume and distribute into 500 µl aliquots. Stock viruses are stored in 2 ml screw-cap tubes with rubber gaskets in the caps. Ferret inoculations will be performed with a dosage of 1 x 10^6 PFU per animal in 0.5 ml of PBS, and inocula will be prepared in 15 ml conical tubes. Virus titers in ferret nasal washes range in concentration from 0 PFU to 10^7 PFU per ml.

Duration: Generation and titration of reassortant virus stocks is expected to take 1 week. Each ferret transmission study takes 3 weeks, and follow-up analyses of collected samples (including virus titration, plaque-picking, and virus stock preparation and sequence analysis) may take additional weeks to months, depending on the number of plaques that are picked.

Frequency: The frequency of this experiment is dependent on the results.

Alternative approaches: No alternative approaches have been identified or approved by the NIAID for ferret passaging of reassortant viruses described in this section.

D. Generation and characterization of 1918-like avian influenza viruses – Recently, we generated a virus composed of avian influenza viral segments with high homology to the 1918 virus, and we showed that this virus has increased pathogenicity in mice and ferrets compared to an authentic avian H1N1 virus. In addition, passaging the 1918-like virus in ferrets led to the accumulation of mutations that increased transmissibility. These data suggested that contemporary avian influenza viruses with 1918 virus-like proteins may have pandemic potential. The purpose of these experiments is to further characterize the molecular determinants that promote transmission and pathogenicity of 1918-like influenza viruses.

Basic protocol:

- Reassortant viruses, composed of subsets of 1918-like and authentic avian H1N1 genome segments will be generated by reverse genetics. Alternatively, specific mutations that are expected to increase transmission or pathogenicity will be introduced into authentic avian H1N1 influenza virus gene segments by PCR-based mutagenesis and mutant viruses will be generated by using reverse genetics.
- The resultant virus stocks will then be used for ferret transmission studies (described above) or for ferret or mouse pathogenicity studies (see the following points).
- Pathogenicity studies in mice will include mouse lethal dose 50 (MLD_{50}) analysis, in which mice are infected with serial dilutions of virus and monitored for clinical symptoms, body weight loss, and survival over a 14-day time period; and sample collection studies, in which mice are infected with 1 or 2 dosages of virus and then euthanized at various times after infection so that tissues can be collected for additional analyses. Additional analyses may include virus titration of respiratory tissues (nasal turbinates and lungs) or brain, histopathological assessment, measurement of lung cytokine and chemokine levels, global analysis of host response processes (by using OMICs approaches), and other assays. Samples collected for OMICs analyses will be inactivated using established standard procedures (e.g., Trizol lysis or chloroform-methanol treatment) and inactivation will be verified for a representative sample for each batch of inactivations that are performed prior to removal from BSL-3 containment.
- Pathogenicity studies in ferrets may be performed for selected viruses. For these studies, ferrets will be inoculated with a single virus dosage and euthanized at several time points after infection, and samples will be collected for the same types of analyses described for mice.

Containment: All experiments involving generation or manipulation of viruses may be performed in BSL-3Ag containment (for viruses with mutations that are expected to increase transmissibility and/or pathogenicity) or ABSL-3+ containment (for viruses that are expected to increase only pathogenicity). Viruses that must be transferred between rooms in the BSL-3Ag suite are transported using locked plastic containers (whose outer surfaces have been soaked with 70% ethanol) according to approved protocols. Nasal wash RNA extractions are initiated in BSL-3Ag containment and inactivated samples are transferred to BSL-2 containment for the remainder of the RNA isolation procedure. Virus-containing samples that need to be centrifuged are placed in gasket-sealed centrifuge containers or rotors, and centrifuges are fitted with HEPA filters. All procedures using virus are performed inside of a BSC. Inactivated specimens are removed from BSL-3 containment for additional analyses in BSL-2 containment following verification of successful inactivation.

Number of animals: At least 3 pairs of inoculated and contact ferrets (6 ferrets in total) will be used for each transmission study with each virus. For MLD_{50} studies, groups of at least 6 mice will be inoculated with up to 5 virus dosages (e.g., 10^2, 10^3, 10^4, 10^5, 10^6 PFU/animal) of each virus. For mouse sample collection experiments, groups of 6 mice will be infected with 1 or 2 virus dosages (for each virus), and for each dosage, groups of mice will be euthanized at multiple time points after inoculation (e.g., 1, 2, 4, 7 and 12 days). For ferret sample collection experiments, groups of 3 animals will be inoculated with a single virus dosage (for each virus), and animals will be euthanized at 2-3 time points to collect samples for follow-up analyses.

Amount of pathogen: Influenza virus stocks typically range in concentration from 10^5 PFU to 10^9 PFU per ml. For all virus stocks needed for this work, we expect to prepare a 25 ml volume and distribute into 500 µl aliquots. Stock viruses are stored in 2 ml screw-cap tubes with rubber gaskets in the caps. Ferret inoculations will be performed with a dosage of 1 x 10^6 PFU per animal in 0.5 ml of PBS, and inocula will be prepared in 15 ml conical tubes. Virus titers in ferret nasal washes range in concentration from 0 PFU to 10^7 PFU per ml. Mouse inocula will encompass a wide range of dosages (from 10^0 through 10^6 PFU/mouse), and are prepared in 50 µl of PBS per mouse in 2 ml screw-cap tubes with rubber gaskets in the caps. Virus titers in mouse and ferret lung or brain tissues typically range from 0 to 10^8 PFU per gram.

Duration: Generation and titration of reassortant virus stocks is expected to take 1 week. The duration of ferret transmission studies is about 3 weeks. Ferret pathogenicity experiments take 1-2 weeks for sample collections, and mouse pathogenicity experiments (both MLD_{50} and sample collection) take about 2 weeks. After sample collections are completed, it may take another 1-3 weeks to perform follow-up analyses and procedures (i.e, virus titration, sample inactivation, etc.).

Frequency: The frequency of these experiments will vary depending on the results.

Alternative approaches: Mutations expected to increase pathogenicity will be introduced into viruses with modified HA genes/proteins that no longer encode a multibasic sequence at the HA cleavage site. Pathogenicity studies will be performed with these viruses exactly as described in this section.

E. Introduction of mutations expected to increase pathogenicity or transmissibility of influenza viruses. The purpose of these experiments is to determine how individual or combinations of mutations in influenza virus proteins contribute to pathogenicity and/or transmission of influenza viruses. Specific mutations identified in other studies (e.g., those identified in the virus passaging experiments in ferrets described above, or those identified by sequence analysis of natural influenza virus isolates) will be introduced into different influenza virus backgrounds by using reverse genetics. Mutations that are expected to promote transmission will then be tested in the ferret model, while mutations that are expected to increase pathogenicity will be tested in the mouse model. These studies will provide important insight into mechanisms of increased transmission and pathogenicity, both of which are essential for evaluating pathogenic and pandemic potential of viruses in nature and rational design of more effective countermeasures against influenza virus disease in humans. This work is described in more detail below.

Basic protocol:

- Specific mutations will be introduced into influenza virus gene segments by PCR-based mutagenesis, mutant viruses will be generated by using reverse genetics, and virus stocks will be generated.
- The resultant virus stocks will then be used for ferret transmission studies or for ferret or mouse pathogenicity studies (described above).

Containment: Containment procedures are the same as that described for generation and characterization of 1918-like influenza viruses.

Number of animals: The number of animals that will be used for these experiments is the same as that described for generation and characterization of 1918-like influenza viruses.

Amount of pathogen: The amount of pathogen that will be used for these experiments is the same as that described for generation and characterization of 1918-like influenza viruses.

Duration: The duration of these experiments is the same as that described for generation and characterization of 1918-like influenza viruses.

Frequency: The frequency of these experiments will vary depending on the results.

