To: The NSABB Board (in advance of the September 28, 2015 meeting)

From: Lynn C. Klotz, PhD
Senior Science Fellow and member of the Scientist Working Group on Biological and Chemical Weapons
Center for Arms Control and Non-proliferation, Washington, DC, USA

Home contact Information:
5 Duley Street
Gloucester MA 01930
978-281-6015
lynnklotz@live.com

The following document is a first draft of a literature-research project that started in the summer of 2015. The project is in an early stage, but has gone far enough to make its point: There is an urgent need for international proactive oversight of influenza research that might increase the pathogenicity of influenza viruses. Some of this gain-of-function research may create lab-made potential pandemic influenza viruses.

Even if the probability is small for an escape from a lab in a single year for such a virus, the fact that there are a large number of research projects underway throughout the world, projects that will be conducted for many years, the overall probability of escape from at least one lab is uncomfortably high.

The Potential Pandemic Influenza Research Enterprise

In a recent Letter to the Editor titled *Danger of Potential-Pandemic-Pathogen Research Enterprises* (http://intl-mbio.asm.org/content/6/3/e00815-15.full), I argued that there are likely many labs throughout the world, many not funded by the NIH, that are developing mammal-contagious influenza viruses. Research that makes avian, mammalian, or human influenza viruses more virulent, increases their transmissibility, alters their host range, or evades countermeasures is potentially dangerous and may create potential pandemic pathogens.

Influenza viruses are more likely to fuel an uncontrollable outbreak because of their long history of doing just that. This kind of research got considerable attention in 2011 when Professor Ron Fouchier announced that his laboratory had made the H5N1 highly pathogenic avian influenza virus (HPAI) airborne transmissible by respiratory aerosols from ferret to ferret.

In the context of this analysis of recent publications reported in Pub Med (references (1) through (35)), the larger category of Experiments of Concern (EoC) is used as a guide to look for potentially dangerous research. In 2004, the National Academy of Sciences published a report *Biotechnology Research in an Age of Terrorism* (http://www.nap.edu/catalog/10827.html). The so-called Fink Committee that produced the report was asked to "consider ways to minimize threats from biological warfare and bioterrorism without hindering the progress of biotechnology, which is essential for the health of the nation." The committee recommended that the "Department of Health and Human

Services…create a review system for seven classes of experiments (the Experiments of Concern) involving microbial agents that raise concerns about their potential for misuse." Specifically, the EoC are:

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

These seven classes of experiments "will require review and discussion by informed members of the scientific and medical community before they are undertaken [proactive oversight] or, if carried out, before they are published in full detail." For experiments making deadly avian influenza viruses airborne transmissible, many scientists think they should not be carried out at all.

An excellent system for reviewing potentially dangerous experiments, *Controlling Dangerous Pathogens: A Prototype Protective Oversight System*, was developed in 2007 by The Center for International and Security Studies at Maryland (http://drum.lib.umd.edu/bitstream/1903/7949/1/pathogens_project_monograph.pdf). It recommends a tiered review, from most to least dangerous research. Paraphrased from the Maryland paper:

> International Oversight: *Activities of Extreme Concern* – An international body would be charged with approving and monitoring all research projects of extreme concern. That authority would be narrowly focused only on those ... that could put an appreciable fraction of the human species at risk, such as research with potential pandemic pathogens.
> National Oversight: *Activities of Moderate Concern* – National oversight bodies would be responsible for research activity of moderate concern, such as work with anthrax and other agents already identified as having biological weapons potential.
> Local Oversight: *Activities of Potential Concern* – Concern—This "encompasses those activities that may increase the destructive potential of biological agents that otherwise would not be considered a threat.
> No oversight: *All other research*

In my opinion, there should be two levels of local oversight. The first level is the currently employed Institutional Biosafety Committee (IBC), and the second is an outside committee. There is concern that IBCs will simply rubber-stamp research proposals from labs in their own institution, so I suggest proactive oversight by a committee outside the institution (perhaps at the state level in the US) for experiments of concern on influenza viruses that do not carry an immediate threat of an outbreak from an escape from the laboratory (e.g., vaccine viruses and other attenuated and inactivated viruses).

Because of the potential for some strains of lab-made influenza viruses to cause international outbreaks, research mutagenizing these viruses that could result in increased pathogenicity (gain of function) should be subject to external oversight. At present, there is little national and no international proactive oversight with any authority to guide or ban experiments. See for instance: Gronvall GK, Rozo M. Synopsis of Biological Safety and Security Arrangements. UPMC Center for Health Security.July 2015. Available at http://www.upmchealthsecurity.org/ourwork/publications/synopsis-of-biological-safety-and-security-arrangements.

