Prepared by:
Corey Meyer, Ryan Ritterson, Emily Billings and Rocco Casagrande
Gryphon Scientific
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Case Studies to Inform Discussions about Defining and Evaluating GoF Studies of Concern

This document describes several notional research cases related to Gain of Function studies of concern (GoFSoC) as defined by the NSABB Working Group draft recommendations. These projects may be useful for investigating the boundaries of what constitutes GoFSoC, including identifying types of studies that should be subject to additional scrutiny, levels of oversight, and/or denied funding. In addition, discussing how the risks and benefits of these projects could be evaluated may help to identify key points that need further discussion prior to operationalizing a policy for the oversight of GoF research.

Case Study #1a: Sequence-based discovery and experimental confirmation of mutations associated with mammalian transmissibility of avian influenza (AI) viruses.

Researchers characterized the airborne transmissibility of several wild type (WT) human isolates of avian influenza (AI) H7N9 in ferrets and found that while most isolates were not transmissible, one isolate was moderately transmissible. The researchers then compared the sequences of the moderately transmissible and non-transmissible isolates to identify amino acid residues present exclusively in the moderately transmissible isolate. The researchers propose to introduce these mutations, individually and in combination, into the non-transmissible H7N9 isolates using reverse genetics and then to characterize the airborne transmissibility of the modified H7N9 isolates in ferrets. Their goal is to identify which mutations are necessary and sufficient to promote the transmissibility of H7N9 viruses in mammals. The researchers will then conduct follow-up biochemical experiments to determine how the mutations cause phenotypic changes that enhance mammalian transmissibility.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate modified H7N9 viruses containing mutations identified in WT H7N9 isolates that are associated with mammalian transmissibility.
    - *In vivo*: Characterization of the airborne transmissibility of WT and modified H7N9 viruses in ferrets (single round of transmission, no selection).
- Virus strains used:
    - Starting strains: human isolates of H7N9 viruses that are not transmissible in ferrets.
    - Ending strains: modified variants of the above strains, which contain mutations present in WT H7N9 isolates that are associated with mammalian transmissibility and may exhibit enhanced transmissibility. These modified strains do not exist in nature but only contain mutations that are found in WT strains (albeit in a slightly different genetic context) – i.e. the modified strains are similar but not identical to WT strains.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).

- Points for discussion:
    - Does the introduction of genetic traits (either mutations, as described above, or genes, through reassortment) that are associated with enhanced transmissibility or virulence in mammals and found in other WT viruses into new strains constitute GoFSoC?
    - What if the mutations are introduced into a similar strain (as described above), essentially re-creating or partially re-creating a WT pathogen?

Case Study #1b: Determination of whether mutations associated with mammalian transmissibility of AI viruses are functionally conserved across different AI strains.

Researchers carried out the experiment described above and identified three point mutations that enhance the airborne transmissibility of H7N9 viruses in ferrets. The researchers propose to introduce these mutations into a WT H5N1 isolate that is not airborne transmissible and to characterize the transmissibility of the modified virus in ferrets, in order to determine whether the same mutations promote mammalian transmissibility in H5N1 and H7N9 AI viruses. Their goal is to better understand whether the mechanisms underlying mammalian transmissibility are conserved across AI strains or whether these mechanisms are virus-specific.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate modified H5N1 viruses containing mutations identified in WT H7N9 isolates that were previously shown to promote airborne transmission of H7N9 viruses.
    - *In vivo*: Characterization of the airborne transmissibility of WT and modified H5N1 viruses in ferrets (single round of transmission, no selection).
- Virus strains used:
    - Starting virus: a human isolate of HPAI H5N1 that is not airborne transmissible in ferrets.
    - Ending virus: modified H5N1 viruses that contain mutations found in WT H7N9 viruses that were previously shown to promote airborne transmission of H7N9 viruses in ferrets, and thus may exhibit enhanced transmissibility relative to the WT H5N1 starting strain.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Points for discussion:
    - Does the introduction of genetic traits (either mutations, as described above, or genes, through reassortment) that are associated with enhanced transmissibility or virulence in mammals and found in WT viruses into new strains constitute GoFSoC?
    - What if the mutations are introduced into a different virus clade? Into a different HA and/or NA sub-type (as described in case study #2)?