Alternative approaches: Mutations expected to increase pathogenicity will be introduced into viruses with modified HA genes/proteins that no longer encode a multibasic sequence at the HA cleavage site. Pathogenicity studies will be performed with these viruses exactly as described in this section.

F. *Passaging wild-type and 'mutant library viruses' (possessing random mutations in PB2 and/or NP) in MxA- or Mx1-expressing cell lines to obtain Mx1/MxA resistant viruses –* MxA/Mx1 is a potent host antiviral restriction factor for influenza virus. Our preliminary data indicate that different influenza viruses exhibit variable abilities to counteract MxA/Mx1 activity. The purpose of these experiments is to identify molecular mechanisms through which influenza viruses evolve to evade antiviral restriction by MxA/Mx1. Since MxA/Mx1 antiviral activity is mediated through effects on the viral PB2 and NP proteins, we will generate and screen PB2 and NP mutant virus libraries in the background of an MxA/Mx1-sensitive influenza virus strain, and select viruses that exhibit enhanced growth properties in cells that express the MxA/Mx1 protein for further analysis. These studies will provide important insight into influenza virus-host interactions, which may be exploited for development of novel countermeasures (i.e., antiviral compounds). Additionally, mutations identified in these studies will be useful for identifying natural influenza virus isolates that may have increased ability to replicate in humans.

Basic protocol:

- To generate virus libraries containing random mutations in PB2 or NP proteins, plasmids carrying these genes would be subjected to PCR-based random mutagenesis. A virus stock will then be generated by using reverse genetics with PB2 or NP libraries used in place of individual PB2 or NP gene plasmids.
- The resultant virus library stocks will be screened in cell lines expressing Mx1/MxA to select viruses with high growth characteristics, which indicates resistance to the Mx1/MxA gene. Subsequently, viruses will be plaque-purified and sequenced, and a set of viruses derived from each library will be selected for in-depth follow-up studies. Virus stocks would be created for selected clones prior to the start of follow-up studies.

Containment: PCR-based mutagenesis for PB2 and NP library generation will be conducted in BSL-2 containment. All experiments involving manipulation of virus will be performed in BSL-3Ag or ABSL-3+ containment. RNA extractions are initiated in BSL-3Ag containment and inactivated samples are transferred to BSL-2 containment for the remainder of the RNA isolation procedure. Virus-containing samples that need to be centrifuged are placed in gasket-sealed centrifuge containers or rotors, and centrifuges are fitted with HEPA filters. All procedures using virus are performed inside of a BSC.

Number of animals: No animals are used for these studies.

Amount of pathogen: For selection experiments, cells would be inoculated at a multiplicity of infection (MOI) of 0.01, 0.001 or 0.0001 PFU per cell of the mutant virus libraries. Typically, we use T175 flasks for growing virus stocks. If one T175 flask has about 20 million cells, we would need between 20,000 and 200,000 PFU for inoculation of a single flask. Virus stocks generated by this method are expected to have a concentration of 10^5 to 10^9 PFU per ml.

Duration: Generation and titration of the mutant virus library stocks (not including mutagenesis) is expected to take 1 week (the majority of this being incubation time). For all virus stocks needed for this work, we expect to prepare a 25 ml volume and distribute into 500 µl aliquots. Stock viruses are stored in 2 ml screw-cap tubes with rubber gaskets in the caps. Selection experiments in MxA/Mx1-expressing cells may take 1-2 months, depending on the number passages that will be performed.

Frequency: The frequency of these experiments will vary depending on the results.

Alternative approaches: Mutant NP and PB2 virus libraries will be generated using a replication-incompetent virus that lacks the pathogenicity- and transmissibility-determining HA gene. Such a virus does not replicate in wild-type cells but can be propagated in a cell line expressing HA, which is available in our group. Cell line passaging experiments for identification of mutations that confer influenza virus resistance to MxA/Mx1 will be carried out with the replication-incompetent virus libraries exactly as described in this section.

Other Alternatives to GoF Research

- We will perform loss-of-function studies, in which mutations that are expected to reduce pathogenicity are introduced into highly pathogenic viruses, followed by assessment of the effects on pathogenicity in mice. Mouse pathogenicity experiments will be carried out in a manner similar to that described for 1918-like viruses (Section D, above).
- We study the host response (transcriptome, proteome, metabolome, lipidome) to natural influenza virus isolates in cell types and tissues that are targeted by influenza viruses *in vivo*, and use computational analyses to predict host factors that are critical for the regulation of influenza virus replication and/or pathogenicity. These predictions are then tested (also with natural virus isolates) in cells using RNA interference (RNAi) or protein overexpression, or in knock-out or knock-in mice. Sample collection studies are performed in cells infected at MOIs of 1-3 PFU per cell, and at various times after infection, cell monolayers are collected and inactivated prior to OMICs analysis. Sample collection experiments in mice are performed in a manner similar to that described for 1918-like viruses (Section D, above). To validate computational predictions in cell cultures, cells with perturbed host gene expression are infected with influenza viruses (MOIs of 0.001 or 1 PFU per cell) and 12-48 h later culture supernatants are collected to assess virus titers by plaque assay. Validation studies in knockout mice are performed in a manner similar to that described for 1918-like viruses (Section D, above), except that twice as many mice are used because both wild-type and knockout mice need to be assessed in parallel. All host response experiments with H5N1 viruses are carried out in ABSL-3+ containment, and all host response experiments with H7N9 influenza viruses are carried on in BSL-3Ag containment. Information obtained from these studies is expected to aid in the identification of novel therapeutic targets.
- We are performing a host genetic screen to identify host factors that regulate susceptibility to influenza virus pathogenicity in populations of mice that are genetically unique at the individual level. In brief, mouse sample collection experiments are performed in a manner similar to that described for 1918-like viruses (Section D, above), except that mice are infected with a single virus dosage and tissues are collected at a single time point (only one virus dosage and one sample collection time point can be used since each mouse is genetically unique from all other mice in the population). In addition, tail biopsies are collected from every mouse in the screen for single nucleotide polymorphism (SNP) array analysis; SNP data will be used for identifying host genes that are linked to different pathogenicity parameters (e.g., virus replication in lungs, lung inflammation, lung pathology, expression of lung cytokines, etc.). For these studies, groups of 132 mice are inoculated for sample collections three times per year, and all experiments are carried out in the BSL-3Ag facility.

3) Please describe logistical aspects of preparing and storing stocks of virus for experiments.

Influenza virus stocks can be prepared in several different ways:

- Existing viruses (wild-type or lab-created) may be amplified in cell cultures (usually Madin-Darby canine kidney [MDCK] cells) or in the allantoic tissues of embryonated chicken eggs.
- Viruses also may be created by using reverse genetics. For reverse genetics experiments, 293T cells are transfected with plasmid DNAs encoding influenza virus genomic cDNA and influenza virus polymerase proteins. Following 12-72 h incubation periods, the resultant supernatants (containing virus) are used to infect MDCK cultures or embryonated chicken eggs for virus stock amplification.

For all Select Agent viruses, following the preparation of a virus stock, the stock is tracked in a non-permanent experimental inventory until the stock virus titer (and sometimes other properties) can be tested. Once a virus stock has been determined to be useful for future experiments (and within 30 days from the time point of generation), the virus stock is entered into permanent inventory. The experimental inventory is verified quarterly and the permanent inventory is verified monthly by two researchers.

a. Please walk through the protocol, including the containment level in which each step is conducted.

We recently published a detailed protocol of our infection procedures for MDCK cell cultures and embryonated chicken eggs (please see PMID: 25321410 for additional details; Note: This paper is freely available to the public). These procedures are standard for all influenza virus research. We describe them *in brief* below.