The literature analysis

To date, only one general Pub Med search term, "avian influenza virus mutagenesis," has been used here to identify potentially dangerous research that might fall under the Experiments of Concern (EoC). To focus on the most recent research, only research over the last two years (September 1, 2013 through August 29, 2015) published Pub Med abstracts were read. Thirty-five potential EoC were identified in 136 abstracts for this single search term. Many of the 136 abstracts (136-35=101) described research that did not constitute EoC; for the most part, they did not employ live viruses.

For each of the 35 abstracts that seemed to describe EoC, parts of the full research papers were read to confirm their EoC status. Since I have only a modest grasp of molecular virology, I may have labeled a few that are not EoC, and I may have missed a few that are EoC.

The actual number of EoC research being carried out today is likely much greater than 35 because of the following:

- Only a single avian influenza search term was used; other influenza search terms would yield additional EoC. In particular, viruses that have already caused pandemics such at the 2009 H1N1 virus.
- Expanding the search back to 2012, and even before that, would yield more EoC.
- There are surely some EoC that are not yet published.
- Search terms involving other pathogens such as SARS, MERS and Ebola would yield more EoC.

A summary of the 35 EoC found from the search is provided in Table 1. Titles and citations for the reference numbers are in the reference list at the end.

Reference Number	Countries of Authors	Viruses	Biosafety Level	EOC Category
1	USA, Korea	H1N1 vaccine strain	?	2
2	**China**	Avian, human H6N1	BSL3	5
3	**China**	H5N1 HPAI	not reported	1, 3
4	USA, Egypt	H5N1 HPAI	?	1, 3
5	**China**	H9N2 avian	BSL3	3, 5
6	**Japan**, USA	H5N1 HPAI	BSL3	3, 5
7	**Netherlands**, UK	H1N1 2009	BSL2	1, 3
8	**China**	H5N1 HPAI	Not reported	3
9	**China**	H7N1 avian	BSL3	3, 5
10	**China**	H9N2, H1N1 2009, H5N1 HPAI	BSL3, BSL3+	3
11	**USA**, Japan	H5N1 HPAI	BSL3	3
12	**China**	H6N1 avian	??	3, 5
13	**Japan**, Thailand	H5N1 HPAI	BSL3	3
14	**China**	H9N2 duck	ABSL3+	3, 5
15	**France**	avian H1N1	BSL3+	3, 5
16	**USA**	A/WSN/1933 H1N1	likely BSL2	5
17	**Netherlands**	airborne trans H5N1 HPAI	animal BSL3+	3, 4
18	**China**	H7N9 HPAI	ABSL3	3
19	**Japan**	H7N9 HPAI	BSL3+	3
20	**China**	H1N1 2009 pandemic	not reported, BSL2?	3
21	**China**	H1N1 2009 pandemic	not reported, BSL2?	2
22	Spain, UK	influenza A vaccine strains	assume BSL2	3
23	**Netherl.**, Germany	HPAI H5N1	BSL3+	1
24	**USA**	H1N1 vaccine strain	assume BSL2	1
25	**USA**	H3N2	BSL2?	2
26	**Germany**	HPAI H5N1	BSL3+	1
27	**China**, USA	HPAI H5N1	BSL3, ABSL3	2
28	**Russia**	nonpath H5N2, HPAI H5N1	not reported, BSL2?	1
29	**Germany**	1968 pandemic H3N2	not reported, BSL2?	3?
30	**USA**	H1N1 vaccine strain	not reported, BSL2?	1
31	**USA**	HPAI H5N1	BSL3	3, 5
32	**UK**	HPAI H5N1	BSL3	3, 5
33	**USA**	H3N2, H1N1	not reported, BSL2?	3?
34	**USA**	HPAI H5N1	ABSL3+	3, 4
35	**USA**	human H3N2, HPAI H5N1	ABSL3+	1

Table 1: The 35 EoC. The boldface in the Countries of Authors column indicates the country where the BSL2, BSL3 research was performed. Much of that research is being carried out in Asia, particularly China.

The 35 published research listed in the Table are described briefly below. The descriptions are a combination of quotes from the Pub Med abstracts and full papers, often paraphrased to make them readily understandable with regard to EoC. The numbers, 1 through 35, at the beginning of each entry below correspond to the numbered reference citations at the end of this document. The **bold-face highlighted** descriptions are the greatest concern in my opinion because the mutated viruses are often more pathogenic than the wild-type strains and are potentially airborne transmissible from human to human.