Case Study #1c: Sequence-based discovery and experimental confirmation of mutations associated with mammalian transmissibility of avian influenza (AI) viruses, variation of case study 1a.

Researchers compare the sequences of a canine H3N2 influenza virus that originated in birds and its avian precursor strain to identify mutations that are associated with transmissibility in dogs. The canine H3N2 virus is not known to transmit to humans but transmits efficiently in ferrets via the airborne route. By

performing LoF experiments (mutating the canine H3N2 virus) and GoF experiments (mutating the avian-origin H3N2 strain), the researchers determine that four mutations are necessary and sufficient to promote the transmissibility of the canine H3N2 virus in ferrets. The researchers propose to introduce these mutations into an AI H5N1 strain and to test the transmissibility of the modified H5N1 virus in ferrets, in order to determine whether the same mutations promote mammalian transmissibility in avian H3N2 and H5N1 viruses.

- Virus strains used:
    - Starting virus: human isolate of AI H5N1 that is not airborne transmissible in ferrets.
    - Ending virus: modified H5N1 viruses that contain mutations found in WT canine H3N2 viruses that were previously shown to promote airborne transmission of canine H3N2 viruses in ferrets, and thus may exhibit enhanced transmissibility.
- Point for discussion: The mutations of interest promote influenza transmissibility in dogs but may not promote transmissibility in people. Does this constitute a GoFSoC?

Case Study #2a: Forward genetic screen to identify novel mutations that alter phenotypes underlying mammalian transmissibility of AI viruses.

Researchers randomly mutagenize the H7 gene from a human isolate of AI H7N9. The researchers then screen the mutant H7 proteins to identify those with a preference for binding "human-like" α2,6 SA receptors over "avian-like" α2,3 SA receptors, using an *in vitro* assay for SA receptor binding specificity. The researchers identify three different mutations that confer an α2,6 binding preference to H7 proteins *in vitro* and would like to confirm that each mutation is also sufficient to alter the SA receptor binding specificity of the full H7N9 virus.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate modified H7N9 viruses that contain H7 mutations previously shown to enhance the binding of H7 proteins to α2,6 SA receptors *in vitro*; *in vitro* SA receptor binding assays using whole H7N9 viruses.
- Virus strains used:
    - Starting virus: a WT H7N9 virus exhibiting a preference for binding to "avian-like" α2,3 SA receptors over "human-like" α2,6 SA receptors.
    - Ending virus: a modified H7N9 virus that contains mutations associated with α2,6 SA receptor binding, which may exhibit enhanced binding to α2,6 SA receptors.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Point for discussion: The mutations of interest were identified through a forward genetic screen and may not exist in natural, WT strains. Does this constitute a GoFSoC?

Case Study #2b: Computational studies to predict mutations associated with phenotypes underlying mammalian transmissibility.

Researchers use *in silico* approaches to identify H7 mutations that may promote H7 binding to α2,6 SA receptors. The researchers would like to experimentally confirm whether these mutations are sufficient to alter the SA receptor binding specificity of the full H7N9 virus.

- Point for discussion: The mutations of interest were identified through *in silico* modeling approaches and may not exist in natural, WT strains. Does this constitute a GoFSoC?

Case Study #3a: Selection experiment to identify novel mutations that enhance airborne transmission of AI viruses in ferrets.

Researchers serially passage a human isolate of H7N9 in ferrets with selection for airborne transmission, in order to generate modified H7N9 viruses that transmit more efficiently in ferrets via the respiratory route. Their goal is to identify mutations that enhance airborne transmissibility of H7N9 viruses and to characterize their underlying phenotypes, in order to better understand the mechanistic basis of airborne transmission of AI viruses.

- Assays and model systems:
    - *In vivo*: Serial passaging of H7N9 viruses that are not airborne transmissible in ferrets with selection for airborne transmission, in order to select for viruses that are more efficiently transmitted via the respiratory route.
- Virus strains used:
    - Starting virus: a human isolate of H7N9 that is not airborne transmissible in ferrets.
    - Ending virus: if the experiment is successful, a modified H7N9 virus that more efficiently transmits between ferrets via the airborne route.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Point for discussion: How likely are these types of selection experiments to generate viruses with enhanced airborne transmissibility in ferrets?

