The ongoing battle against influenza

Influenza viruses can cause a broad spectrum of disease severity, including devastating cases in some people. Several factors influence the epidemiological success of the virus; the mechanisms of transmission and the strategies for prevention and treatment have an impact on the disease outcome and the incidence of flu infection in the population. Understanding how and why the viruses spread so efficiently among people and determining possible ways to harness this transmission have been arduous tasks, given the limitations of flu animal models. In 'Bedside to Bench', Kanta Subbarao and Seema S. Lakdawala peruse a study that used a human challenge model to assess influenza transmission; this experimental approach shows how transmission can be studied in humans and emphasizes factors that are different compared to animals, such as distinct disease severity and incidence. Lessons can be taken to optimize animal studies. Another issue that dictates the severity of flu episodes is the potential emergence of drug-resistant strains in treated individuals. In 'Bench to Bedside', Anne Kelso and Aeron C. Hurt discuss another concern—the presence of drug-resistant viruses with additional permissive mutations that make them fit to infect and compete with wild-type strains. The fact that these strains can be found in untreated people and can spread poses a public health concern and a challenge for scientists to find new drugs and assess antiviral combinations.

■ BEDSIDE TO BENCH

The challenge of flu transmission

Seema S Lakdawala & Kanta Subbarao

The epidemiological success of seasonal and pandemic influenza viruses depends on their ability to transmit efficiently from person to person. The size of infectious virus–containing particles released by infected individuals and the relative importance of fomites and airborne transmission is a matter of debate. Additionally, the contributions of viral, host and environmental factors on the transmissibility of influenza viruses are poorly understood. For the last decade, investigators have relied upon animal models such as ferrets and guinea pigs to assess this complex process. Conventional studies use small numbers of animals with exposure lasting 14 d that includes contact transmission, in which the infected donor and naive recipient animals are co-housed, or airborne transmission, where perforated panels separate them (**Fig. 1**).

In a recent study, Killingley *et al.*[1] sought to characterize influenza transmission in humans using the human challenge model that has been used to study disease progression and vaccine efficacy[2–4]. The authors examined the transmission of influenza from donor volunteers who were experimentally infected by intranasal administration of an influenza virus to healthy subjects who shared living conditions in a quarantine facility. The donors and recipients were separated except during exposure events that took place over several hours. The clinical signs and symptoms in the experimentally infected volunteers, which are known to be milder than in natural infection[5], were runny nose, stuffy nose and cough. The authors assessed the deposition of influenza virus in the environment, on inanimate surfaces and in the air, which was sampled and size fractionated to assess the size of exhaled particles containing influenza virus. This proof-of-concept study establishes the feasibility of studying influenza transmission in humans and highlights several important considerations for future studies in human subjects, such as a low incidence of illness and lack of severe symptoms in infected donors. Notably, for bench researchers, this study also suggests crucial ways by which animal models of influenza transmission can and should be refined.

Although 77% of the donors were infected, the virus only transmitted to 25% of recipients; in contrast, the transmission efficiency of seasonal influenza viruses is 100% in animal models[6–8]. There are potential explanations for this discrepancy. First, humans often have prior experience with circulating influenza viruses, whereas influenza-naive animals are used in animal studies. Preexisting immunity is known to prevent or limit virus replication and might therefore limit the spread of influenza. During the 2009 H1N1 pandemic, elderly individuals were protected from severe disease because of prior exposure to H1N1 viruses that circulated before 1950 (ref. 9). Similarly, prior infection of donor guinea pigs with a seasonal influenza virus decreased the transmissibility of the pandemic H1N1 virus to recipient animals[8]. In addition to antibody-mediated immunity, T cells have been implicated in limiting disease symptoms and early viral clearance in humans[4]; these factors could reduce the likelihood of transmission to contacts. However, the role of memory T cells in transmission has not been explored in animal models owing to a lack of appropriate immunological reagents.

Second, human exposure events are limited to a few hours, as in the study by Killingley *et al.*[1] in which exposure events included sharing meals, playing games and watching TV. Transmission studies in animals, however, involve continuous exposure, with the recipient and donor animals housed a few feet apart for 14 d. Possible modifications of animal studies could better mimic human transmission events (**Fig. 1**).

