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Influenza A Virus Coinfection through Transmission Can Support High Levels of Reassortment

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ABSTRACT

The reassortment of gene segments between influenza viruses increases genomic diversity and plays an important role in viral evolution. We have shown previously that this process is highly efficient within a coinfected cell and, given synchronous coinfection at moderate or high doses, can give rise to ~60 to 70% of progeny shed from an animal host. Conversely, reassortment *in vivo* can be rendered undetectable by lowering viral doses or extending the time between infections. One might also predict that seeding of transmitted viruses into different sites within the target tissue could limit subsequent reassortment. Given the potential for stochastic factors to restrict reassortment during natural infection, we sought to determine its efficiency in a host coinfected through transmission. Two scenarios were tested in a guinea pig model, using influenza A/Panama/2007/99 (H3N2) virus (wt) and a silently mutated variant (var) thereof as parental virus strains. In the first, coinfection was achieved by exposing a naive guinea pig to two cagemates, one infected with wt and the other with var virus. When such exposure led to coinfection, robust reassortment was typically seen, with 50 to 100% of isolates carrying reassortant genomes at one or more time points. In the second scenario, naive guinea pigs were exposed to a cagemate that had been coinoculated with wt and var viruses. Here, reassortment occurred in the coinoculated donor host, multiple variants were transmitted, and reassortants were prevalent in the recipient host. Together, these results demonstrate the immense potential for reassortment to generate viral diversity in nature.

IMPORTANCE

Influenza viruses evolve rapidly under selection due to the generation of viral diversity through two mechanisms. The first is the introduction of random errors into the genome by the viral polymerase, which occurs with a frequency of approximately 10^{-5} errors/nucleotide replicated. The second is reassortment, or the exchange of gene segments between viruses. Reassortment is known to occur readily under well-controlled laboratory conditions, but its frequency in nature is not clear. Here, we tested the hypothesis that reassortment efficiency following coinfection through transmission would be reduced compared to that seen with coinoculation. Contrary to this hypothesis, our results indicate that coinfection achieved through transmission supports high levels of reassortment. These results suggest that reassortment is not exquisitely sensitive to stochastic effects associated with transmission and likely occurs in nature whenever a host is infected productively with more than one influenza A virus.

The segmented nature of the influenza virus genome allows for ready exchange of genetic material between two viruses that coinfect one cell (1). If the parental viruses differ in all eight gene segments, 256 different progeny viruses can be produced in a single reassortment event. Reassortment between two very distinct strains is typically associated with marked genotypic and phenotypic changes and is well described by the term "genetic shift." The substantial impact of genetic shift on the epidemiology of influenza has been documented repeatedly. Genetic shift contributed to the emergence of the 1957, 1968, and 2009 pandemic influenza A viruses (IAV) (2–4). It was important to the establishment of the highly pathogenic H5N1 lineage now endemic in Southeast Asian poultry and continues to play a critical role in the rapid evolution of this lineage (5–8) and the emergence of related H5 subtype viruses (9, 10). Similarly, reassortment was central to the emergence of the H7N9 subtype IAV that gave rise to an ongoing zoonotic outbreak in China starting in 2013 (11, 12). Reassortment between human 2009 pandemic strains and IAV endemic to swine hosts has produced a plethora of new genotypes in swine, including the H3N2v viruses, which appear to transmit to humans more readily than previously circulating swine viruses (13–16). Intrasubtype reassortment of IAV in human hosts was shown to underlie the emergence of viruses that caused unusually severe seasonal outbreaks in 1947, 1951, and 2003–2004 (17–19). Reassortment between human H1N1 and H3N2 lineages is detected more rarely but gave rise to an H1N2 virus that circulated widely in the United Kingdom in 2001–2002 (20, 21). Reassortment among avian influenza viruses in birds is highly prevalent and has a major impact on viral population structure in avian reservoirs (22–30). Thus, reassortment between IAV from two distinct sources occurs in nature and can have major consequences for the epidemiology of the virus in humans and other natural hosts. Nevertheless, the reassortant viruses that are detected in the wild are most often those that are evolutionarily successful. For this reason, the prevalence of reassortment in naturally infected hosts cannot be extrapolated from the detection of circulating reassortant viruses.