Case Study #3b: **Selection experiment using reassortant viruses to identify novel mutations that enhance airborne transmission of AI viruses in ferrets.**

Researchers perform the selection experiment described in Case Study #3a but start with a reassortant virus comprising the HA and NA gene segments from H7N9 and the remaining gene segments from the human seasonal H1N1 strain. (The starting reassortant virus is not airborne transmissible in ferrets.) Their goals are two-fold: (1) to identify reassortants that have the capacity for airborne transmission should they arise in nature, and (2) to identify mutations in HA and NA that enhance the airborne transmissibility of the reassortant virus in ferrets.

- Virus strains used:
    - Starting virus: a reassortant virus comprising the HA and NA genes from a human H7N9 isolate and the remaining six genes from a human seasonal H1N1 isolate.
    - Ending virus: if the experiment is successful, a modified H7N9/H1N1 reassortant virus that transmits between ferrets via the airborne route.
- Point for discussion: Given that the transmissibility of the starting H7N9/H1N1 reassortant virus in people is unknown and there is no quantitative assay for transmissibility in ferrets, do you anticipate that the resulting virus will be “highly transmissible”?

Case Study #3c: GoF forward genetic screen to identify novel mutations that enhance airborne transmission of AI viruses in ferrets.

Researchers randomly mutagenize the HA and PB2 genes, two genes known to play a role in airborne transmissibility in mammals, from a human isolate of H7N9. The researchers then create modified H7N9 viruses that contain the mutagenized HA and PB2 genes and screen the mutant viruses to identify those with enhanced transmissibility in ferrets (single round of transmission, no selection). As above, their goal is to identify mutations that enhance airborne transmissibility of H7N9 viruses and to characterize their underlying phenotypes, in order to better understand the mechanistic basis of airborne transmission of AI viruses.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate modified H7N9 viruses containing randomly mutagenized HA and PB2 genes.
    - *In vivo*: Characterization of the airborne transmissibility of modified H7N9 viruses in ferrets (single round of transmission, no selection).
- Virus strains used:
    - Starting virus: a human isolate of H7N9 that is not airborne transmissible in ferrets.
    - Ending virus: modified H7N9 viruses, which contain mutations in their HA and PB2 genes, some of which may exhibit enhanced transmissibility in ferrets.
- Point for discussion: How likely are these types of screening experiments to generate viruses with enhanced airborne transmissibility in ferrets?

Case Study #4a: GoF forward genetic screen to identify novel mutations that enhance airborne transmissibility of AI viruses in ferrets.

Researchers randomly mutagenize the HA and PB2 genes, two genes known to play a role in airborne transmissibility in mammals, from a human isolate of H7N9 that is ***moderately*** transmissible in ferrets. The researchers then create modified H7N9 viruses that contain the mutagenized HA and PB2 genes and screen the mutant viruses to identify those with increased transmissibility in ferrets. Their goal is to identify mutations that ***enhance*** the airborne transmissibility of H7N9 viruses in ferrets.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate modified H7N9 viruses containing randomly mutagenized HA and PB2 genes.
    - *In vivo*: Characterization of the airborne transmissibility of modified H7N9 viruses in ferrets (single round of transmission, no selection).
- Virus strains used:
    - Starting virus: a human isolate of H7N9 that is *moderately* airborne transmissible in ferrets.
    - Ending virus: modified H7N9 viruses, which contain mutations in their HA and PB2 genes, some of which may exhibit increased transmissibility in ferrets (and some of which are likely to exhibit reduced transmissibility).

- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Point for discussion: How likely are these types of screening experiments to generate viruses with enhanced airborne transmissibility in ferrets, given the moderate transmissibility of the starting virus?

Case Study #4b: LoF forward genetic screen to identify novel mutations that are required for airborne transmissibility of AI viruses in ferrets.

Researchers randomly mutagenize the HA and PB2 genes, two genes known to play a role in airborne transmissibility in mammals, from a human isolate of H7N9 that is *moderately* transmissible in ferrets. The researchers then create modified H7N9 viruses that contain the mutagenized HA and PB2 genes and screen the mutant viruses to identify those with reduced transmissibility in ferrets. Their goal is to identify mutations that are *required* for airborne transmissibility of H7N9 viruses in ferrets.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate modified H7N9 viruses containing randomly mutagenized HA and PB2 genes.
    - *In vivo*: Characterization of the airborne transmissibility of modified H7N9 viruses in ferrets (single round of transmission, no selection).
- Virus strains used:
    - Starting virus: a human isolate of H7N9 that is *moderately* airborne transmissible in ferrets.
    - Ending virus: modified H7N9 viruses, which contain mutations in their HA and PB2 genes, some of which may exhibit reduced transmissibility in ferrets (and some of which may exhibit enhanced transmissibility).
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Point for discussion: The same experiment (i.e. case studies #4a and #4b) can be framed as a GoF (#4a) or LoF (#4b) experiment, depending on whether researchers seek to identify mutant viruses with enhanced or reduced transmissibility. Does one or both experiments constitute GoFSoC? Does the intent of the experiment matter?