Influenza viruses can transmit between humans via direct or indirect contact and by respiratory droplets, but their relative contribution is unknown[10,11]. Contact transmission refers to viruses transferred by skin contact with secretions from an infected person, by way of contaminated hands or contact with a contaminated inanimate object, to mucous membranes of the eyes or nose. Respiratory droplet transmission is caused by a spray of particles that include large (>5 μm) droplets and small (<5 μm) particles. Large droplets can be deposited on inanimate objects and onto mucous surfaces, whereas small particles remain suspended in the air, can travel more than 1 m from the source to the recipient and can be inhaled into the lower respiratory tract (**Fig. 1**). Respiratory droplet rather than contact transmission is held as the

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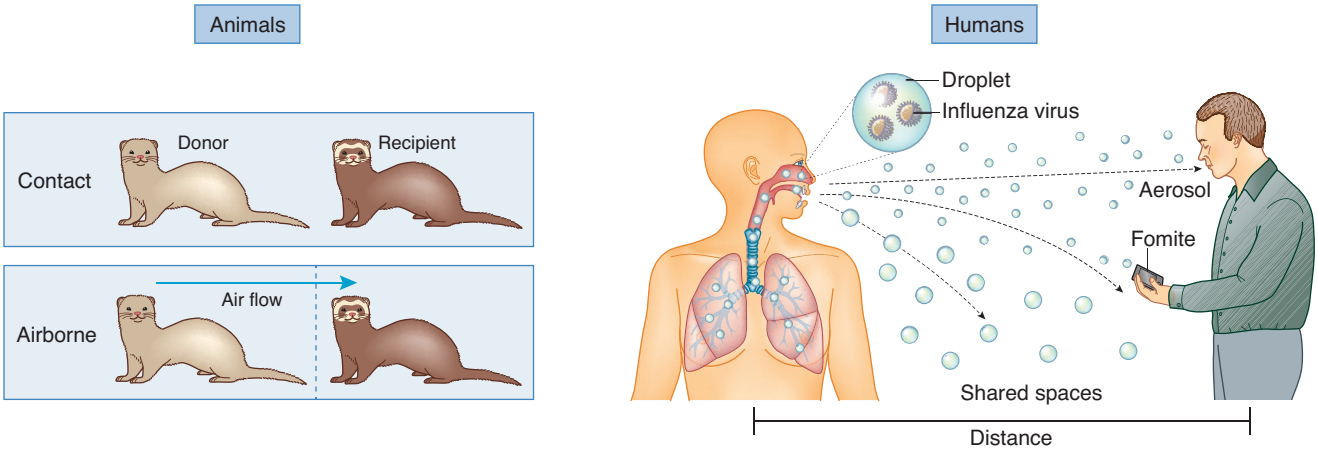

Modifying airborne transmission studies in animals		
Transmission parameters	Commonly used	Proposed modifications
Exposure time	14 d	30 min, 8 h or 1 h d^{-1}
Influenza virus exposure history	Seronegative	Vaccine versus prior infection in donors and/or recipients
Air flow/particle movement	Directional, from donor to recipient	Variable or nondirectional
Distance from aerosol plume	24–60 in	Design cages with larger distances (up to 1 m) between animals

Figure 1 Transmission of influenza viruses in animal models and humans. The design of animal transmission studies is not standardized, and they include either contact or airborne transmission. In humans, influenza viruses are transmitted in shared spaces such as classrooms, healthcare settings, work spaces and public transport, by direct or indirect contact via contaminated surfaces (fomites) and by respiratory droplets. These droplets include a wide range of particle sizes that are released from different parts of the respiratory tract; large particles fall out of the air quickly and can be deposited onto surfaces. Certain parameters that probably influence animal transmission by respiratory droplets could be modified to better mimic transmission in humans.

gold standard for assessing the transmissibility of influenza viruses in ferrets and guinea pigs because animal contact behavior differs from humans, and influenza viruses that are epidemiologically unsuccessful in humans are able to transmit efficiently between ferrets via contact[12]. Contact transmission studies in animals require co-housed animals to share water bottles and food, and social and playful animals, such as ferrets, maintain close contact with their cage mates. Disregarding contact transmission in animal models might result in the loss of some potentially important information about influenza transmission. Therefore, the importance of this mode of transmission needs to be studied in the human challenge transmission model. Understanding the relative contribution of each mode of transmission will help tailor the use of nonpharmaceutical interventions, such as hand washing, social distancing measures and cough etiquette, and infection control measures, such as bed spacing and personal protective equipment, to limit the spread of influenza.