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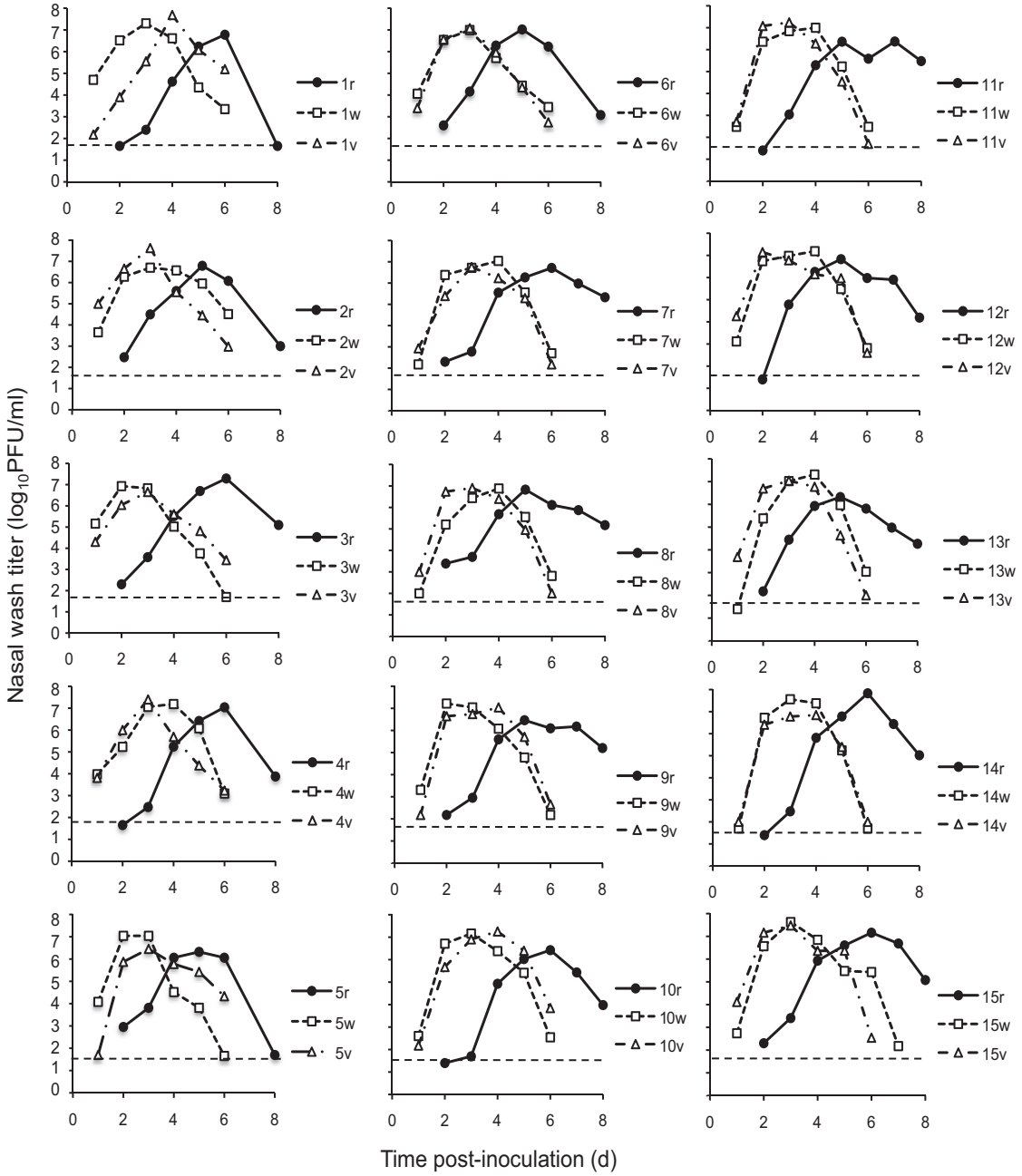

FIG 1 Nasal-wash titers of donor and recipient guinea pigs in a dual-exposure model. Each cage of three guinea pigs is represented by a separate graph. The symbols in each graph indicate the identification (ID) number of each animal, which comprises the cage number followed by "r" for a recipient guinea pig, "w" for a wt-virus-infected donor, or "v" for a var-virus-infected donor. Transmission in cages 1 to 6 was performed in parallel and involved Pan/99wt and Pan/99var6 viruses. Transmission in cages 7 to 15 was performed in parallel at a later time and involved Pan/99wt and Pan/99var15 viruses. The titers of recipient guinea pigs are plotted with solid lines and circles. Donor guinea pigs are shown with open symbols and dashed lines. The limit of detection (50 PFU/ml) is indicated with a horizontal dashed line, and titers below the limit of detection were plotted as 45 PFU/ml. d, day(s).

Since coinfection of a host with two differing strains is a prerequisite for reassortment, some insight into the frequency of reassortment in nature can be gleaned from data on the incidence of coinfections in natural hosts. Efforts to characterize intrahost viral genetic diversity are valuable in this regard, and a number of such studies have been performed using samples collected during natural outbreaks or from experimental transmission chains (31–37). Importantly, these efforts have revealed high levels of mixed infection and point to two ways that such infections arise. Natural transmission was found to be associated with loose transmission bottlenecks, allowing the cotransmission of multiple virus variants (32–36, 38, 39). In addition, evidence for infection with distinct IAV strains through two independent transmission events was observed in some hosts (31, 33, 34, 37). Due to the nature of the viral sequence data used in these studies, reassortment cannot be tracked readily and was not examined. Thus, although mixed

infection is not uncommon in natural settings, the outcome of these infections in terms of reassortment is unclear.