MDCK cultures: To prepare virus stocks in MDCK cells, we first prepare MDCK cell cultures in appropriately-sized tissue culture flasks (T25, T75, T125). Prior to the infection, the cells are washed twice with 1X PBS, and the cell growth medium (MEM containing 5% normal calf serum) is replaced with virus growth medium (MEM containing 0.3% BSA and TPCK-treated trypsin [typically ~ 1 µg/ml]). The virus inoculum is then added to the MDCK cells, and the culture is allowed to incubate for 24-96 h in a tissue culture incubator. When cytopathic effects (CPE) – typically characterized by cell rounding and detachment from the flask surface – are observed in >90% of the culture, the medium is collected and debris is pelleted by centrifugation. The resultant supernatant is then aliquoted into screw-cap tubes (with rubber gaskets in the caps), and the tubes are placed at -80°C until use at a later time. Containment: Initial MDCK cell culture preparation is performed in BSL-2 containment. All other steps are carried out in BSL-3+, ABSL-3+ or BSL-3Ag containment.

Embryonated eggs: To prepare virus stocks in eggs, we first prepare embryonated eggs by candling them to ensure the embryo is viable, and to identify the placement of the allantoic membrane. Subsequently, a small puncture is made in the egg at top of the allantoic membrane. A blunt-ended needle is then used to inoculate the allantoic cavity with virus inoculum, the puncture is sealed (with Elmer's glue, clear tape or wax) and inoculated eggs are allowed to incubate in an egg incubator for 24-72 h. Eggs are then removed from the incubator and placed at 4°C for 1-16 h, after which the top of the shell is removed and the allantoic fluid is extracted with a micro-pipette or a serological pipette. Debris is pelleted by centrifugation, and the resultant allantoic fluid supernatants are then aliquoted into screw-cap tubes (with rubber gaskets in the caps), and the tubes are placed at -80°C until use at a later time. Containment: Initial egg preparation, including egg puncturing, is performed in BSL-2 containment. All other steps are carried out in BSL-3+, ABSL-3+ or BSL-3Ag containment.

Reverse genetics experiments: Reverse genetics transfections are performed using lipid-based transfection reagents (e.g., LT1 from Mirus Bio LLC). When performing reverse genetics experiments to generate viruses that require BSL-3 containment (i.e., highly pathogenic H5N1 avian influenza viruses, H7N9 viruses and H2N2 viruses), DNA dilutions and lipid reagent preparation are carried out in BSL-2. These two components are then brought into a BSL-3+/ABSL-3+/BSL-3Ag laboratory and are mixed and added to cells only in BSL-3 containment. At 6-16 h post-transfection, medium containing transfection complexes are removed from the cells, the cells are washed, and the medium is replaced with Opti-MEM containing 0.3% BSA. Following 12-72 h further incubation, TPCK-treated trypsin is added to the cell culture medium and the cultures are incubated at 37°C for ≥30 minutes. Subsequently, the supernatants are cleared of debris by centrifugation, and they are either used directly for MDCK or egg inoculation, or they are aliquoted into screw-cap tubes (with rubber gaskets in the caps), and are placed at -80°C until use at a later time. Containment: As described above, the initial preparation of DNA/lipid dilutions and cell cultures and eggs are carried out in BSL-2, but all other steps are carried out in BSL-3+, ABSL-3+ or BSL-3Ag containment. Note: Reverse genetics experiments for low pathogenicity viruses are carried out entirely in BSL-2 containment.

b. What type of PPE are you wearing during each step?

For cell culture and egg preparations (performed in BSL-2), researchers use the following PPE:

- Lab coats
- Gloves
- Eye protection

The following PPE is always used in all of our BSL-3 facilities:

- Dedicated scrubs
- Shoe covers over feet
- Dedicated shoes (garden clogs)

- Shoe covers over shoes
- Tyvek booties or sleeve covers over feet/ankles
- Tyvek suit
- Tyvek shroud
- Powered air purifying respirator (PAPR)
- One pair of nitrile gloves under Tyvek suit sleeve (sleeve hooked over thumb)
- One pair of nitrile gloves over the Tykev suit sleeve

When working with (potentially) mammalian-transmissible influenza viruses, the following PPE is required in addition to each of the items listed above:

- Tyvek sleeve covers

c. What volume and at what concentration of virus do you typically produce?

Virus stock volumes may vary from 2 ml to 100 ml, but a typical virus stock is approximately 25 ml.

The stock virus concentration is dependent on the growth properties of each individual virus, and may vary from 10^4 to 10^9 plaque forming units (PFU) per ml.

d. What amount of virus is typically in each aliquot?

Aliquot volumes may range from 100 µl to 1 ml. Most commonly, people use 250-500 µl aliquots.

e. How often do you prepare virus stocks?

This varies, depending on the activities of the laboratory. In general, we prepare enough stock of a particular virus so that the same stock can be used for at least one year of experiments. It is possible to go months at a time without the generation of any new virus stocks; however, it is also possible that we may prepare 10 or more virus stocks in a single month.

4) Please describe logistical aspects related to animal facilities.

a. Are infected and uninfected animals caged in the same area or in different areas? Can asymptomatic infected and uninfected animals be distinguished in some way? How?

General animal husbandry is performed in facilities other than the IRI. Animals that will be used for experiments (which are either commercially purchased or generated in existing colonies at the UW-Madison) are brought into BSL-3 containment and acclimated for one week prior to infection. Thus, uninfected animals are not kept in BSL-3 containment for extended time periods.

Infected and uninfected animals that are part of the same experiment are kept in the same room with each other, but are in different cages. It is standard procedure for any mock-infected (i.e., uninfected) animals to be handled first on a given day, prior to handling any infected animals, to prevent cross-contamination. There are also standard procedures for decontaminating biosafety cabinets (BSCs) and any materials or equipment inside of BSCs (including hands) prior to removing them from the BSC and/or changing between groups of experimentally infected animals. It should be noted that mock infections are never used for ferret transmissibility studies that are performed at the IRI. Indeed, mock infections are typically only performed in animal experiments if the analyses that will be performed require some kind of baseline assessment (e.g., transcriptome analysis of lung tissues after infection).

The only way to distinguish between asymptomatic infected animals and uninfected animals is to perform some type of sample collection (e.g., nasal wash from ferrets or lung/nasal turbinate collection from mice) followed by virus titration, hemagglutination assay, an RT-PCR assay to detect viral components, etc. Serological analysis can also be used to assess whether asymptomatic or uninfected animals have been

exposed to influenza virus, but since it takes time for any animal to develop antibodies, this type of analysis is only useful after the infection has been resolved.

Importantly, at the end of any experiment, the carcasses of mock-infected animals are disposed of in the same manner as infected animal carcasses, and no live animals are ever removed from any BSL-3 containment laboratory at the IRI.

b. Given the typical flux of experiments in your facility, how many infected animals are typically caged in the facility at a given point in time?

The number of infected animals that are caged in our facility varies from 0 to several hundred at any given time, depending on the flux of required experiments and the type of animals needed for these experiments.

Ferret transmission studies may include as few as 6 ferrets (3 pairs of infected and contact animals) or as many as 36 ferrets (18 pairs of infected and contact animals). A 'typical' ferret experiment usually consists of 24 ferrets, and such an experiment might be performed once per month (or less) in the absence of any research moratorium.

- **Ferrets used at IRI in 2013:** 142
- **Ferrets used at IRI in 2014:** 66 (the number is reduced relative to 2013 due to the GoF research pause)
- The number of animals includes all animals used for both GoF and non-GoF research. For example, some of the ferrets included in the numbers provided above were used to generate antisera, and were never involved in any transmission study.

Mouse studies may include as few as 6 mice, or up to 160 mice. If several mouse experiments are ongoing at the same time, it is possible to have more than 200 mice in the facility. It is important to emphasize that mice infected with highly pathogenic viruses of different subtypes are never housed in the same room, and that strict separation practices are observed by the participating researchers.

- **Mice used at IRI in 2013:** 829
- **Mice used at IRI in 2014:** 1468
- The number of animals includes all animals used for both GoF and non-GoF research.

c. How are animals inventoried and traced over the course of an experiment?