Air samplers developed by the US National Institute for Occupational Safety and Health fractionate aerosols by size, and the presence of viral nucleic acid can be determined by PCR[13]. Analysis of aerosols produced in clinical settings and from individuals who cough suggests that humans predominantly release submicron particles containing influenza[14,15]. Respiratory droplet transmission in ferrets correlated with release of submicron particles containing influenza RNA, and this release was linked to specific viral properties[16]. Infected ferrets also exhale aerosol plumes similar to humans, during tidal breathing and induced sneezing[17]. However, the duration and distribution of aerosol release may differ between intranasally inoculated animals and naturally infected humans. It is important to understand the similarities and differences between aerosol generation in humans and animal models.

To take the findings from the clinical study back to the bench, the design of transmission studies in animal models can be modified by changing environmental conditions, assessing aerosol release, using partially immune animals and mimicking human contact behavior (**Fig. 1**). Human exposure events, such as a short bus ride (30-min exposure), a long flight (8-h exposure), or intermittent daily exposure to a co-worker (1 h exposure every day for 5 d) can be mimicked. In addition, the timing of exposure of naive animals to experimentally infected animals can be limited to exposure early or late in the course of infection. Although such modifications will probably decrease the transmission efficiency in animal models and will require the use of larger numbers of animals per study, the experiments will better reflect the transmissibility of viruses in humans. A new, alternative approach to studying transmission without animals is the use of simulated coughing and breathing machines, which showed that a properly fitted N95 respirator or surgical mask was effective in blocking entry of the virus into the mouth[18].

Understanding the mechanism and molecular basis of influenza transmission is essential for assessing the pandemic potential of novel influenza viruses and for the development of public health intervention strategies for influenza. Influenza virus transmission can be interrupted by the use of nonpharmaceutical interventions as well as by vaccination and antiviral prophylaxis if implemented early[2,19]. Human challenge studies will extend our understanding of human transmission events in controlled settings and should guide modification of animal models; the latter are of great value, as they allow us to tease apart molecular and viral properties that contribute to transmission using engineered viruses. A two-pronged

approach that combines clinical research with the use of animal models will allow scientists to understand how we catch the flu.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Killingley, B. *et al. J. Infect. Dis.* **205**, 35–43 (2012).
2. Killingley, B. *et al. Lancet Infect. Dis.* **11**, 879–886 (2011).
3. Powell, T.J. *et al. J. Infect. Dis.* **205**, 20–27 (2012).
4. Wilkinson, T.M. *et al. Nat. Med.* **18**, 274–280 (2012).
5. Little, J.W., Douglas, R.G. Jr., Hall, W.J. & Roth, F.K. *J. Med. Virol.* **3**, 177–188 (1979).
6. Belser, J.A., Maines, T.R., Tumpey, T.M. & Katz, J.M. *Expert Rev. Mol. Med.* **12**, e39 (2010).
7. Maines, T.R. *et al. Proc. Natl. Acad. Sci. USA* **103**, 12121–12126 (2006).
8. Steel, J. *et al. J. Virol.* **84**, 21–26 (2010).
9. Itoh, Y. *et al. Nature* **460**, 1021–1025 (2009).
10. Brankston, G., Gitterman, L., Hirji, Z., Lemieux, C. & Gardam, M. *Lancet Infect. Dis.* **7**, 257–265 (2007).
11. Tellier, R. *J. R. Soc. Interface* **6 Suppl 6**, S783–7S90 (2009).
12. Wan, H. *et al. PLoS One* **3**, e2923 (2008).
13. Blachere, F.M. *et al. Clin. Infect. Dis.* **48**, 438–440 (2009).
14. Fabian, P. *et al. PLoS One* **3**, e2691 (2008).
15. Lindsley, W.G. *et al. PLoS One* **5**, e15100 (2010).
16. Lakdawala, S.S. *et al. PLoS Pathog.* **7**, e1002443 (2011).
17. Gustin, K.M. *et al. Proc. Natl. Acad. Sci. USA* **108**, 8432–8437 (2011).
18. Noti, J.D. *et al. Clin. Infect. Dis.* **54**, 1569–1577 (2012).
19. Ferguson, N.M. *et al. Nature* **442**, 448–452 (2006).