1. Recombinant influenza viruses were made that have single or double substitutions in neuraminidase N3, N7 and N9 subtypes in a background of an H1N1 vaccine strain. N3, N7 and N9 subtypes have caused human infections. The research discovered resistance to neuraminidase inhibitors in some strains. [Comment: Mutagenesis of vaccine strains are not of the highest concern, unless there is reason to believe that the mutagenesis could make the strain virulent.]

2. Avian H6N1 virus was adapted to human receptor-binding. Receptor-binding was analyzed using isolated H6 proteins. Binding was confirmed using two avian and one human-derived H6N1 recombinant viruses. The research found two HA substitutions important to acquire the human receptor-binding. [Comment: Only one case of human H6N1 infection has been reported to date. Could increasing receptor binding in humans lead to more human cases?]

3. Site-directed mutagenesis was used to generate different patterns of stem glycans on the HA protein of an HPAI H5N1. The results indicated that some glycans were dispensable for the generation of replication-competent influenza viruses. Some combinations of glycans led to a significant decrease of

the growth rates of the mutant viruses in animal cells in comparison to wild type virus. Furthermore, most of the mutant viruses were more sensitive to neutralizing antibodies than the WT virus. [Comment: Could researchers predict results in advance? These are experiments that should be proactively reviewed, as some mutations could have increased virulence or avoided existing vaccines. The outcome is, however, reassuring]

4. Variant H5N1 viruses with five mutations in the HA gene were made. The research indicated that targeted mutation in the HA may be effectively used as a tool to develop broadly reactive influenza vaccines to cope with the continuous antigenic evolution of viruses. [Comment: Could researchers predict results in advance? These are experiments that should be proactively reviewed, as some mutations could have increased virulence or avoid existing vaccines. As viral mutant population sizes are huge, the probability of finding an adaptive mutation is pretty large for RNA viruses.]

5. The research found three mutations in HA, N and PB2 proteins that after four passages conferred high virulence to H9N2 virus in mice. Adaption in mice enhanced the viral polymerase activity and receptor-binding ability, which resulted in a virulent phenotype in mice but not a transmissible phenotype to guinea pigs. [Comment: This additional guinea pig experiment was useful to reduce concern or fear over increased host range.]

6. Mutations made in the PA protein enhanced HPAI H5N1 virus growth capability in human lung cells and increased pathogenicity in mice, suggesting that they contribute to adaptation to mammalian hosts.

7. Mutants made with substitutions in the hemagglutinin of a strain of 2009 H1N1 pandemic influenza virus revealed that single substitutions affecting the loop adjacent to the receptor binding site caused escape from ferret and human antibodies elicited after the 2009 H1N1 pandemic influenza virus infection. The majority of these substitutions resulted in similar or increased replication efficiency *in vitro* compared to that of the virus carrying the wild-type hemagglutinin. However, none of the substitutions was sufficient for escape from the antibodies in sera from individuals that experienced <u>both</u> seasonal and pandemic H1N1 virus infections. [Comment: This is the virus that infected 25% of the world population world-wide in 2009 and killed thousands of people. Any experiment that increases replication efficiency or escapes antibodies should not be carried out in BSL2.]

8. Mutant HPAI H5N1 viruses made with loss of two HA protein glycosylation sites showed increased pathogenicity, systemic spread and pulmonary inflammation in mice compared to the wild-type H5N1 virus.

9. Two mouse-adapted variants of wild-type avian H7N9 made by independent serial passages in mice confer enhanced virulence in mammals. [Comment: This virus has infected and caused fatalities in humans from direct contact with poultry. It would have been informative if the researchers had carried out a single ferret to ferret transmission experiment to see if this mouse-passaged virus has increased host range and virulence in a species (ferrets) that is perhaps a model for humans.]

10. Mouse-adapted PB2 gene reassortants with a phenylalanine-to-leucine mutation contributes to enhanced polymerase activity, enhanced replication, pathogenicity of H9N2 in mice, increased

virulence of H5N1 and 2009 pandemic H1N1. [Comment: Could increasing virulence in the 2009 pandemic flu cause a new outbreak among humans?]

11. The introduction of an arginine residue into PA of HPAI H5N1 significantly increased the viral polymerase activity in mammalian cells and its virulence and pathogenicity in mice.