At the beginning of each experiment, we record the number of animals receiving a specific virus and the dosage of that virus in an animal inventory binder; one binder is kept in each of the common areas of the ABSL-3+ and BSL-3Ag suites. The same information is also recorded in a lab notebook. Over the course of the experiment, individual animal body weights and clinical symptoms are collected and recorded (in a table) on a daily basis. If an animal drops below a pre-specified body weight or exhibits a specific constellation of symptoms, that animal will be euthanized, and its fate will be recorded in the weight loss/clinical symptom table. The animal carcass is then double-bagged and placed in a -20°C freezer until autoclaving; a label on the outside of the bag indicates the number of animals inside the bag and the virus that was used to infect these animals. At the conclusion of the experiment, the animal carcasses are thawed and autoclaved, and at this time, the animal inventory binder is updated to reflect the removal of these carcasses from the laboratory.

5) Consider a lab-created avian-origin influenza strain that is transmissible between ferrets (i.e. an H5N1 strain that was lab-evolved to become airborne transmissible between ferrets through serial passaging with selection for airborne transmission).

   a. This may be outside your area of expertise, but relative to the original strain, what is the likely transmissibility of this strain:

   i. Between wild birds?
   ii. Between wild birds and poultry?
   iii. Between wild birds and swine?

iv. Between poultry and humans?

Influenza viruses that are adapted for replication and transmission in mammals undergo changes in receptor binding that prevent replication and transmission in birds. Therefore, any transmission route that involves avian species (avian-avian or avian-mammal) is unlikely to occur with these viruses.

v. Between swine and humans?

It is possible that a lab-evolved strain that is transmissible in ferrets could also be transmissible between swine and humans. However, for transmission to occur, the virus must be able to replicate well in both humans and swine. Without knowledge of the replicative abilities of such viruses in different species, it is not possible to provide a definitive answer to this question.

6) Please describe the standard monitoring procedures in place to detect potential loss of containment events prior to illness, if any are in place. (For example, swabbing and testing of laboratory surfaces).

We do not have any standard laboratory monitoring procedures aimed at detecting potential loss of containment prior to a researcher exhibiting influenza-like symptoms or the report of an inadvertent exposure. It is important to note that influenza viruses are not viable for long on surfaces.

However, all containment laboratories (and all engineering barriers that are part of the building/containment laboratories) are monitored at all times by a building automation system (BAS), and the BAS sends alarms to multiple recipients as soon as any problem is detected (e.g., a room's pressure falls out of the acceptable range).

Additionally, during the course of any experiment performed in our BSL-3 facilities, extensive precautions are taken to ensure that all virus manipulations are carried out within primary containment (e.g., a biosafety cabinet). In the BSL-3Ag facility, the use of primary containment amounts to double containment because each room inside of the BSL-3Ag facility is considered primary containment itself. Moreover, the following standard procedures make the need for swabbing and testing laboratory surfaces for the presence of virus unnecessary:

- Full air exchanges are performed in each room >13 times per hour.
- Comprehensive disinfection procedures are carried out at the conclusion of all work sessions.
- In the BSL-3+ and ABSL-3+ facilities, floors are mopped on an as-needed basis (but at least weekly); in the BSL-3Ag suite, floors of animal procedure rooms are mopped at the conclusion of each work session, and all other floors are mopped on an as-needed basis (but at least weekly while a room is in use).
- BSCs, backdraft and downdraft tables are certified annually, and each BSL-3 researcher is trained on how to ascertain whether airflow is working properly in a BSC/backdraft table/downdraft table.
- All aerosol-generating equipment are fitted with HEPA filters (e.g., centrifuges, vacuum aspirators), housed in a BSC (e.g., hematology machine), or have other aerosol minimizing equipment that are required for their use (e.g., flow cytometer).
- All researchers that work in BSL-3 containment perform extensive disinfection procedures on their PPE prior to exiting a laboratory. In addition, in the BSL-3Ag suite, researchers that move between rooms disinfect PPE with 70% ethanol and remove outer gloves and shoe covers, and don new outer gloves and shoe covers in the clean hallway.

a. If a surface comes back contaminated with a potential pathogen, what would be the standard response to such an incident?

Not applicable.

i. Would workers be isolated and/or quarantined prior to symptoms appearing?

With regard to the identification of a release of pathogen based on swabbing, this question is irrelevant.

However, in the event that a break in PPE occurs, or if a researcher is thought to have been potentially exposed to virus in some other manner, then that researcher would be immediately isolated from other workers/friends/family until an assessment can be made by University and local or state public health officials.

ii. Would the laboratory be shut down for a period of time?

Not applicable.

b. What procedures are in place for monitoring workers?

- All workers are required to give a baseline blood sample prior to initiating work in any BSL-3 facility.
- All BSL-3 workers are required to self-monitor and report influenza-like symptoms to the principal investigator (PI; Dr. Kawaoka) and other laboratory staff as soon as they are identified, if they occur within 10 days of the last entry into a BSL-3 laboratory. This information is immediately relayed to the Select Agent Program Responsible Official (RO).
- Researchers that have not entered a BSL-3 laboratory within the preceding 10 days or that work only with BSL-2 viruses are also required to report influenza-like symptoms to the PI and other laboratory staff, who will notify the RO.
- All BSL-3 researchers are taught to identify influenza-like symptoms during training with their BSL-3 mentor. No researcher is allowed to enter any BSL-3 facility without first knowing this information.
- Identification of influenza-like symptoms is reviewed with all BSL-2 and BSL-3 researchers yearly, prior to the start of the annual influenza infection "season," for all laboratory staff.
- All BSL-3 researchers have been given a thermometer to enable regular body temperature self-monitoring.

i. Are laboratory personnel routinely vaccinated?

All researchers that will enter the BSL-3 suites must obtain (and provide evidence thereof) an annual seasonal influenza vaccine. BSL-3 researchers are also strongly encouraged to obtain available H5N1 vaccine(s) when available. Other researchers and laboratory staff that do not work in the BSL-3 facility are strongly encouraged (but not required) to obtain the annual seasonal influenza vaccine.

ii. Do personnel monitor their health routinely (e.g. through regular temperature monitoring)?

As stated above,

- All BSL-3 workers are required to self-monitor and report influenza-like symptoms to the PI and other laboratory staff as soon as they are identified, if they occur within 10 days of the last entry into a BSL-3 laboratory. This information is immediately relayed to the Responsible Official.
- Researchers that have not entered a BSL-3 laboratory within the preceding 10 days or that work only with BSL-2 viruses are also required to report influenza-like symptoms to the PI and other laboratory staff, who will notify the RO.
- All BSL-3 researchers have been given a thermometer to enable regular body temperature self-monitoring.

iii. Are laboratory personnel's interactions with swine or poultry restricted?

All researchers that enter the BSL-3 facilities at the IRI are subject the "Quarantine Policy for Highly Pathogenic Avian Influenza (HPAI)," which must be reviewed and signed on an annual basis. Individuals subject to this policy are prohibited from coming into contact with susceptible avian species for a minimum of 5 days after the last possible contact with HPAI viruses in a facility where highly pathogenic avian influenza virus is used or stored. Susceptible species include, but are not limited to, commercial poultry operations and privately-owned poultry flocks, pet birds, birds at county/state fairs and petting zoos, zoological collections (i.e., zoos), and wild birds (e.g., ducks, swans). This quarantine policy is a requirement of APHIS for any researcher that will work with HPAI viruses.

Currently, there are no other restrictions on laboratory personnel interactions with swine or any other species that is susceptible to influenza infection.

7) Imagine that a potential laboratory exposure has occurred to a pathogen of pandemic potential, for example, highly pathogenic influenza. Please elaborate on the steps that are taken following that exposure.

   a. Do people that have been potentially exposed have any restrictions placed on their movements/are they taken directly to a clinic or isolated?