Case Study #5a: Evaluation of reassortment compatibility of two virus strains.

Researchers propose to perform comprehensive reassortment of the human seasonal H3N2 strain and the HPAI strain H5N1 and to evaluate the *in vitro* fitness of all reassortant strains. Their goal is to determine whether H3N2/H5N1 reassortants are viable and if so, which gene combinations are viable, as reassortment could occur in people who are co-infected with H3N2 and H5N1 viruses. The researchers do not plan to evaluate the transmissibility or virulence of the reassortant viruses.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate all possible H3N2 and H5N1 reassortant viruses; *in vitro* viral growth assays to evaluate the fitness of reassortant strains.

- Virus strains used:
    - Starting viruses: a human seasonal H3N2 strain and an AI H5N1 strain.
    - Ending viruses: reassortant strains comprising all possible combinations of genes from the H3N2 and H5N1 parental strains; some may be unviable, some may have reduced fitness relative to the parental strains, and some may have increased fitness relative to the parental strains.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the experimental manipulations are not expected to alter antiviral susceptibility of the reassortant strains.
- Points for discussion: The proposed reassortment study may generate strains with enhanced virulence or transmissibility relative to the parental viruses, though the phenotypic consequences of reassortment are currently impossible to predict. The researchers do not propose to measure the virulence or transmissibility of the reassortant strains.
    - Is this experiment considered likely to generate a strain with enhanced virulence/transmissibility?
    - Does it matter that the researcher won't know whether their strains possess enhanced virulence/transmissibility?

Case Study #5b: Evaluation of reassortment compatibility of two strains, variation of case study 5a.

The researchers propose to conduct the experiment described in Case Study #5a, but using different strains. Do these experiments constitute GoFSoC?

- (1) Human seasonal H3N2 plus AI H9N2
    - Point for discussion: AI H9N2 has infected fewer humans than AI H5N1 and AI H7N9 and typically causes mild infections. How would you consider this case differently from the reassortment study involving AI H5N1 (#5a), which has infected several hundred people and has a higher mortality rate?
- (2) Human seasonal H3N2 plus AI H5N2
    - Point for discussion: AI H5N2 (the strain that caused the massive outbreak in the US poultry industry in the summer of 2015) has not infected humans. How would you consider this case differently from the reassortment studies involving AI strains that have infected people (#5a and #5b)?

Case Study #6: Antigenic drift of AI H5N1 viruses.

Researchers serially passage a clade 2.2 avian H5N1 virus (a clade which contains viruses that have caused human infections) in chicken cells in the presence of sera from chickens that were vaccinated against H5N1. As vaccination of chickens is an outbreak control strategy used by some countries, the researchers' goal is to determine whether antibody pressure induces antigenic drift that causes the viruses to escape neutralization from the vaccine. The researchers expect to generate H5N1 viruses with altered antigenicity in chickens.

- Assays and model systems:
    - *In vitro*: Serial passaging of H5N1 virus in chicken cells in the presence of sera collected from H5N1-vaccinated chickens.

- Virus strains used:
    - Starting virus: a clade 2.2 avian H5N1 strain (a clade which contains viruses that have caused human infections).
    - Ending virus: an avian H5N1 strain with HA mutations that alter its antigenicity in chickens (i.e. confer escape from the chicken H5N1 vaccine); whether the HA mutations alter virulence or transmissibility of the strain in people is unknown.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Points for discussion: The experimental manipulation is not expected to alter the transmissibility or virulence of the strain in people. That is, the experiment results in the creation of a novel virus that is expected to be highly virulent but not transmissible in people, similar to the parental strain.
    - Do these strains rise above the threshold level of concern for GoFSoC?
    - Does the fact that the resulting strain is novel, albeit not phenotypically different from WT strains from a human perspective, matter?