12. A substitution in the PB2 protein and a substitution in the PA protein enhance virulence and expand the tropism of H6N1 virus in mice. [Comment: Only one case of human H6N1 infection has been reported to date. Could increasing virulence and tropism in humans lead to more human cases?]

13. Introduction of a single substitution into PB1 polymerase of an HPAI H5N1 increased both polymerase activity in chicken cells and the pathogenicity of the recombinant viruses in chickens. [Comment: This translates to humans.]

14. A nonpathogenic duck-origin H9N2 virus was serial-passaged in mouse lungs. Increased virulence was detectable after five passages, and a highly pathogenic mouse-adapted strain was obtained after 18 passages. There were eight amino-acid substitutions in six viral proteins. [Comment: Since serial passage was in lungs, this kind of research could lead to airborne transmission. A single ferret to ferret passage experiment should have been carried out to see if airborne transmission was achieved.]

15. A deletion in the NS segment of a duck-origin avian H1N1 virus showed both increased replication potential and an increased pathogenicity in chicken embryonated eggs and in a chicken lung epithelial cell line.

16. Mutants created in the PB2 subunit identified critical residues required for general polymerase function and specific residues preferentially required in human but not avian cells. [Comment: It is unclear what virus was used in the study. It may have been PB2 mutants reassorted into A/WSN/1933 H1N1 virus. A/WSN/1933 is a derivative of 1918 flu virus and is not around today. This is a mouse brain adapted virus so not a threat.]

17. Five substitutions proved to be sufficient to retain the airborne-transmissible phenotype of HPAI H5N1. [Comment: A large number of substitution experiments on an airborne transmissible, deadly virus were carried out in this study, and a large number of nose and throat swabs and blood samples were taken, all increasing significantly the likelihood of an LAI. This is follow-up research from the Fouchier lab.]

18. An H7N9 virus from a fatal case was used as the recombination background to study the contribution of the E627K mutation in PB2 and of other mutations to the pathogenicity of H7N9 virus infection in mammals. All the mutant viruses generated were likely to be loss-of-function mutants with regard to pathogenicity, compared to the wild-type H7N9. [Comment: The research appears to yield less pathogenic H7N9. Nonetheless, it is not possible to predict pathogenicity at the outset of the experiments. Since the background virus is a fatal case; proactively, the generated viruses could have been more virulent humans. It would have been informative if the researchers had carried out a single

ferret to ferret transmission experiment to see if this virus was more virulent in ferrets, the model for human lung.]

19. Potentially mammalian adapting amino acids were converted individually and in combination to their avian virus-type counterparts in a H7N9 virus. Several mutants were slightly more virulent in mice than the wild-type A(H7N9) virus and exhibited increased polymerase activity in human cells.

20. A single "consensus" PB2 mutation common to swine and the 2009 H1N1 pandemic virus increased pathogenicity. Mutant virus prepared by recombination of a 2009 H1N1 pandemic virus with a segment containing the single PB2 mutation significantly enhanced polymerase activity in mammalian cells. Also, the virus exhibited increased growth properties and induced significant weight loss in a mouse model compared to the wild type. [Comments: This more pathogenic virus could win the battle with the immune system, so cause significant illness.]

21. Reduced sensitivities to oseltamivir were observed in three mutant H1N1 2009 pandemic viruses. A double mutant showed a large increase of IC-50 for the drug Oseltamivir from 0.7 nM for WT to 4,000 nM for the double mutant, a 5,700-fold difference [Comment: Such a large increase in IC-50 would almost certainly make the drug unusable in humans.]

28. A non-pathogenic avian H5N2 was adapted to mice by lung-to-lung passage. Also, the reverse genetics-derived influenza virus containing the HA and NA genes of an HPAI H5N1 in the genetic background of a high-growth H1N1 vaccine strain was obtained. Antibody escape mutants using these two viruses were obtained. Monitoring of effects of HA mutations found in H5 segment escape mutants is essential for accurate prediction of mutants with pandemic potential. [Comment: While H5N2 does not appear to have caused any human infections, adapting it to mice by lung to lung passage could have made it virulent in humans and even airborne transmissible.]

29. Influenza A viruses circulating in humans from ~1950 to ~1987 featured a nonstructural (NS1) protein with a C-terminal amino acid extension present in the H3N2 1968 pandemic flu virus. This research deleted the NS1 extension in the H3N2 in order to compare the wild type H3N2 with the virus with the NS1 deletion. The replication kinetics of the wild-type H3N2 and the deletion mutant were indistinguishable in most experimental systems. However, wild-type virus out-competed the mutant during mixed infections, suggesting that the NS1 extension conferred minor growth advantages. [Comment: The resurrection/rescue of an historical pandemic virus is potentially as dangerous as a lab-made PPP if it escapes from the laboratory, provided that the virus employed is identical to or very close to the 1968 pandemic strain.]