People who have experienced a known potential exposure to a pathogen in an IRI BSL-3 facility are treated as follows:

- Following any potential exposure, the individual is immediately quarantined within the conference room at the IRI until notification of university and local/state public health officials occurs and reception at an appropriate quarantine location has been arranged. During this period, arrangements are made for immediate initiation of anti-viral treatment.

- A high-risk mucous membrane or respiratory exposure to a mammalian transmissible avian influenza virus or a recombinant virus containing one or more genome segments from the 1918 influenza virus would result in the immediate quarantine of the individual in an isolation room at the University of Wisconsin Hospital. The individual would be transported to the hospital by an IRI on-call scientist while wearing an N-95 respirator without an exhalation valve after notifying the RO and subsequent notification of the Infectious Disease Physicians and Emergency Room.

- A high-risk mucous membrane or respiratory exposure to a non-transmissible influenza virus of avian origin would result in quarantine at the individual's personal residence, provided that antiviral treatment has been initiated within 5 hours of the exposure. Other household members are required to relocate for the duration of the quarantine. IRI employees sign annual acknowledgement of this requirement and arrangements are available through the university to provide housing and other support to displaced family members.

- A low-to-moderate-risk, non-mucous membrane/respiratory exposure to any BSL-3 influenza virus would result in quarantine at the individual's personal residence, provided that antiviral treatment has been initiated within 5 hours of the exposure. Other household members are required to relocate for the duration of the quarantine. IRI employees sign annual acknowledgement of this requirement and arrangements are available through the university to provide housing and other support to displaced family members.

   b. Would the laboratory be shut down for a period of time as a precaution?

There are no procedures in place that absolutely require the shutdown of the BSL-3 laboratories in the event of a potential (known or unknown source) exposure. However, such a measure may be considered based on the situation in which an exposure or potential exposure occurs, so that potential routes of exposure may be assessed, additional staff training can be performed, etc..

   c. If the number of people exposed was not immediately clear (e.g. an accident created a splash on several workers), how would you determine which individuals to monitor and/or treat?

Laboratory workers would not make this decision; rather, the decision would be made by the RO after consultation with university and local/state public health officials, the PI and laboratory staff members.

In the event that such an event was deemed an exposure, the procedures outlined in item a) would be followed.

   d. If multiple people were potentially exposed is the response different in a significant way from exposure by a single individual?

The response would be the same for multiple people as for a single person.

i. Are people potentially exposed treated differently from those certainly exposed (e.g. those who have suffered a needle stick)?

No. People with a known potential exposure would be immediately quarantined as described above. Following any potential exposure, the individual is immediately quarantined until there is consultation with university and local/state public health officials, the PI and laboratory staff members. In the event it is determined that there is a credible concern for a potential exposure, the procedures described in 7a are followed.

People with potential exposures, as indicated by the development of influenza-like illness symptoms in any person working within the BSL-3 facility within 10 days of symptoms onset, are immediately isolated at home (or at work, if applicable) until a diagnostic test can be performed that excludes the presence of lab-acquired influenza virus strains (the test performed also identifies the presence of other community acquired respiratory pathogens). University Health Services staff are available on-call to travel to the location to any ill individual and collect appropriate samples. Samples are immediately transported to the Wisconsin State Laboratory of Hygiene (WSLH) where on-call staff is also immediately available to perform diagnostic testing. The WSLH uses CDC developed influenza RT-PCR assays that detect all influenza A and influenza B viruses. CDC-developed subtyping PCR assays are available for the identification of influenza A seasonal H1, pandemic H1, H3, H5, and H7 strains, but cannot detect 1918 influenza virus. If laboratory personnel have been working with 1918 influenza viruses directly or in parallel to other researchers that are working with 1918 influenza viruses, then a portion of the sample will be provided to the IRI, where a 1918 influenza virus-specific RT-PCR assay is available. In the event that a sample is found to be positive for any lab-acquired strain or for an influenza virus of an unknown subtype, a portion of this sample will be sent to the CDC for independent verification.

The family or housemates of individuals with a potential exposure are also required to remain at home and in isolation until the diagnostic test can be completed. If the diagnostic test suggests a potentially lab-acquired strain, then the individual exhibiting symptoms would receive further treatment at UW Hospital in consultation with UW Infectious Disease and local/state public health. Arrangements and plans are in place to allow for reception of the individual using appropriate infection control procedures through the UW Emergency Department.

ii. Are potentially exposed individuals treated with countermeasures preventatively?

Yes. Following any potential exposure, the individual is immediately quarantined until there is consultation with university and local/state public health officials, the PI and laboratory staff members. In the event it is determined that there is a credible concern for a potential exposure, the procedures described in 7a) are followed including initiation of anti-viral therapy.

Are there standard procedures for contacting local public health officials?

Yes. In the event of a known exposure, or the development of influenza-like illness in a BSL-3 researcher, the researcher first notifies a designated on-call scientist; the on-call scientist immediately notifies the RO/ARO and the PI. The RO/ARO will directly contact UW Infectious Disease physicians, University Health Services, local/state public health officials, the CDC Division of Select Agents and Toxins, and the CDC Influenza Branch. This is described in more detail in the "Select Agent Influenza Virus Exposure Control Overview."

iii. What conditions initiate those procedures?

Either the reporting of a known or credible potential exposure or the reporting of influenza-like illness symptoms in a researcher at risk for exposure initiates these procedures.

iv. What information is shared with local public health officials?

Local public health officials would receive information on the researcher's work (e.g., last known BSL-3 entry, virus strains they are working with and specific biological features associated with these strains [i.e., potential for mammalian transmissibility]), the date of onset and type of symptoms if applicable, details of anti-viral treatment initiated, results from any diagnostic testing, and quarantine/isolation procedures to be followed.

e. Does the research facility have any procedures to encourage reporting or discourage non-reporting, for example, a work-in-pairs requirement?

All work that is carried out with mammalian transmissible viruses of avian origin requires that at least two individuals are present at all times. Additionally, all procedures with sharps (i.e., needles and razor blades) require the presence of at least two individuals at all times. While other procedures do not require pairs of individuals, working in a buddy system is encouraged whenever possible. Additionally, it should be noted that for researchers to receive antivirals in a timely manner, they must report any potential exposures.

8) Now, imagine that laboratory workers are falling ill without a known route of exposure, with a pathogen suspected to be of laboratory origin. Please elaborate on the steps that would be taken following this type of discovery.

a. Are people that have been potentially exposed immediately isolated or taken to a clinic?

Any worker exhibiting influenza-like symptoms without a known exposure would be isolated as indicated above until diagnostic testing is performed. If the diagnostic test indicates a potentially laboratory-acquired strain, the worker would be immediately be treated and further treatment initiated as indicated above.

b. How would the institution attempt to find the source of the infection?

Diagnostic testing would include the use of RT-PCR with strain-specific primers, which would be carried out by the WSLH and/or the Kawaoka laboratory. If an H2, H5, H7 or 1918 influenza virus strain is identified by the initial diagnostic test, then specimens would be split and referred to the CDC. Subsequent analyses would include full genome sequencing of virus(es) isolated from infected researchers to determine the genetic characteristics. This information would then be used to compare against laboratory isolates in order to determine if the infection was laboratory-acquired.

In such a scenario, it is highly likely that the UW-Madison Emergency Operations Center (EOC) would be activated to determine what is going on and what resources are needed for investigation and control purposes. This would bring key university players, resources, and services to help address the situation. Local and state public health agencies would be involved, as well as (probably) the UW Hospital. Depending on the severity of the situation, a unified command with key state agencies could be initiated.

c. Would the laboratory be shut down for a period of time as a precaution?

It would depend on the situation. For a potential exposure, it is unlikely that the laboratory would be closed unless decontamination procedures are required. In the event of an environmental release, the laboratory most likely would be closed to determine what happened, and to run tests on the facility. In the event that laboratory workers are falling ill without a known route of exposure with a pathogen suspected to be of laboratory origin, it is highly probable that the laboratory would be closed to determine what is going on.

d. What types of information would be communicated to local public health officials, and when?