■ BENCH TO BEDSIDE

Drug-resistant influenza viruses: why fitness matters

Anne Kelso & Aeron C Hurt

Influenza viruses can spread explosively and cause devastating disease. Of the few drugs we have to reduce the impact of influenza, oseltamivir is the most widely used and stockpiled. However, just over four years ago, A(H1N1) influenza viruses circulating in humans rapidly acquired a mutation that conferred oseltamivir resistance without a loss of viral fitness—their ability to infect, replicate in and be transmitted by the host—in the absence of the drug[1,2]. This lineage of seasonal H1N1 viruses vanished from human circulation as the pandemic H1N1 virus spread around the world in 2009–2010. In the two years before their disappearance, however, the former seasonal H1N1 viruses gave us an important lesson in influenza virology. That lesson is relevant again today.

Since 1999, the influenza neuraminidase (NA) inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza) have been widely available for the treatment of influenza, and many countries have acquired stockpiles for use in a pandemic. Before 2008, few countries other than Japan and the US had used oseltamivir on a large scale, and resistance to the drug was detected only rarely. Resistance occurred most commonly in oseltamivir-treated patients, particularly the immunosuppressed who received extended drug treatment because they could not clear the infection quickly. Many oseltamivir-resistant H1N1 viruses had an amino acid substitution near the enzyme active site of their NA, from histidine to tyrosine at position 275 (H275Y) (274 in N2 numbering), which impaired the ability of NA to undergo the rearrangement that is necessary for effective oseltamivir binding (summarized by Moscona[3]). Initial studies showed that H1N1 viruses with the H275Y mutation were less fit than wild-type viruses *in vitro* and in animals in the absence of the drug[4,5], suggesting that sustained transmission between untreated people was unlikely (**Fig. 1**).

This comfortable notion was overturned in early 2008 when Norway reported that a high proportion of H1N1 viruses analyzed in that country were oseltamivir resistant and carried the H275Y mutation, despite negligible clinical use of oseltamivir. Over the next year, H275Y variants progressively became predominant among H1N1 viruses circulating in both hemispheres[1,2], apparently arising independently in several locations[1]. Although it is not known whether they originated in oseltamivir-treated patients, there was no escaping the conclusion that these viruses could compete effectively with their oseltamivir-sensitive counterparts and that one of the few drugs available for influenza prophylaxis and treatment was no longer useful for H1N1 viruses.

The unexpected ability of the new drug-resistant variants to spread between untreated people was partly explained by studies investigating the function of the resistant NA[6,7]. Bloom *et al.*[6] showed that the reduced expression of H275Y NA at the infected cell surface and concomitant loss of viral fitness in the absence of drug were overcome by substitutions at positions 222 and 234 of the NA. These 'permissive' mutations had preceded the widespread emergence of H275Y in circulating H1N1 viruses, apparently enabling acquisition of oseltamivir resistance without loss of viral fitness in the absence of the drug (**Fig. 1**). The fact that most oseltamivir-resistant (and many sensitive) H1N1 viruses circulating in 2007–2008 belonged to a new antigenically distinct group (represented by the vaccine virus A/Brisbane/59/2007) may be relevant, particularly if the rapid spread was assisted by their antigenic novelty or other functional changes in the hemagglutinin[1,7].

Why does this story matter now that the former seasonal H1N1 lineage is no longer circulating? For the first year after their emergence in early 2009, the pandemic H1N1 (H1N1pdm09) viruses behaved like the former seasonal H1N1 viruses before 2008 with only sporadic oseltamivir resistance, usually in treated patients. In 2010 and 2011, however, oseltamivir-resistant H275Y H1N1pdm09 viruses were detected in several countries at elevated frequencies among untreated community cases with no known contact with treated patients[8–10]. The largest cluster identified to date occurred in and around the city of Newcastle in eastern Australia between May and August 2011 (31 cases), with a single case in September about 4,000 km away[8,11]. High sequence similarity suggested the spread of a single variant. No further cases have been detected. It is not known whether the cluster died out because the influenza season came to an end or because these viruses were less fit than oseltamivir-susceptible viruses concurrently circulating in the same region.

These observations raise the possibility that recently circulating H1N1pdm09 viruses that acquire tyrosine at position 275 may not suffer much loss of fitness in untreated patients. It is notable that several candidate permissive NA mutations, predicted by computational modeling to offset the negative effect of the H275Y substitution on NA stability, are now present in these currently circulating viruses[11,12].

Against the background of the experience in 2008, the Newcastle cluster and other recent

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