To address this knowledge gap, we have evaluated here the prevalence of reassortment in animal hosts where coinfection with two different IAV strains occurred through transmission. Mixed infections achieved through cotransmission of multiple variants and through multiple independent transmission events were analyzed. To perform these experiments in a streamlined and unbiased manner, we employed our recently developed coinfection system based on two parental viruses that differ only by silent nucleotide changes in each segment (1). Studying reassortment between well-matched wild-type (wt) and silently mutated variant (var) viruses allows us to eliminate the confounding effects of fitness differences among parental and reassortant progeny viruses. In this way, we study the process of reassortment itself rather than the genetic compatibility of a particular pair of influenza viruses. At the same time, the silent differences between wt and var gene segments allow them to be differentiated using high-resolution melt (HRM) analysis (1, 40); thus, reassortants can be detected without full or partial sequencing of all eight gene segments. Using this system, we have detected high frequencies of reassortment in guinea pigs coinfected through transmission. Our results indicate that, at least in a guinea pig model, reassortment potential is not markedly reduced by stochastic effects inherent in viral transmission.

TABLE 1 Detection of wt and var HA segments in guinea pig nasal-wash fluids by RT-qPCR

Guinea pig no.[a]	C_T[b] Day 5 wt	Day 5 var	Day 6 wt	Day 6 var	Virus detected
1r	35.1	—	32.2	—	wt
2r	—	28.0	—	29.5	var
3r	33.6	—	29.8	—	wt
4r	**36.8**	**36.4**	**30.9**	**31.6**	**Both**
5r	34.9	—	34.0	—	wt
6r	**33.3**	**34.9**	**33.1**	**35.5**	**Both**
7r	33.6	—	32.1	35.5	Both
8r	—	33.6	—	31.0	var
9r	36.4	33.6	33.3	29.4	Both
10r	—	—	32.4	35.4	Both
11r	**36.5**	**33.4**	**37.0**	**30.7**	**Both**
12r	**34.5**	**32.3**	**34.5**	**31.6**	**Both**
13r	—	32.7	—	30.3	var
14r	**32.6**	**34.1**	**30.3**	**30.4**	**Both**
15r	**33.4**	**34.9**	**30.7**	**29.4**	**Both**

[a] The animals analyzed for reassortment are indicated in boldface.
[b] C_T values obtained from nasal washes collected on day 5 and day 6 postinfection are shown. —, values of >37.0, which were considered negative.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. These cells were used to determine viral titers in guinea pig nasal washes and to isolate plaque clones from these samples by standard plaque assay.

Viruses. Recombinant A/Panama/2007/1999 (H3N2) (rPan/99wt, or wt) and rPan/99var6 viruses were described previously (1, 41). Here, we also used, for the first time, rPan/99var15 virus. These viruses were generated by reverse genetics (41, 42) and propagated in embryonated hens' eggs for one (var15), two (var6), or three (wt) passages. rPan/99var6 virus contains the following silent mutations relative to rPan/99wt virus (nucleotide numbering is from the 5′ end of the cRNA): NS, C329T, C335T, and A341G; M, C413T, C415G, and A418C; NA, C418G, T421A, and A424C; NP, C537T, T538A, and C539G; hemagglutinin (HA), T308C, C311A, C314T, A464T, C467G, and T470A; PA, A342G and G333A; PB1, C288T and T297C; and PB2, C354T and C360T. rPan/99var15 virus differs from rPan/99var6 virus only in the PB1 and M segments and carries PB1 A540G and M G586A mutations relative to the wt strain. Collectively, these mutations were shown not to attenuate the growth of rPan/99var viruses relative to rPan/99wt virus in guinea pigs and to allow distinction of wt and var gene segments using HRM analysis (reference 1 and this study). Analysis of the frequency of incorporation of wt and var segments into reassortant viruses, furthermore, does not reveal segment biases that might arise if var mutations affected packaging signals (data not shown). For simplicity, both Pan/99var6 and Pan/99var15 viruses are referred to as "var" here, but we have indicated which var virus was used in each experiment in Results below and the figure legends. The var15 virus was generated recently, and we switched from var6 to var15 part way through the study since, in the PB1 and M segments, it gives clearer separation in HRM between the wt and var viruses.