Case Study #7a: GoF experiment to identify virulence determinants of the 1918 H1N1 pandemic virus.

Researchers propose to create reassortant strains comprising genes from the 1918 H1N1 pandemic virus and the 2009 H1N1 pandemic virus. Specifically, researchers propose to add 1918 H1N1 genes to the 2009 H1N1 virus, singly and in combination, starting with single 1918 genes (i.e. to create a 2009:1918 7:1 reassortant strain) and progressing up to seven 1918 genes (i.e. to create a 2009:1918 1:7 reassortant strain). The virulence of each reassortant strain will be assessed in ferrets, compared to the parental 2009 and 1918 strains. The researchers' goal is to understand which 1918 genes contribute to virulence and how different 1918 genes interact to enhance virulence (i.e. epistasis effects).

- Assays and model systems:
    - *In vitro*: Reverse genetics to create 2009:1918 H1N1 reassortant viruses.
    - *In vivo*: Evaluation of the virulence of the reassortant strains in ferrets.
- Virus strains used:
    - Starting strains: 1918 H1N1 pandemic virus and 2009 H1N1 pandemic virus.
    - Ending strains: Reassortant strains comprising genes from the 1918 and 2009 H1N1 pandemic viruses, ranging from a 2009:1918 gene ratio of 7:1 to 1:7.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Points for discussion:
    - Does this constitute a GoFSoC? Which parts of the study?
    - Is the virulence of the reassortant strains likely to exceed the virulence of the 1918 H1N1 pandemic strain (which will be used as a positive control in all experiments characterizing virulence)? Does that matter?
    - If some of the reassortments were highly likely to exhibit reduced virulence compared to the 1918 H1N1 virus, would researchers be allowed to generate those reassortants but not others?

Case Study #7b: LoF experiment to identify virulence determinants of the 1918 H1N1 pandemic virus.

Researchers propose to replace genes from the 1918 H1N1pdm virus with genes from the 2009 H1N1pdm virus, i.e. to create reassortant strains comprising seven genes from 1918 H1N1pdm and one gene from 2009 H1N1pdm. They will then characterize the virulence of the reassortant strains in ferrets. Their goal is to determine which 1918 genes are necessary for its enhanced virulence. Subsequently, the researchers will use biochemical and immunological assays to probe the function of the identified genes, to gain mechanistic insight into viral virulence.

- Assays and model systems:
    - *In vitro*: Reverse genetics to create 1918:2009 H1N1 reassortant viruses.
    - *In vivo*: Evaluation of the virulence of the reassortant strains in ferrets.
- Virus strains used:
    - Starting strains: 1918 H1N1 pandemic virus and 2009 H1N1 pandemic virus.
    - Ending strains: Reassortant strains comprising genes from the 1918 and 2009 H1N1 pandemic viruses in a 7:1 ratio.
- Points for discussion: Case Studies #7a and 7b present the same experiment. #7a frames the experiment as a GoF study involving 2009 H1N1pdm, whereas #7b frames the experiment as a LoF study involving 1918 H1N1pdm.
    - Is either or both of concern?
    - Does the researchers' intent matter?

Case Study #8: Creation of a GFP-expressing SARS-CoV.

Researchers propose to insert the GFP gene into the genome of SARS-CoV, to create a modified SARS-CoV strain that constitutively expresses GFP. This strain can be used for imaging studies, including fixed cell/tissue and *in vivo* imaging, to gain insight into the biology of SARS-CoV infection.

- Assays and model systems:
    - *In vitro*: Molecular biology to insert the GFP gene into the SARS-CoV genome; reverse genetics to rescue the modified virus; use of GFP-SARS-CoV for *in vitro* infection experiments.
    - *In vivo*: Use of GFP-SARS-CoV for *in vivo* infection experiments.
- Virus strains used:
    - Starting strains: WT SARS-CoV.
    - Ending strains: GFP-expressing SARS-CoV.
- Point for discussion: This experiment proposes to create a highly virulent and highly transmissible pathogen that does not exist in nature, but (if done correctly) GFP-SARS-CoV is not expected to display altered virulence or transmissibility relative to the WT virus. Would this experiment constitute GoFSoC according to the current definition?