31. A particular point mutation in the PB2 protein of HPAI H5N1 virus, PB2 627K, has been identified as a virulence and host range determinant for infection of mammals, and is present in strains capable of airborne transmission. This mutation in the PB2 gene appeared from day 4 and 5 along the respiratory tracts of mice inoculated intranasally and was complete by day 6 post-inoculation. The mutation correlated with efficient replication of the virus in mice. [Comment: This kind of experiment may be on a path to an airborne transmissible strain.]

32. This research focused on the particular PB2 point mutation in Reference 31, just above. Viruses constructed by reverse genetics were made to contain converse PB2 627K/E mutations in a Eurasian HPAI H5N1 virus and, for comparison, a historical pre-Asian HPAI H5N1 virus that naturally bears PB2 627E. Effects on viral fitness were observed in *in vitro* or *in vivo* experiments. Results suggest that the PB2 627K mutation supports viral fitness in Eurasian-lineage viruses; in contrast, the mutation carries a significant fitness cost in a historical pre-Asian virus.

34. Influenza virus entry is mediated by the acidic-pH-induced activation of HA protein. This research investigated how a decrease in the HA activation pH influences the properties of highly pathogenic H5N1 influenza virus in mammalian hosts. Viruses containing either wild-type HA or an acid-stabilizing point mutation were prepared. Wild-type and viruses with the mutation promoted similar levels of morbidity and mortality in mice and ferrets. The mutation was found to enhance the growth of an H5N1 influenza virus in the mammalian upper respiratory tract, and yet it was insufficient to enable contact transmission in ferrets. Neither virus transmitted efficiently to naive contact cage-mate ferrets. [Comment: It is fortunate that contact transmission was not found.]

35. The research focused on an antigenic cluster associated with a natural single hemagglutinin (HA) substitution that occurred between 1992 and 1995 in the H3N2 virus. Reverse-genetics experiments demonstrated that the HA mutation increases viral receptor binding avidity. The mutation does not prevent antibody binding; rather, viruses possessing this mutation escape antisera simply because the virus attaches to cells more efficiently. [Comment: The H3N2 virus has caused human infections when transmitted from swine. In a 2012 small outbreak, there was no evidence of community transmission. Nonetheless, the virus is an immune escape strain.]

While the search term was not designed to pick up the 2009 human pandemic H1N1 virus, it did pick up a few experiments involving mutagenesis of that strain. While some of this research is carried out at BSL2, it could be classified as research of great concern because that virus is airborne transmissible.

For research involving mutagenesis of vaccine strains, biosafety level was generally not reported. It is assumed that it is BSL2, as vaccine strains are attenuated or inactivated viruses. One concern is that some mutagenesis research could make a vaccine strain virulent. Researchers should be prepared to argue for the safety of their particular proposed vaccine-strain mutagenesis research to defend the lower BSL2 containment.

Several of the EoC (references 7, 10, 14, 17, 20, 21, 28, 29) are lab-made potentially dangerous influenza viruses that could spread from human to human by the airborne route.

Proactive review at the local, national, or international level that considers risk and value (benefits) should be considered before allowing any mutagenesis and related research that might result in Experiments of Concern to go forward, and under what conditions.

Conclusion

Research that employs, makes, or could make airborne transmissible strains is of the greatest concern. All this research should be subject to proactive international review and oversight. There is an urgent need for a binding international process. While the NSABB mandate is likely restricted to NIH-funded research or perhaps any research in the United States, it behooves the NSABB to urge the State Department to seek a binding international agreement for proactive review and oversight of potential pandemic research.

I would like to thank Simon Wain-Hobson for comments and insights.

REFERENCES

1. Song MS(1), Marathe BM(2), Kumar G(3), Wong SS(2), Rubrum A(2), Zanin M(2), Choi YK(4), Webster RG(2), Govorkova EA(2), Webby RJ(5). Unique determinants of neuraminidase inhibitor resistance among N3, N7, and N9 avian influenza viruses. JVI.01514-15. [2015, Epub ahead of print]

2. Wang F(1), Qi J(2), Bi Y(2), Zhang W(2), Wang M(1), Zhang B(3), Wang M(4), Liu J(4), Yan J(2), Shi Y(5), Gao GF(6). Adaptation of avian influenza A (H6N1) virus from avian to human receptor-binding preference. EMBO J. 2015 Jun 12;34(12):1661-73. doi: 10.15252/embj.201590960.