Local public health officials would receive information on the researcher's work (e.g., last known BSL-3 entry, virus strains they are working with and specific biological features associated with these strains [i.e., potential for mammalian transmissibility]), the date of onset and type of symptoms, and results from any diagnostic testing. This information would be conveyed to public health officials as soon as it is available.

If the EOC is activated, local and state public health would be involved and would have a presence at the command center.

9) How many full-time equivalent research employees do you have in your laboratory typically, and what percentage of their time is spent within the containment suite, on average, per year?

In 2014, 16 researchers worked in the BSL-3Ag suite for roughly 1,340 hours, and 17 researchers worked in the ABSL-3+ for roughly 1,250 hours.

a. Please describe wait periods or demonstration of competence requirements for new hires prior to being allowed access to the high-containment suite.

New hires must go through the following procedures before being allowed to work on their own in the IRI BSL-3 laboratories:

- They must first undergo and pass an FBI background check (fd-961).
- They must complete a general, online Select Agent training module (provided by the UW-Madison and including both general program information and biosafety and biosecurity elements) and complete and pass two written exams.
- Once these first two steps have been completed, researchers can begin training with a BSL-3 training mentor. Prior to initiating this training, trainees must first exhibit competence in biosafety and biocontainment at the BSL-2 level.
- Each trainee is required to complete 10-20 escorted entries into the BSL-3 labs (with the training mentor(s)). The number of escorted entries will vary on a person-to-person basis, and the final required number for each trainee will be determined at the discretion of the trainer.
- During the escorted entries, trainees are taught about basic concepts of containment in the BSL-3 laboratory, how to assess if the facility is safe to use, proper entry and exit procedures, what to do in the case of emergencies (e.g., medical, fire, etc.), how to address biohazardous spills; how to clean, disinfect and maintain general laboratory operations; how to use the autoclave and how to communicate in and about the laboratory. These tasks are classified under the Tier 1 component of the training program.
- Trainees may also be taught aspects of Tier 2 (working with viruses in cell culture) and/or Tier 3 (working with viruses in animal models) during their escorted entries.
- Typically, subjects that are covered as part of Tier 1, 2 and 3 training program in the BSL-3 lab are reviewed outside of the BSL-3 lab to ensure that each researcher has a full understanding of all procedures.
- At the conclusion of the escorted entry process, trainees must perform a lab practical to demonstrate knowledge of Tier 1 training components. This lab practical is typically carried out by an experienced BSL-3 worker other than the trainer. The trainee must also take and pass a written exam covering Tier 1 training topics. Both trainers and the trainee sign a document to indicate the trainee's performance on the Tier 1 lab practical and the written exam.
- If the trainee has demonstrated the necessary competency in Tier 2 or Tier 3 training tasks, the trainer may also certify the trainee in these training levels at the conclusion of the escorted entries. If sufficient proficiency in Tier 2 or Tier 3 has not yet been demonstrated, then a trainee will be required to work with one other trained researcher while performing Tier 2 or Tier 3 tasks until competency has been demonstrated. Training for common Tier 2 and tier 3 procedures is documented in each individual's training record.
- Researchers may not perform any task for which they have not been specifically trained, and must obtain permission to be trained on any new task or procedure. The responsibility for acquiring training for new procedures is partially dependent on the individual researcher; however, other checks in the system also prevent researchers from performing tasks they are not trained to do. Specifically,
    - As part of the virus stock inventory tracking process, all researchers must ask for permission from designated IRI scientists to use any virus for any procedure. It is the responsibility of the designated scientists to determine whether a researcher has been properly trained in the planned procedure prior to granting permission for virus use.
    - Designated scientists are copied on all orders for research animals (i.e., mice and ferrets), which are typically placed a minimum of 1-2 weeks prior to the start of an experiment. It is the responsibility of the designated scientist to ensure that a researcher is trained to perform the animal experiment.

- Ferret (and many mouse) infection procedures require the use of anesthetic drugs, which can only be obtained with permission from a designated scientist. Prior to granting permission for the use of these drugs, the designated scientist must determine whether a researcher is trained to perform the planned procedure.
- Many procedures used in animal experiments require a minimum of two researchers.

We realize that some of this information may be sensitive, such as information relating to the number of animals used in your experiments and housed in your facilities. To alleviate your potential concerns, we want to let you know that in our final report, we have no need (and no plans) to show data on a lab by lab basis. Instead, we will use the data from all facilities to provide a range of parameter values related to laboratory activities (e.g. experiments with infected animals occur from 1.4-3.8x a month with of a mode of 2.3; these experiments last from 4-12 days--mode 6--before the last animal is sacrificed, etc). Of course, we would collect these data on a lab-by-lab basis and the raw data would exist in some form somewhere (but would not be in the final report).

Finally, the RA will also be comparative, in that we will be assessing the net risk posed by research involving GoF pathogens relative to research involving wild-type pathogens.

Questions Related to Research Proliferation

Finally, an additional component of our RA will be evaluation of the risks posed by proliferation of the research in additional US laboratories. Our questions will address the current size of the GoF research community in the US, as well as how the debate surrounding GoF research has influenced your interest in doing this work in the future.

Biosafety Risk Assessment - Questions for Biosafety Officers

Understanding the SOPs for responding to laboratory exposures and LAIs is essential to parameterize a model of outbreak spread following a laboratory release. Biosafety officers may have insights into these SOPs. They can also provide information on institutional connections to local public health resources. Information here feeds into the parameterization of the probability of detection of an outbreak or loss of containment event.

1) Please describe the standard monitoring procedures in place to detect potential loss of containment events prior to illness, if any are in place. For example, swabbing and testing of laboratory surfaces.

We do not have any standard laboratory monitoring procedures aimed at detecting potential loss of containment prior to a researcher exhibiting influenza-like symptoms or the report of an inadvertent exposure. It is important to note that influenza viruses are not viable for long on surfaces.

However, all containment laboratories (and all engineering barriers that are part of the building/containment laboratories) are monitored at all times by a building automation system (BAS), and the BAS sends alarms to multiple recipients as soon as any problem is detected (e.g., a room's pressure falls out of the acceptable range).

Additionally, during the course of any experiment performed in our BSL-3 facilities, extensive precautions are taken to ensure that all virus manipulations are carried out within primary containment (e.g., a biosafety cabinet). In the BSL-3Ag facility, the use of primary containment amounts to double containment because each room inside of the BSL-3Ag facility is considered primary containment itself. Moreover, the following standard procedures make the need for swabbing and testing laboratory surfaces for the presence of virus unnecessary:

- Full air exchanges are performed in each room >13 times per hour.
- Comprehensive disinfection procedures are carried out at the conclusion of all work sessions.

- In the BSL-3+ and ABSL-3+ facilities, floors are mopped on an as-needed basis (but at least weekly); in the BSL-3Ag suite, floors of animal procedure rooms are mopped at the conclusion of each work session, and all other floors are mopped on an as-needed basis (but at least weekly while a room is in use).
- BSCs, backdraft and downdraft tables are certified annually, and each BSL-3 researcher is trained on how to ascertain whether airflow is working properly in a BSC/backdraft table/downdraft table.
- All aerosol-generating equipment are fitted with HEPA filters (e.g., centrifuges, vacuum aspirators), housed in a BSC (e.g., hematology machine), or have other aerosol minimizing equipment that are required for their use (e.g., flow cytometer).
- All researchers that work in BSL-3 containment perform extensive disinfection procedures on their PPE prior to exiting a laboratory. In addition, in the BSL-3Ag suite, researchers that move between rooms disinfect PPE with 70% ethanol and remove outer gloves and shoe covers, and don new outer gloves and shoe covers in the clean hallway.

a. If a surface comes back contaminated with a potential pathogen, what would be the standard response to such an incident?