Case Study #9: Generation of recombinant bat CoV/SARS-CoV strains to identify determinants of cross-species transmission of CoVs.

Researchers have identified a SARS-like bat CoV that can infect bat cell lines but not human cell lines. The researchers propose to "swap" individual SARS-CoV genes that are thought to promote infection of human cells into the bat-CoV genome, generating recombinant bat-SARS CoVs. The researchers will then compare the ability of the recombinant viruses to infect and replicate within human cells, relative to the parental bat virus, in order to identify SARS-CoV proteins that promote cross-species adaptation. The researchers will then perform follow-up biochemical and cell biological assays to elucidate the function of those SARS-CoV proteins and gain insight into the mechanisms underlying adaptation of bat CoVs to humans.

- Assays and model systems:
    - *In vitro*: Reverse genetics to create recombinant CoVs; *in vitro* infection assays to test the ability of the recombinant viruses to infect and replicate within human cells.
- Virus strains used:
    - Starting strains: WT SARS-like bat CoV, WT SARS-CoV.
    - Ending strains: recombinant bat-SARS CoVs, in which single bat CoV genes have been replaced with corresponding SARS genes.
- Point for discussion: The proposed experiment may enhance the infectivity and virulence of the bat CoV in humans but is unlikely to generate a virus that is more virulent than WT SARS-CoV. However, it may generate new virulent and potentially transmissible viruses that do not exist in nature. Does this experiment constitute GoFSoC?

Case Study #10: Generation of recombinant Ebola strains to identify virulence determinants.

The Zaire species of Ebola virus was responsible for the West African outbreak in 2014 – 2015 that resulted in more than 28,000 cases and more than 11,000 deaths. In contrast, the Reston species of Ebola virus causes Ebola virus disease in non-human primates but does not infect people. In order to identify Ebola Zaire virus genes that promote human infection and pathogenesis, researchers propose to replace individual Ebola Reston virus genes with their corresponding Ebola Zaire virus genes, to generate recombinant Ebola Reston viruses. The researchers will then evaluate the ability of the recombinant viruses to infect human cells, mice, and non-human primates, relative to the parental viruses.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate recombinant Ebola viruses; *in vitro* infection assays to test the ability of the recombinant viruses to infect and replicate within human cells.
    - *In vivo*: Mouse and non-human primate infection experiments to characterize the virulence of the recombinant Ebola viruses.
- Virus strains used:
    - Starting strains: WT Ebola Zaire virus and WT Ebola Reston virus.
    - Ending strains: recombinant Ebola Reston viruses, in which single Ebola Reston virus genes have been replaced with corresponding Ebola Zaire virus genes.
- Points for discussion:
    - The proposed experiment may enhance the infectivity and virulence of the Ebola Reston in humans and mice but is unlikely to generate a virus that is more virulent than the WT Ebola Zaire virus. Does this experiment constitute a GoFSoC?

- Experiments with Ebola will be restricted to BL4 conditions. Does this reduce the level of concern?

Case Study #11: Forward genetic screen to identify *Vibrio cholerae* genes involved in intestinal colonization.

The mechanisms underlying colonization of the intestine by the bacterium *Vibrio cholerae*, the first step in establishing infection, are poorly understood. Researchers propose to use the CRISPR-Cas system to randomly knock out genes throughout the *V. cholerae* genome and subsequently to screen mutants for their ability to colonize the intestine using an infant rabbit model of infection. The researchers expect that most mutants will display no change in colonization or will display colonization defects, but some may exhibit increased colonization relative to the WT bacterium ("hypercolonizers"). Researchers will then study the function of those genes found to promote or suppress colonization in order to better understand the mechanistic basis of intestinal colonization and how colonization efficiency influences disease pathogenesis.

- Assays and model systems:
  - *In vitro*: Use of the CRISPR-Cas system to create pools of mutant bacteria with individual genes knocked out.
  - *In vivo*: Use of the infant rabbit model of infection to characterization the ability of different bacterial strains to colonize the intestine.
- Virus strains used:
  - Starting strains: a WT cholera strain.
  - Ending strains: a pool of recombinant cholera strains in which individual genes have been randomly knocked out.
- Points for discussion:
  - The proposed experiment takes a LoF approach (i.e. by knocking out genes) but may yield mutants with an enhanced ability to colonize the intestine, relative to WT strains. Is this type of experiment considered likely to generate strains with enhanced virulence?
  - *V. cholerae* is not considered a pandemic pathogen in the US or other developing countries with good plumbing infrastructure, but causes epidemics throughout the developing world. Does this matter? How does the role of potentially curative antibiotics change the assessment of the third GoFSoC criterion relative to viral pathogens?