Guinea pigs. Animal work was performed in compliance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and was approved by the Emory Institutional Animal Use and Care Committee under protocol number DAR-2002738-051317GA. Female Hartley strain guinea pigs weighing 300 to 350 g were obtained from Charles River Laboratories. Prior to intranasal inoculation, nasal lavage, or CO_2 euthanasia, the guinea pigs were sedated with a mixture of ketamine and xylazine (30 mg/kg of body weight and 4 mg/kg, respectively). Inoculation and nasal lavage were performed as described previously (43), with phosphate-buffered saline (PBS) as the diluent/collection fluid in each case. Following inoculation and recovery from sedation, donor guinea pigs were housed in Caron 6040 environmental chambers (fitted with the optional dryer package) set to 5°C and 20% relative humidity. At 24 h postinoculation of the donor animals, exposed guinea pigs were introduced into the same cage with the donor animal(s). Conditions of 5°C and 20% relative humidity were maintained throughout the exposure period, which ended on day 8 postinoculation.

Identification of coinfected guinea pigs by detection of HA segments in bulk nasal-wash fluids. As described previously (1), the HA segments of rPan/99wt and rPan/99var viruses differ by 6 nucleotides in two clusters: T308C/C311A/C314T and A464T/C467G/T470A. Forward and reverse primers encompassing these mutation clusters were designed: HAwt 295F/HAwt 481R and HAvar 295F/HAvar 481R. These primers specifically amplify portions of the wt or var HA segment, respectively, allowing their quantification by conventional quantitative-PCR (qPCR) methods. Thus, RNA extracted directly from nasal-lavage fluids was subjected to reverse transcription (RT) followed by qPCR using SsoFast Evagreen Supermix (Bio-Rad), according to the manufacturer's instructions. qPCR was performed with a CFX384 Real-Time PCR detection system, and the results were analyzed using CFX Manager software (Bio-Rad). Threshold cycle (C_T) values of <37 were considered a positive indication that the wt or var virus was present in the sample.

Genotyping of viral isolates. Virus genotypes were determined by HRM analysis essentially as described previously with minor modifications as noted here (1, 44). Plaque isolates were obtained by plaque assay of guinea pig nasal-wash fluids. RNA was extracted from agar plugs using the Zymo Research ZR-96 Viral RNA kit, with the following modification to the manufacturer's protocol: 40 μl water was used for the elution step. Twelve microliters of RNA was reverse transcribed using Maxima reverse transcriptase (Fermentas) according to the manufacturer's instructions. cDNA was used as the template in qPCRs with the appropriate primers (1) and Precision Melt Supermix (Bio-Rad) in wells of a white, thin-wall, 384-well plate (Bio-Rad). qPCR and melt analyses were carried out in a CFX384 Real-Time PCR detection system, according to the instructions provided with the Precision Melt Supermix. Data were analyzed using