Not applicable.

i. Would workers be isolated and/or quarantined prior to symptoms appearing?

If a researcher has a potential exposure then yes, an individual will be quarantined for the incubation period and tested on a daily basis to determine if the individual was exposed.

ii. Would the laboratory be shut down for a period of time?

This question is not applicable in regard to contaminated surfaces, since we do not perform any swab testing.

In the event of a potential exposure, it is unlikely the laboratory would be shut down. However, if an individual developed influenza infection from a laboratory strain, then it is possible the lab could be shut down until the source of infection could be determined.

b. What procedures are in place for monitoring workers?
i. Are laboratory personnel routinely vaccinated?

Yes, personnel receive the seasonal influenza vaccine annually and a H5N1 vaccine when available.

ii. Do personnel monitor their health routinely?

Yes, they do. Please see above comments.

iii. Are laboratory personnel's interactions with swine or poultry restricted?

Yes, there is an avian quarantine policy, which is described above. This is a requirement from APHIS for working with HPAI. There are no restrictions on interactions with swine.

2) Imagine that a potential laboratory exposure has occurred for a pathogen of pandemic potential, for example, highly pathogenic influenza. Please elaborate on the steps that are taken following that exposure.

a. Do people that have been potentially exposed have any restrictions placed on their movements/are they taken directly to a clinic or isolated?

Yes, they will be quarantined immediately. Please see above for more specifics.

b. Would the laboratory be shut down for a period of time as a precaution?

That would depend on the situation. If there was a _**potential**_ (i.e., known) exposure, then most likely not. If an individual developed influenza infection with a laboratory strain and the exposure was unknown, then it is possible.

c. If multiple people were potentially exposed, are the same steps taken, or is the response different in a significant way?

The response is the same.

d. Are there standard procedures for contacting local public health officials?

Yes. The RO or ARO contacts public health officials and provides at least daily updates during a quarantine.

i. What conditions initiate those procedures?

A known potential exposure or the development of influenza infection with a laboratory strain

ii. What information is shared with local public health officials?

The nature of the potential exposure, specifics about the virus, diagnostic test results, when the first dose of Tamiflu was initiated, and the individual's body temperature

3) Now, imagine that laboratory workers are falling ill without a known route of exposure, with a pathogen suspected to be of laboratory origin. Please elaborate on the steps that would be taken following this type of discovery.

a. Are people that have been potentially exposed immediately isolated or taken to a clinic?

Yes, they are isolated and put into quarantine. Please see above for more details.

b. How would the institution attempt to find the source of the infection?

Assuming that the researchers are all infected with a laboratory strain, then we would talk with the individuals to determine when each last worked in a BSL-3 laboratory and whether they have anything in common. Based on that information, we might start running tests. It is highly likely that the university EOC would be activated to provide support, leadership, and decision-making.

c. Would the laboratory be shut down for a period of time as a precaution?

It depends on the situation and what is known at that time. It is possible based upon this scenario or if it was potentially an environmental release.

d. What types of information would be communicated to local public health officials, and when?

Public health officials would be notified as soon as it is determined that the laboratory workers are infected with a lab strain. If the EOC was activated, they would have a vital role in the decision making process, and most likely be part of the incident command.

4) Now, please imagine that an infection is occurring in the community and evidence suggests it is of a laboratory origin, potentially from a lab at your institution. Please describe the response to such a discovery.

a. Are you in regular contact with local public health officials? At what point would you expect local public health officials to reach out to you if they suspected a case or outbreak caused by a laboratory acquired infection?

Yes, we are in regular contact with local public health officials. We assume that they would reach out to us immediately if diagnostic tests suggested that the pathogen could be of laboratory origin. In this scenario, the university EOC probably would be activated.

b. Would all workers using this pathogen be monitored, isolated and/or subjected to diagnostic testing?

It depends on the situation. Most the researchers would be monitored and not isolated. Most likely, all researchers would be tested for seroconversion and results would be compared to their baseline serum sample.

c. How would the investigation into the potential sources of the infection be carried out?

The university EOC would lead the investigation, with the university and public health playing key roles in the incident command. Epidemiologists would start interviewing researchers and look for connections to the community. A list of researcher contacts would be compiled and given to public health as soon as possible. Most likely the facility would be closed for testing to determine if there was a containment issue.

d. What resources would your institution add for epidemiological investigation and contact tracing, or would your efforts be focused on identifying the source?

The university's resources would be used for both. University Health Services has epidemiologists and experience to aid in contact tracing. Additional resources would be used to look at the facility's functionality.

Risk Assessment - Questions for Local Hospital Personnel

Modeling the stochastic phase of a lab-caused infectious disease outbreak will require information about disease surveillance and reporting measures, in order to determine how fast lab-acquired and secondary infections are likely to be detected, as well as the type and level of local public health responses, in order to understand how control measures will influence outbreak spread. The two overarching questions we are trying to understand in this section are: (1) What is the likelihood of diagnosing an infection with a loss-of-containment strain and (2) How quickly does that happen?

Questions

1) Imagine that an individual in the community arrives at a clinic with a respiratory illness with flu-like symptoms. Please elaborate on the treatment and diagnostics that a typical individual may receive.

    a. What infection control measures are typically used for patients with ILI prior to a definitive diagnosis?

        i. At an outpatient clinic? At a hospital ER?

Standard respiratory precautions in ambulatory care include use of a surgical face mask by all patients with a febrile respiratory illness and/or who are coughing. Precautions are increased during times of increased influenza activity in the community. Patients are typically not isolated in an ambulatory care setting unless there is reason to believe that there is an increased risk of specific communicable diseases, which does not include influenza.

Healthcare workers caring for an individual with an ILI typically follow standard respiratory precautions which include use of a face mask at all times and use of a gown, face shield, and gloves during aerosol generating procedures including collection of respiratory swabs.

ii. How many other people (including healthcare workers and other patients) would you expect the sick patient to interact with in either setting prior to isolation?

The exact numbers of exposed individuals depends upon the setting. In a crowded care setting, up to 30-40 patients could be exposed within a waiting room and 5-10 members of the healthcare team.

b. Would an individual be tested, and if so, what diagnostic test or tests would be performed?
    i. At an outpatient clinic? At a hospital ER?

Influenza is most often a clinical diagnosis during periods of increased influenza activity within the community. Definitive testing is typically reserved for patients with severe symptoms, atypical symptoms, during periods of low influenza activity, for purposes of community surveillance, or when a risk factor for novel influenza (such as international travel) or other pathogens is present. An international travel history is a standard part of the history for febrile respiratory illnesses, however, an occupational exposure history is not. All UW-Madison researchers, however, are trained to identify their occupational risk factors when presenting for care. In the event of a potential exposure or development of ILI symptoms, researchers are not allowed to go to their personal physicians and must follow the exposure control plan.

University Health Services, as well as several clinics within the Madison community, participate in influenza surveillance activities coordinated through the Wisconsin State Laboratory of Hygiene (WSLH) and Wisconsin Department of Public Health (DPH). Within this virologic surveillance system, specimens from patients with respiratory signs and symptoms are collected and submitted to the WSLH for testing. The WSLH uses the FDA-approved CDC influenza RT-PCR Diagnostic panel which enables detection and typing of influenza A and B. Influenza A is further subtyped as seasonal H1, pandemic H1, H3, H5 (Asian lineage), or H7. If an influenza A is not able to be subtyped, CDC is immediately contacted and the specimen forwarded to them for further characterization. Specimens that are negative for influenza can be further tested using a molecular respiratory virus panel capable of identifying 16 other commonly identified respiratory viruses.

ii. How quickly would results be delivered back to the clinic?

This is dependent upon the test utilized and the reference laboratory. Rapid influenza tests are available at the point of care, but do not distinguish between subtypes. If circumstances warrant rapid subtype-specific testing (i.e., in the case of a potential laboratory-acquired infection), WSLH can perform testing 24/7 and have results available within a few hours.

c. If diagnostic testing came back negative, what would be the next steps?