3. Zhang X(1), Chen S(1), Yang D(1), Wang X(1), Zhu J(1), Peng D(2), Liu X(1). Role of stem glycans attached to haemagglutinin in the biological characteristics of H5N1 avian influenza virus. J Gen Virol. 2015 Jun;96(Pt 6):1248-57. doi: 10.1099/vir.0.000082.

4. Ibrahim M(1), Sultan HA(2), Razik AG(2), Kang KI(3), Arafa AS(4), Shehata AA(2), Saif YM(5), Lee CW(6). Development of broadly reactive H5N1 vaccine against different Egyptian H5N1 viruses. Vaccine. 2015 May 28;33(23):2670-7. doi: 10.1016/j.vaccine.2015.04.023.

5. Sang X(1), Wang A, Chai T, He X, Ding J, Gao X, Li Y, Zhang K, Ren Z, Li L, Yu Z, Wang T, Feng N, Zheng X, Wang H, Zhao Y, Yang S, Gao Y, Xia X. Rapid emergence of a PB2-E627K substitution confers a virulent phenotype to an H9N2 avian influenza virus during adoption in mice. Arch Virol. 2015 May;160(5):1267-77. doi: 10.1007/s00705-015-2383-5.

6. Yamaji R(1), Yamada S(2), Le MQ(3), Ito M(1), Sakai-Tagawa Y(1), Kawaoka Y(4). Mammalian adaptive mutations of the PA protein of highly pathogenic avian H5N1 influenza virus. J Virol. 2015 Apr;89(8):4117-25. doi: 10.1128/JVI.03532-14.

7. Koel BF(1), Mögling R(2), Chutinimitkul S(1), Fraaij PL(3), Burke DF(4), van der Vliet S(1), de Wit E(1), Bestebroer TM(1), Rimmelzwaan GF(1), Osterhaus AD(1), Smith DJ(5), Fouchier RA(1), de Graaf M(6). Identification of amino acid substitutions supporting antigenic change of influenza A(H1N1)pdm09 viruses. J Virol. 2015 Apr;89(7):3763-75. doi: 10.1128/JVI.02962-14.

8. Zhang X(1), Chen S(1), Jiang Y(1), Huang K(1), Huang J(1), Yang D(1), Zhu J(1), Zhu Y(1), Shi S(1), Peng D(2), Liu X(1). Hemagglutinin glycosylation modulates the pathogenicity and antigenicity of the H5N1 avian influenza virus. Vet Microbiol. 2015 Feb 25;175(2-4):244-56. doi: 10.1016/j.vetmic.2014.12.011.

9. Yu Z(1), Sun W(2), Li X(3), Chen Q(4), Chai H(5), Gao X(2), Guo J(2), Zhang K(2), Wang T(2), Feng N(2), Zheng X(2), Wang H(2), Zhao Y(2), Qin C(6), Huang G(2), Yang S(2), Hua Y(5), Zhang X(7), Gao Y(8), Xia X(9). Adaptive amino acid substitutions enhance the virulence of a reassortant H7N1 avian influenza virus isolated from wild waterfowl in mice. Virology. 2015 Feb;476:233-9. doi: 10.1016/j.virol.2014.11.031.

10. Liu Q(1), Huang J(1), Chen Y(1), Chen H(1), Li Q(1), He L(1), Hao X(1), Liu J(1), Gu M(1), Hu J(1), Wang X(1), Hu S(1), Liu X(1), Liu X(2). Virulence determinants in the PB2 gene of a mouse-adapted H9N2 virus. J Virol. 2015 Jan;89(1):877-82. doi: 10.1128/JVI.01775-14.

11. Fan S(1), Hatta M(1), Kim JH(1), Le MQ(2), Neumann G(1), Kawaoka Y(3). Amino acid changes in the influenza A virus PA protein that attenuate avian H5N1 viruses in mammals. J Virol. 2014 Dec;88(23):13737-46. doi: 10.1128/JVI.01081-14.