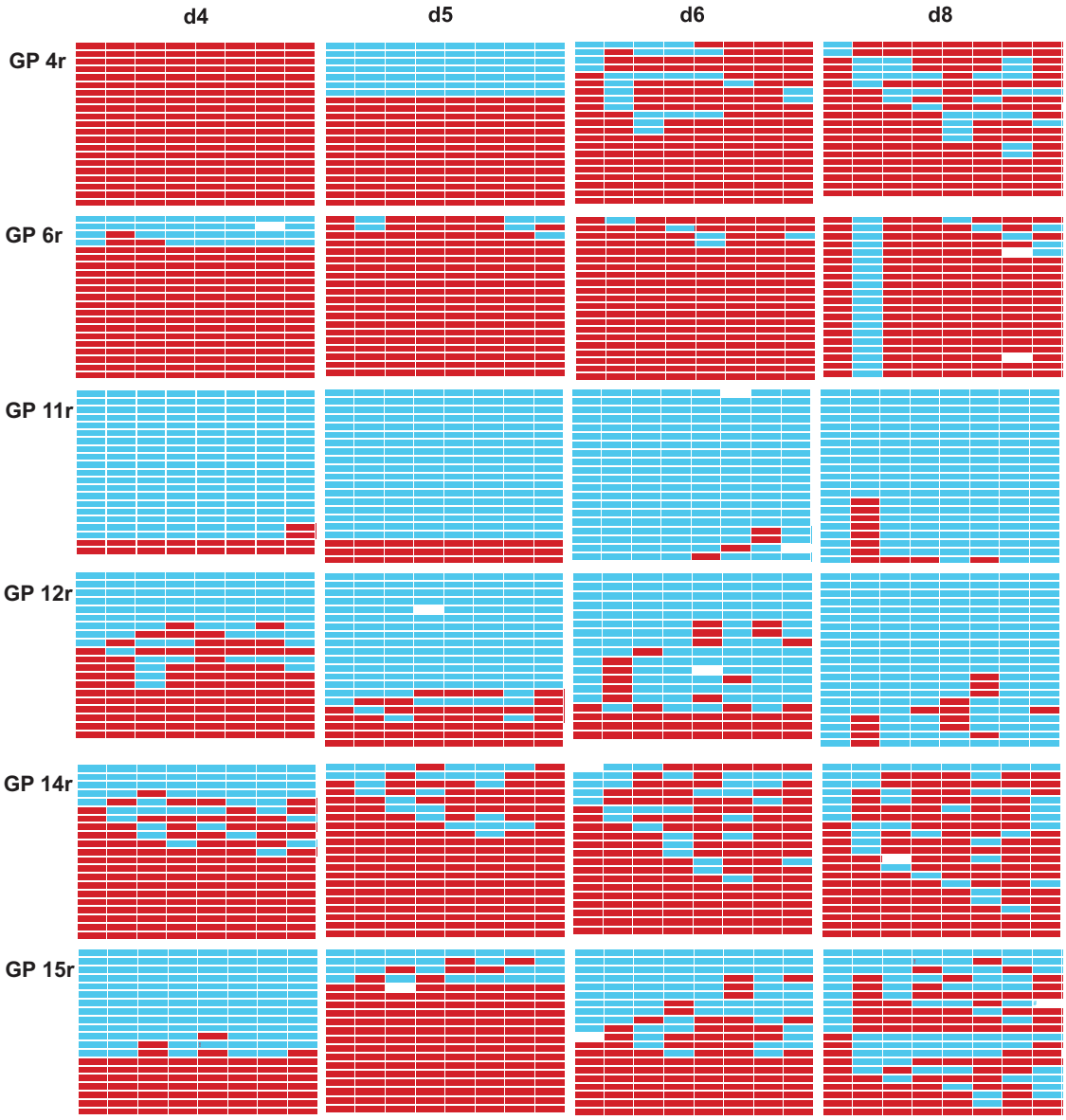

FIG 2 Viral genotypes sampled from nasal washes of guinea pigs coinfected through two independent transmission events. Genotype tables are shown for six guinea pigs coinfected with wt and var viruses through contact exposure to singly inoculated donor hosts. The day postinoculation on which each nasal wash was collected is indicated at the top and refers to the day after inoculation of the donor guinea pigs. The guinea pig (GP) ID numbers are shown at the left and correspond to those in Table 1 and Fig. 1. Each genotype table shows PB2 in the leftmost column, followed by PB1, PA, HA, NP, NA, M, and NS segments. Each row of a genotype table corresponds to a single plaque clone isolated from the indicated nasal-wash sample ($n = 18$ to 21). The red bars indicate segments derived from the wt parental strain, and the turquoise bars indicate segments derived from the var virus. White bars are shown where segments could not be typed unambiguously.

Precision Melt Analysis software (Bio-Rad). Viruses were scored as reassortant if the genome comprised a mixture of wt and var gene segments. Infrequently, unclear results were obtained for one or more gene segments. Isolates with one unclear segment were genotyped based on the remaining seven segments; isolates with >1 unclear segment were discarded from the analysis.

RESULTS

Reassortment was prevalent following dual-transmission events. To evaluate the potential for reassortment between two IAV introduced into the same host through independent transmission events, we modeled this situation experimentally in guinea pigs. Three animals were placed into each cage: one donor animal that had been inoculated 24 h previously with the Pan/99wt virus, a second donor animal that had been inoculated 24 h previously with a Pan/99var virus, and one naive recipient guinea pig. A total of 15 cages were set up in this way: 6 in December 2014 using Pan/99wt and Pan/99var6 viruses and 9 in March 2015 using Pan/99wt and Pan/99var15 viruses. Transmission occurred rapidly to all recipient guinea pigs (Fig. 1), and in 9 of the 15 recipient guinea pigs, infection with both wt and var viruses was detected by RT-qPCR of nasal-wash fluids collected on days 5 and 6 (Table 1). The differing transmission outcomes seen in cages 1 to 6 (2/6 recipi-

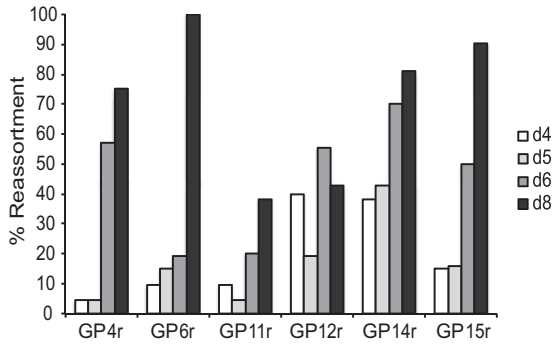

FIG 3 Prevalence of reassortant viruses in nasal washes collected from guinea pigs coinfected through two independent transmission events. The percentage of virus isolates that were found to carry reassortant genomes is plotted for each guinea pig and each time postinoculation that was examined in detail. Guinea pig ID numbers are shown as categories on the *x* axis and correspond to those in Fig. 1 and 2 and Table 1. The day postinoculation is indicated by the legend. The data shown correspond to genotypes displayed in Fig. 2.