This would be dependent upon the clinical scenario and presentation. Based on the risk factors, clinical severity, and symptoms additional diagnostic testing would be warranted including evaluation for other viral and bacterial processes. If a rapid flu test was used as the initial diagnostic test, follow-up testing with a PCR assay would be appropriate as the rapid flu tests are insensitive and cannot rule out influenza infection. Furthermore, even a negative PCR test does not rule out influenza infection. Laboratory tests are interpreted in conjunction with patient history, clinical presentation, and epidemiologic considerations.

d. Would the control measures and diagnostic tests you just described vary between cases appearing during the flu season versus those outside of it?

This has been discussed above. Definitive diagnostic testing would typically be more likely outside of the flu season.

2) Imagine now that an infection with an atypical disease is suspected, for example SARS or highly pathogenic avian influenza H5N1, either because of the patient's symptoms and/or negative results on a previous diagnostic test. Please describe what steps would be taken next.

a. What infection controls measures would be put into place? Would any special procedures be put into place or measures be taken?

The infection controls implemented would depend upon the agent suspected. This requires knowledge of the patient's risk factors, clinical symptoms, and prevalence of various diseases. This can range from the use of the face mask described above, use of an N95 respirator, and placement in a negative pressure room. Negative pressure rooms are available at all inpatient facilities and within University Health Services.

b. Where would samples be sent, and what diagnostic tests would be performed? How quickly would results be delivered back to the clinic?

The reference laboratory utilized varies based upon the health system. The WSLH serves as a resource throughout the state of Wisconsin for these situations and maintains advanced capabilities to identify novel and emerging pathogens such as MERS-CoV, Ebola virus, and influenza A subtypes seasonal H1, pandemic H1, H3, H5, and H7, and potential bioterrorism agents. If circumstances require, results for these agents would be available within 5-6 hr of receipt at WSLH. If further characterization of an agent is required, specimens are sent to CDC.

3) How would the possibility that a nearby laboratory was creating novel strains of pathogens affect the tests, control measures, or treatment protocols, if at all?

It is unlikely to have an immediate impact on the care of an individual patient over the short-term. Nevertheless, all UW-Madison employees working with communicable agents such as influenza viruses receive appropriate training to always provide an occupational exposure history when requiring health care for a suspect process. UW-Madison has developed, in collaboration with Infectious Disease physicians, extensive medical response plans for over 34 agents utilized in research settings on our campus. The medical response plans guide healthcare providers in the evaluation and initial treatment of UW-Madison employees with the exposure to and/or possible development of lab-acquired infections. The medical response plans are available to employees and are within all emergency departments within the City of Madison. Emergency Department staff at all three community hospitals have been oriented to the scope and nature of agents utilized in research at UW-Madison. Researchers working in the Select Agent Program are not allowed to see their personal physician if they have a potential exposure or develop symptoms of the agents they work with. In the event either of those situations occurs, the UW Hospital Infectious Disease physicians, in conjunction with the University Health Services, will provide medical care for the individual.

Risk Assessment - Questions for Local Public Health Personnel

Modeling the stochastic phase of a lab-caused infectious disease outbreak will require information about disease surveillance and reporting measures, in order to determine how fast lab-acquired and secondary infections are likely to be detected, as well as the type and level of local public health responses, in order to understand how control measures will influence outbreak spread. The two overarching questions we are trying to understand in this section are: (1) What is the likelihood of diagnosing an infection with a loss-of-containment strain and (2) How quickly does that happen?

Questions

1) Imagine that a local clinic or hospital has alerted you of a potential case of disease caused by an atypical transmissible respiratory pathogen, such as highly pathogenic avian influenza, SARS, or MERS.

a. Would contact tracing, quarantine, or other special measures be implemented?

Yes, the specific actions would depend on the disease and the exposure history.

i. If so, what are the trigger points for those actions to be taken?

Notification of suspected disease

ii. How many personnel would immediately be available for this effort? How quickly would additional personnel arrive (federal or through MOUs), and how much manpower would you expect?

- Business hours: Acute & Communicable Disease Team (3-8 people), epidemiologist, preparedness coordinator, manager
- After hours: Acute & Communicable Disease Team (1 person), epidemiologist, preparedness coordinator, manager
- Potential to draw up to 100 Public Health Madison and Dane County (PHMDC) employees into response with just-in-time training
- Additional assistance can be requested via Dane County Emergency Management.
- State Health officials are immediately available for consultation at any time.
- Contract with Home Health United is a force multiplier if needed.
- Federal assistance is requested by the state health department.

2) Now imagine that a confirmed outbreak with an atypical, communicable, respiratory pathogen such as SARS or novel influenza has started in your community. Please describe the local capacity to respond to such an outbreak, and what steps would be taken to combat it.

a. If contact tracing were necessary, how many personnel would be immediately available for this effort? How quickly would additional personnel arrive (federal or through MOUs) and how much manpower would you expect? Given this level of manpower, approximately how many contacts could be traced and isolated?

See above.

b. Would special measures be taken, such as the closing of schools or workplaces, or at-home isolation or quarantine?

This decision would be made in consultation with University, State, and Federal officials. The local health officer has broad authority to order special measures.

i. What are the thresholds for implementing these measures?

1. How large would an outbreak be before these steps would be taken?

Depends on the situation and the disease

2. Do the thresholds changed based on the disease?

Yes

3. How fast could these measures be implemented?

Within 4-6 hours

c. Would you seek assistance from the Federal government?

Yes, if the situation expands beyond the capabilities of the local and state governments.

i. What type of assistance would you seek, and from what agency?

Consultation and contact tracing from CDC; possibly OSHA.

ii. What would be the thresholds for seeking Federal support?

If the situation is beyond the capabilities of the local and state governments

iii. How quickly would additional federal personnel arrive, and how many?

Within 24-72 hours

3) Now imagine a similar outbreak, but this time with a novel pathogen engineered in a laboratory, such as a new flu strain, with unknown effects on the community. Please explain whether any of the above measures would change if the disease were novel.

   a. Does the source of the infection matter it and of itself, or only the pathogen itself?

Pathogen only

   b. How would you approach decisions for implementing community-level interventions for a pathogen with unknown properties? Would measures be put into place earlier and/or would more aggressive measures be implemented? Or would you wait until epidemiological investigations had been conducted to have a stronger evidence base for your interventions, given that such interventions are disruptive?

More aggressive measures would be put into place earlier.

4) Do you maintain regular contact with your local colleges and/or universities about the strains they are working with?

Yes

   a. What types of information are exchanged?

The UW-Madison SA Program has provided information about all agents that are in use in SA labs, as well as "IRI Select Agent Influenza Virus Exposure Control Overview," which describes, in detail, the protocol for handling known or potential exposures to laboratory-acquired influenza viruses. Similar exposure control protocols are in place for other SA pathogens used in UW-Madison laboratories other than the IRI. In addition, UW-Madison University Health Services has compiled and shared information about non-SA pathogens that are in use throughout the university.

The IRI has provided lab access and presentations on the work they are doing.

   b. If a potential lab exposure occurred, what information about the incident would you expect to receive?

- Personal information for people exposed
- Name of other people working in the lab at the time of exposure
- Route of exposure
- Specific agent involved
- Time of exposure
- Time of illness onset if applicable
- Potential for release to the environment
- Control measures already in place

   c. To where is this information distributed? For example, would local clinics be notified?

This information is distributed between the university, university hospital, and public health. Local clinics would not be notified because the individual in question would be in quarantine and would not be taken or seen at a

clinic. If medical care is needed, this would be done in the quarantine location or at the UW Hospital ER. However, we have the capacity to notify local health care providers if necessary.

d. Does your community have a cache of influenza MCM, and if so, what?

Yes, Tamiflu is available through a state-administered program.