12. Cheng K(1), Yu Z(2), Chai H(3), Sun W(4), Xin Y(4), Zhang Q(5), Huang J(4), Zhang K(4), Li X(4), Yang S(4), Wang T(4), Zheng X(4), Wang H(4), Qin C(6), Qian J(4), Chen H(5), Hua Y(7), Gao Y(8), Xia X(9). PB2-E627K and PA-T97I substitutions enhance polymerase activity and confer a virulent phenotype to an H6N1 avian influenza virus in mice. Virology. 2014 Nov;468-470:207-13. doi: 10.1016/j.virol.2014.08.010.

13. Suzuki Y(1), Uchida Y(2), Tanikawa T(1), Maeda N(1), Takemae N(2), Saito T(3). Amino acid substitutions in PB1 of avian influenza viruses influence pathogenicity and transmissibility in chickens. J Virol. 2014 Oct;88(19):11130-9. doi: 10.1128/JVI.01564-14.

14. Liu Q(1), Chen H, Huang J, Chen Y, Gu M, Wang X, Hu S, Liu X, Liu X. A nonpathogenic duck-origin H9N2 influenza A virus adapts to high pathogenicity in mice. Arch Virol. 2014 Sep;159(9):2243-52. doi: 10.1007/s00705-014-2062-y.

15. Trapp S(1), Soubieux D(2), Marty H(1), Esnault E(1), Hoffmann TW(3), Chandenier M(3), Lion A(1), Kut E(1), Quéré P(1), Larcher T(4), Ledevin M(4), Munier S(5), Naffakh N(5), Marc D(6). Shortening the unstructured, interdomain region of the non-structural protein NS1 of an avian H1N1 influenza virus increases its replication and pathogenicity in chickens. J Gen Virol. 2014 Jun;95(Pt 6):1233-43. doi: 10.1099/vir.0.063776-0.

16. Kirui J(1), Bucci MD, Poole DS, Mehle A. Conserved features of the PB2 627 domain impact influenza virus polymerase function and replication. J Virol. 2014 Jun;88(11):5977-86. doi: 10.1128/JVI.00508-14.

17. Linster M(1), van Boheemen S(1), de Graaf M(1), Schrauwen EJ(1), Lexmond P(1), Mänz B(1), Bestebroer TM(1), Baumann J(2), van Riel D(1), Rimmelzwaan GF(1), Osterhaus AD(1), Matrosovich M(2), Fouchier RA(3), Herfst S(1). Identification, characterization, and natural selection of mutations

driving airborne transmission of A/H5N1 virus. Cell. 2014 Apr 10;157(2):329-39. doi: 10.1016/j.cell.2014.02.040.

18. Mok CK(1), Lee HH, Lestra M, Nicholls JM, Chan MC, Sia SF, Zhu H, Poon LL, Guan Y, Peiris JS. Amino acid substitutions in polymerase basic protein 2 gene contribute to the pathogenicity of the novel A/H7N9 influenza virus in mammalian hosts. J Virol. 2014 Mar;88(6):3568-76. doi: 10.1128/JVI.02740-13.

19. Yamayoshi S(1), Yamada S, Fukuyama S, Murakami S, Zhao D, Uraki R, Watanabe T, Tomita Y, Macken C, Neumann G, Kawaoka Y. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses. J Virol. 2014 Mar;88(6):3127-34. doi: 10.1128/JVI.03155-13.

20. Zhao Z(1), Yi C, Zhao L, Wang S, Zhou L, Hu Y, Zou W, Chen H, Jin M. PB2-588I enhances 2009 H1N1 pandemic influenza virus virulence by increasing viral replication and exacerbating PB2 inhibition of beta interferon expression. J Virol. 2014 Feb;88(4):2260-7. doi: 10.1128/JVI.03024-13.

21. Huang L(1), Cao Y, Zhou J, Qin K, Zhu W, Zhu Y, Yang L, Wang D, Wei H, Shu Y. A conformational restriction in the influenza A virus neuraminidase binding site by R152 results in a combinational effect of I222T and H274Y on oseltamivir resistance.

22. Pérez-Cidoncha M(1), Killip MJ(2), Asensio VJ(3), Fernández Y(1), Bengoechea JA(4), Randall RE(2), Ortín J(1). Generation of replication-proficient influenza virus NS1 point mutants with interferon-hyperinducer phenotype. PLoS One. 2014 Jun 2;9(6):e98668. doi: 10.1371/journal.pone.0098668.

23. Sitaras I(1), Kalthoff D(2), Beer M(2), Peeters B(3), de Jong MC(4). Immune escape mutants of Highly Pathogenic Avian Influenza H5N1 selected using polyclonal sera: identification of key amino acids in the HA protein. PLoS One. 2014 Feb 25;9(2):e84628. doi: 10.1371/journal.pone.0084628.