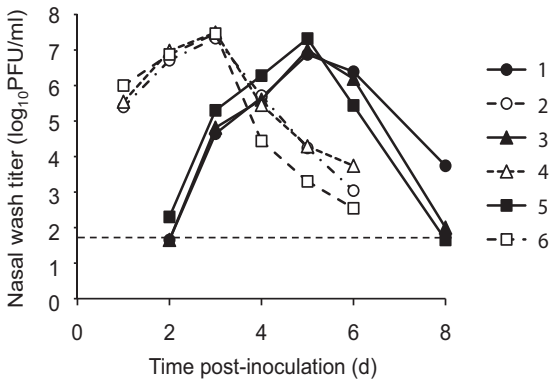

FIG 4 Nasal-wash titers of donor and recipient guinea pigs in a cotransmission model. Donor and recipient guinea pigs were cocaged in pairs at 24 h postinoculation of the donor animals. Guinea pig ID numbers are indicated on the left. Recipient no. 1 was paired with donor no. 2, no. 3 with no. 4, and no. 5 with no. 6. The viral titers in nasal washes of recipient animals are plotted with solid lines and symbols. The titers of donor animals are shown with dashed lines and open symbols. The limit of detection (50 PFU/ml) is indicated with a horizontal dashed line, and titers below the limit of detection were plotted as 45 PFU/ml.

ents coinfected) and cages 7 to 15 (7/9 recipients coinfected) could indicate that the Pan/99var15 virus transmits more readily than the Pan/99var6 virus, but the difference would need to be confirmed with repeat experiments to draw this conclusion with confidence.

To evaluate the prevalence of reassortment in guinea pigs coinfected via dual-transmission events, we focused on four time points: day 4 (when viral titers were increasing), days 5 and 6 (the time of peak shedding), and day 8 (when viral titers were decreasing). Six coinfected recipient guinea pigs were chosen arbitrarily for in-depth analysis: numbers 4r, 6r, 11r, 12r, 14r, and 15r. Twenty-one plaque clones were isolated from the day 4, 5, 6, and 8 nasal-wash fluids of each of these six guinea pigs and genotyped using RT-qPCR followed by high-resolution melt analysis to distinguish between wt and var gene segments. The results, compiled in Fig. 2 and shown graphically in Fig. 3, reveal that reassortment took place in all six guinea pigs. The proportion of viruses that carried reassortant genotypes was typically higher at the later time points (days 6 and 8) than at the early time points, most likely reflecting a need for viral spread in the respiratory tract to reach multiplicities of infection greater than 1 (Fig. 3). In five of the six guinea pigs (11r was the exception), the proportion of viruses carrying reassortant genomes exceeded 50% at one or more time points. This observation went against our prediction that stochastic effects would limit the opportunity for reassortment following dual transmission and indicated that coinfection of target cells within respiratory tissues occurred frequently.

Coinfection of donor hosts led to transmission of multiple variants and high proportions of reassortant viruses in recipient animals. In multiple host species, IAV transmission is characterized by a relatively loose bottleneck (33–35, 38, 39). As a result, cotransmission of multiple variants from a single donor host occurs routinely. To assess the prevalence of reassortment following cotransmission of multiple variants, we coinfected donor guinea pigs with 10^4 PFU each of the wt and var viruses to generate mixed infections. At 24 h postinoculation, we introduced a recipient guinea pig into the cage of each donor animal. The nasal washes of all recipient guinea pigs contained $>10^4$ PFU/ml by day 3 postinoculation, indicating that each had contracted infection within 48 h of exposure (Fig. 4). To evaluate viral diversity prior to transmission, we examined reassortment in the donor guinea pigs on days 1 and 2 postinoculation. All three donors showed reassortment at these early time points, with the percentages of viruses with reassortant genotypes $<20\%$ on day 1 and between 30% and 50% on day 2 (Fig. 5 and 6). To evaluate viral diversity following transmission, we determined viral genotypes shed from the recipient animals on days 3 and 5 postinoculation (days 2 and 4 postexposure). Since the nasal-wash fluid of recipient no. 23 was virus positive on day 2 postinoculation, we also examined reassortment in this sample. Reassortant viruses were detected in all recipient nasal washes that were analyzed, but the proportion of viruses with reassortant genotypes exhibited a wide range (5 to 95%) (Fig. 6). With the exception of PA in guinea pig no. 4, both wt and var versions of all segments were present in all recipient guinea pigs, indicating that the transmitted virus population comprised 15 or 16 different gene segments in each case, and therefore, more than one infectious virion (Fig. 5). Again, reassortment levels increased as viral titers rose, presumably due to increased coinfection following virus spread within the tissue. Guinea pig no. 2 showed evidence of an intrahost bottleneck in which the var PB2, PB1, and PA genes switched from being the minority on day 2 to the majority on days 3 and 5 (Fig. 5). In sum, diversity was high following cotransmission of multiple variant viruses and comparable to that seen when coinfection was achieved via dual-transmission events (see above) or by coinoculation by the intranasal route (44).