Case Study #12: Generation of latency-free HIV.

Chronic infection with HIV is caused by latency, a property of the virus whereby some viruses infect cells and integrate their genomes into the host genome but do not undergo replication until some later time, thereby creating a permanent pool of the virus. It has been speculated that one possible way to cure infected individuals is to induce the latent pool of virus to become active while the individual is under treatment with antiretrovirals. This would allow the immune system to clear the permanent pool, and thereby the infection. However, the mechanisms underlying latency and re-activation are poorly understood. To gain insight into the mechanistic regulation of latency, researchers propose to create a latency-free virus via targeted mutations of WT HIV followed by *in vitro* selection. If successful, the researchers will then individually introduce each mutation discovered into WT HIV in order to characterize their effects on latency in greater detail. Because latency-free HIV does not exist in nature,

the field is uncertain whether human infection with such a virus would (a) cause a more severe acute infection that is ultimately cleared by the body without long term consequences, or (b) kill so many host T cells that an infected individual would progress to AIDS rapidly. (Note that although many have previously attempted to generate a latency-free HIV, all such attempts have failed. Latency remains an area of active research, including efforts to generate latency-free viruses.)

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate mutant HIVs; *in vitro* selection assays to select for latency-free HIVs; *in vitro* infection assays to test for latency, virulence, and fitness of mutant viruses.
- Virus strains used:
    - Starting strains: WT HIV.
    - Ending strains: modified HIVs with mutations that potentially alter the prevalence and duration of latency, and which may also modulate virulence.
- Countermeasure efficacy: Antivirals are effective against the starting strains, and the proposed mutations are not expected to alter antiviral susceptibility (which can be confirmed experimentally).
- Points for discussion:
    - Given that latency-free HIVs do not exist in nature and the biology of latency-free viruses is significantly different from that of WT HIV, predicting their virulence in humans is nearly impossible. Does the possibility that latency-free viruses will be more virulent than WT viruses render these experiments GoFSoC?
    - Notably,the per-exposure infectiousness of HIV is significantly lower than many epidemic viruses (such as influenza), though the R_0 (number of other people one infected person will infect) is relatively high due to the chronic nature of the infection. Do these features imply that WT HIV has high or low transmissibility?

Case Study #13: Generation of vaccine escape mutants of poliovirus.

In 2010, an outbreak of polio in the Congo caused infections in some previously vaccinated individuals. Genomic sequencing of the poliovirus variant revealed that it had a never-before-seen combination of two mutations that enabled partial immune escape of the virus. Researchers, concerned that similar phenotypes may arise during the worldwide campaign to eradicate polio, propose to randomly mutagenize poliovirus and then test the infectiousness of the resultant viruses after serum neutralization assays. Their goal is to determine if other mutations may also confer vaccine resistance while preserving some level of infectiousness. If any mutations are found, follow-up experiments will be done to attempt to determine the mechanisms underlying vaccine resistance.

- Assays and model systems:
    - *In vitro*: Reverse genetics to generate mutant polioviruses; *in vitro* serum neutralization assays followed by assays to test for antigenic escape.
- Virus strains used:
    - Starting strains: WT poliovirus.
    - Ending strains: modified polioviruses that contain random mutations, some of which may be vaccine-resistant.

- Countermeasure efficacy: The 2010 Congolese outbreak strain overcame vaccination in some individuals previously vaccinated with the inactivated polio vaccine (IPV), though individuals recently vaccinated with the oral polio vaccine (OPV) were still protected. This study may generate novel viruses that are vaccine-resistant (to either type of vaccine), which will be determined during the course of the study.
- Points for discussion:
    - Would this study constitute GoFSoC? During the Congolese outbreak, individuals who were recently vaccinated with OPV were all protected. The strains generated in this experiment may or may not be resistant to OPV vaccination. If the OPV vaccine remained protective, and all researchers in this study undergo OPV vaccination, would this study still constitute GoFSoC? In this case, the risk of LAI would be minimal, but the consequences to the general population could be severe if a laboratory escape event occurred.