24. Heaton NS(1), Sachs D, Chen CJ, Hai R, Palese P. Genome-wide mutagenesis of influenza virus reveals unique plasticity of the hemagglutinin and NS1 proteins. Proc Natl Acad Sci U S A. 2013 Dec 10;110(50):20248-53. doi: 10.1073/pnas.1320524110.

25. Tamura D(1), Nguyen HT, Sleeman K, Levine M, Mishin VP, Yang H, Guo Z, Okomo-Adhiambo M, Xu X, Stevens J, Gubareva LV. Cell culture-selected substitutions in influenza A(H3N2) neuraminidase affect drug susceptibility assessment. Antimicrob Agents Chemother. 2013 Dec;57(12):6141-6. doi: 10.1128/AAC.01364-13.

26. Kalthoff D(1), Röhrs S, Höper D, Hoffmann B, Bogs J, Stech J, Beer M. Truncation and sequence shuffling of segment 6 generate replication-competent neuraminidase-negative influenza H5N1 viruses. J Virol. 2013 Dec;87(24):13556-68. doi: 10.1128/JVI.02244-13.

27. Zhu X(1), Guo YH, Jiang T, Wang YD, Chan KH, Li XF, Yu W, McBride R, Paulson JC,

Yuen KY, Qin CF, Che XY, Wilson IA. A unique and conserved neutralization epitope in H5N1 influenza viruses identified by an antibody against the A/Goose/Guangdong/1/96 hemagglutinin. J Virol. 2013 Dec;87(23):12619-35. doi: 10.1128/JVI.01577-13.

28. Rudneva IA(1), Timofeeva TA, Ignatieva AV, Shilov AA, Krylov PS, Ilyushina NA, Kaverin NV. Pleiotropic effects of hemagglutinin amino acid substitutions of H5 influenza escape mutants. Virology. 2013 Dec;447(1-2):233-9. doi: 10.1016/j.virol.2013.09.013.

29. Lohrmann F(1), Dijkman R, Stertz S, Thiel V, Haller O, Staeheli P, Kochs G. Emergence of a C-terminal seven-amino-acid elongation of NS1 in around 1950 conferred a minor growth advantage to former seasonal influenza A viruses. J Virol. 2013 Oct;87(20):11300-3. doi: 10.1128/JVI.01271-13.

30. Myers JL(1), Wetzel KS, Linderman SL, Li Y, Sullivan CB, Hensley SE. Compensatory hemagglutinin mutations alter antigenic properties of influenza viruses. J Virol. 2013 Oct;87(20):11168-72. doi: 10.1128/JVI.01414-13.

31. Min JY(1), Santos C, Fitch A, Twaddle A, Toyoda Y, DePasse JV, Ghedin E, Subbarao K. Mammalian adaptation in the PB2 gene of avian H5N1 influenza virus. J Virol. 2013. Oct;87(19):10884-8. doi: 10.1128/JVI.01016-13.

32. Long JS(1), Howard WA, Núñez A, Moncorgé O, Lycett S, Banks J, Barclay WS. The effect of the PB2 mutation 627K on highly pathogenic H5N1 avian influenza virus is dependent on the virus lineage. J Virol. 2013 Sep;87(18):9983-96. doi: 10.1128/JVI.01399-13.

33. Roberts KL(1), Leser GP, Ma C, Lamb RA. The amphipathic helix of influenza A virus M2 protein is required for filamentous bud formation and scission of filamentous and spherical particles. J Virol. 2013 Sep;87(18):9973-82. doi: 10.1128/JVI.01363-13.

34. Zaraket H(1), Bridges OA, Duan S, Baranovich T, Yoon SW, Reed ML, Salomon R, Webby RJ, Webster RG, Russell CJ. Increased Acid Stability of the Hemagglutinin Protein Enhances H5N1 Influenza Virus Growth in the Upper Respiratory Tract but Is Insufficient for Transmission in Ferrets. J Virol. 2013 Sep;87(17):9911-22. doi: 10.1128/JVI.01175-13.

35. Li Y(1), Bostick DL, Sullivan CB, Myers JL, Griesemer SB, Stgeorge K, Plotkin JB, Hensley SE. Single hemagglutinin mutations that alter both antigenicity and receptor binding avidity influence influenza virus antigenic clustering. J Virol. 2013 Sep;87(17):9904-10. doi: 10.1128/JVI.01023-13.