DISCUSSION

Using a system that avoids sampling bias due to fitness differences among parental and reassortant progeny viruses, we have previously shown that reassortment is highly efficient in coinfected cells and occurs readily in guinea pigs coinfected intranasally (1, 44). Here, we addressed whether the conditions necessary for robust reassortment can also arise *in vivo* when animals are infected by a more natural route. The results were clear: when coinfection was achieved through transmission from two singly infected donors or from one donor with mixed infection, reassortment occurred readily. Our data support the idea that reassortment is prevalent in

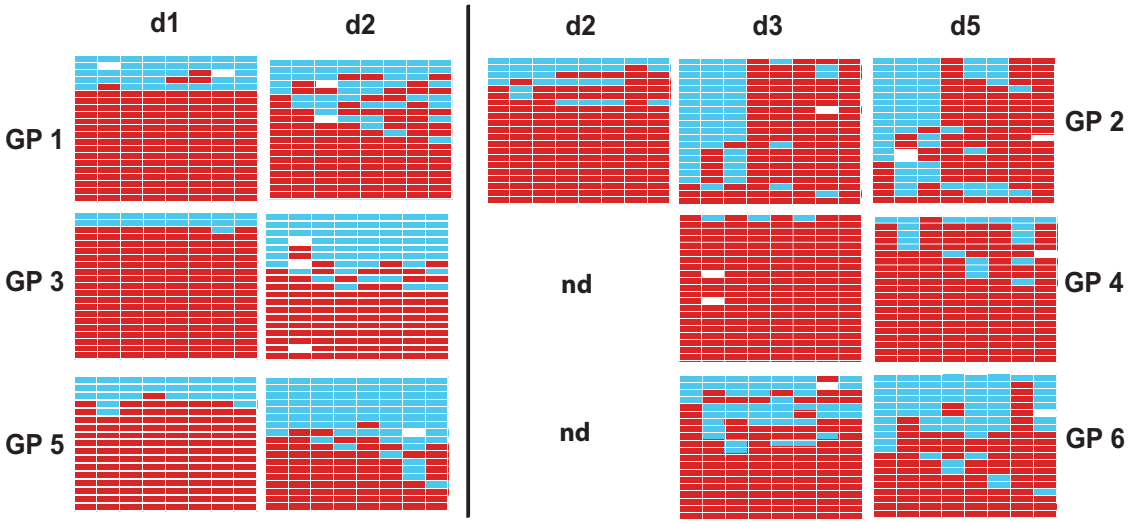

FIG 5 Viral genotypes sampled from nasal washes of guinea pigs coinfected through transmission of multiple variant viruses from a single donor host. On the left of the vertical line, genotype tables are shown for three donor guinea pigs coinfected through intranasal administration of 10^4 PFU Pan/99wt and 10^4 PFU Pan/99var6 viruses. On the right of the line, genotype tables are shown for the corresponding recipient guinea pigs coinfected through contact exposure to the donor hosts with mixed infections. Recipient no. 1 was paired with donor no. 2, no. 3 with no. 4, and no. 5 with no. 6. The day postinoculation on which each nasal-wash sample was collected is indicated at the top. The guinea pig ID numbers are shown on the left or right for the donors and recipients, respectively, and correspond to those in Fig. 3. Each genotype table shows PB2 in the leftmost column, followed by the PB1, PA, HA, NP, NA, M, and NS segments. Each row of a genotype table corresponds to a single plaque clone isolated from the indicated nasal-wash sample ($n = 17$ to 21). The red bars indicate segments derived from the wt parental strain, and the turquoise bars indicate segments derived from the var virus. White bars are shown where segments could not be typed unambiguously. nd, time points at which virus was not detected in nasal washes of the indicated guinea pigs.

naturally infected hosts and therefore allows IAV diversification through genetic exchange on a routine basis.

In the model system used here, reassortment does not lead to phenotypic differences among the progeny. For this reason, any reductions in diversity seen are most likely due to bottleneck events or possibly to guinea pig-adaptive drift mutations arising in a wt or var gene segment. Intrahost bottlenecks are more commonly associated with pathogens that spread across a physical barrier, such as the mucosa, rather than those that replicate within one tissue (45, 46). If indeed the losses of diversity apparent in our data sets are due to bottlenecks rather than adaptive evolution, these bottlenecks could arise if virus replication takes place within discrete foci and clearance of virus from those foci occurs in a temporally heterogeneous manner. Under these circumstances, a stochastic loss of diversity in the total virus population could be seen when replication ceases at one site but continues at another site. In contrast to our experimental system, in coinfections involving two or more genotypically divergent IAV variants, fitness differences follow directly from reassortment (37, 39, 47–59). In this situation, we would expect a viral population to be shaped first by diversification through reassortment and, second, by a reduction in diversity mediated by natural selection (as well as stochastic bottleneck events). In this way, reassortment is predicted to accelerate viral evolution.

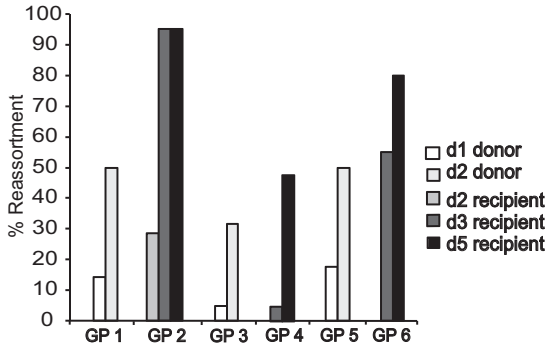

FIG 6 Prevalences of reassortant viruses in nasal washes collected from coinfected donor and recipient guinea pigs. The percentage of virus isolates that were found to carry reassortant genomes is plotted for each guinea pig and each time postinoculation examined. Guinea pig ID numbers are shown as categories on the *x* axis and correspond to those in Fig. 4 and 5. The day postinoculation is indicated by the legend. The data shown correspond to genotypes displayed in Fig. 5.

The transmission model used in our experiments was designed to optimize transmission efficiency in order to achieve mixed infections in recipient hosts. Thus, guinea pigs were housed under cold and dry environmental conditions and donors and recipients were placed together in the same cage. This approach was necessary to efficiently achieve our aim of evaluating reassortment in hosts coinfected through transmission. In the field, however, transmission does not always occur under optimal conditions. Factors that were controlled in our experiments but vary in nature are known to affect the frequency and timing of dual-transmission events. For example, temperature and humidity (60–62), preexisting immunity (63–66), timing and duration of exposure (67), proximity of exposure (68), host species (69), and viral fitness in that host species (41, 70–73) all impact transmission efficiency and are therefore expected to dictate the likelihood of two independent transmission events leading to coinfection.

In those cases where productive coinfection does arise, the timing of superinfection and the dose of incoming viruses are also important determinants of reassortment efficiency that were not controlled in the present study. Our previous data show that when

coinfection is achieved through intranasal inoculation, a short (12-h) delay between infections increases reassortment in guinea pigs, whereas a longer delay (>18 h) prevents superinfection (1). We also found that reassortment levels seen early in infection decreased with the inoculation dose and, where a low dose was used, a bottleneck at infection could lead to clonal rather than mixed infection (44). The number of virions that initiate an infection following a single transmission event has been shown experimentally to vary with the host species and proximity of exposure (38). The contact transmission model used here supports the transfer of a greater number of viruses than would be seen in a model where spread is limited to a respiratory droplet route. Nevertheless, data obtained using respiratory droplet models and from natural outbreaks indicate that transmission typically involves more than one infectious virus (33–36, 38). The results presented here suggest that, at least under the transmission-favorable conditions used, the timing and dose of coinfections achieved through transmission are compatible with robust reassortment.

Considering the factors at play in nature, coinfection with IAV of distinct lineages, which is necessary for genetic shift, may occur relatively rarely due to the need for two exposures within a short time window (1). In contrast, coinfection with related viruses is expected to occur often when multiple variants are cotransmitted (33–35, 38, 39) or via dual exposures during an outbreak (31, 33, 34). Although unlikely to lead to large shifts in genotype or phenotype, reassortment occurring after the latter type of coinfection is expected to be important for viral evolution on a larger time scale. Genetic exchange among related viruses allows the combination of multiple adaptive mutations within a single genome, as well as separation of lethal or fitness-decreasing changes from adaptive ones. In these ways, reassortment is predicted to increase the rate of evolution of a diverse viral population under selection pressure (39, 74).

In summary, the data presented here suggest that reassortment among related variants is most likely a routine feature of IAV infections, which therefore plays a critical role in shaping the evolution of the pathogen.

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