Health, and by the American Lebanese Syrian Associated Charities (ALSAC).

Disclosure

Some data presented within this manuscript was also published in PLoS Pathog. 2010 Jul 29;**6**(7):e1001022; Duan S, Boltz DA, Seiler P, Li J, Bragstad K, Nielsen LP, Webby RJ, Webster RG, Govorkova EA. Oseltamivir-resistant pandemic H1N1/2009 influenza virus possesses lower transmissibility and fitness in ferrets.

References

1 Brownstein JS, Freifeld CC, Madoff LC. Influenza A (H1N1) virus, 2009 – online monitoring. N Engl J Med 2009; 360:2156.

2 Centers for Disease Control and Prevention (CDC). Update: drug susceptibility of swine-origin influenza A (H1N1) viruses, April 2009. MMWR Morb Mortal Wkly Rep 2009; 58:433–435.

3 Bautista E, Chotpitayasunondh T, Gao Z *et al.* Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. N Engl J Med 2010; 362:1708–1719.

4 WHO. Weekly update on oseltamivir resistance to influenza A (H1N1) 2009 viruses. 2010. Available at: http://www.who.int/csr/disease/swineflu/oseltamivirresistant20100820.pdf (Accessed 10 October 2010).

5 European Centre for Disease Prevention and Control. ECDC THREAT ASSESSMENT: First isolation of a secondary oseltamivir-resistant A(H1N1)v strain in Denmark. 9A.D. Available at: http://ecdc.europa.eu/en/healthtopics/documents/0906_influenza_ah1n1_ecdc_threat_assessment_first_isolation_of_a_secondary_oseltamivir_resistant_strain_in_denmark.pdf (Accessed 10 October 2010).

6 Potier M, Mameli L, Belisle M *et al.* Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. Anal Biochem 1979; 94:287–296.

7 van Doorn HR, Hien NT, Horby P. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. N Engl J Med 2009; 362:86–87.

8 Gulland A. First cases of spread of oseltamivir resistant swine flu between patients are reported in Wales. BMJ 2009; 339:b4975.

9 Hamelin ME, Baz M, Abed Y *et al.* Oseltamivir-resistant pandemic A/H1N1 virus is as virulent as its wild-type counterpart in mice and ferrets. PLoS Pathog 2010; 6:e1001015.

10 Kiso M, Shinya K, Shimojima M *et al.* Characterization of oseltamivir-resistant 2009 H1N1 pandemic influenza A viruses. PLoS Pathog 2010; 6:e1001079.

Pathogenicity and transmissibility of the pandemic H1N1 2009-related influenza viruses in mice, ferrets, and pigs

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Keywords ferret, infectivity, mouse, pandemic, pathogenicity, pig, swine influenza virus, transmissibility, zoonotic potential.

Please cite this paper as: Zhu *et al.* (2011) Pathogenicity and transmissibility of the pandemic H1N1 2009-related influenza viruses in mice, ferrets, and pigs. Influenza and Other Respiratory Viruses 5 (Suppl. 1), 79–89.

Introduction

Pigs have been considered as hypothetical ''mixing vessels'' facilitating the genesis of pandemic influenza viruses.[1,2] The pandemic H1N1/2009 virus (pH1N1/09) contained a very unique genetic combination and was thought to be of swine origin, as each of its eight gene segments had been found to be circulating in pig populations for more than a decade.[3] However, such a gene constellation had not been found previously in pig herds all around the world. Only after its initial emergence in humans has this virus been repeatedly detected in pigs, and found to further reassort with other swine influenza virus.[3–5] A primary question remaining to be answered is whether the pH1N1/09-like and their genetically related viruses could become established in pig populations, thereby posing novel threats to public health.

Despite the fact that pH1N1/09 first appeared in Mexico and the United States, and six of its eight gene segments were derived from the established North American triple reassortant swine influenza virus (TRIG), its neuraminidase (NA) and matrix protein (M) genes belonged to the Eurasian avian-like swine lineage (EA), which had never been detected in North America previously.[3,6] Likewise, the

TRIG-like viruses were never reported in Europe.[6] In contrast, both lineages of virus were frequently detected in Asia, and reassortants between them have also been documented in recent years.[3,5] This has given rise to a complicated ecological situation, i.e. the simultaneous prevalence of multiple genotypes of H1N1 and H1N2 viruses in pigs.[3,5] Among them, two representative reassortants showed the most similar genotypic characterization to the pH1N1/09 virus, the Sw/HK/915/2004 (H1N2) and Sw/HK/201/2010 (H1N1), which respectively harbor seven and six gene segments closely related to the pandemic strains.[3,5]

To understand their *in vivo* characteristics and zoonotic potential, these two viruses, together with a human prototype strain and a swine pH1N1/09-like isolate, were chosen for a study of their pathogenicity and transmissibility in domestic pigs, ferrets, and mice.

Materials and methods

Viruses

The prototype pH1N1/09 virus, A/California/04/2009 (CA04), was provided by the World Health Organization Collaborating Centers for Reference and Research on Influenza (Atlanta, GA, USA). Three pH1N1/09-related swine influenza viruses were isolated through our surveillance program in South China as previously described.[3,5] The A/Swine/Guangdong/106/2009 (H1N1, GD106) virus was a pH1N1/09-like swine isolate. A/Swine/Hong Kong/915/2004 (H1N2, HK915), the closest pandemic ancestor known to date, possesses an M gene derived from the EA lineage, with the other gene segments from TRIG viruses.[3] A/Swine/Hong Kong/201/2010 (H1N1, HK201), a recent pandemic reassortant progeny, had a pH1N1/09-like NA gene (also belonging to the EA lineage), an EA-like hemagglutinin (HA) gene, and six TRIG-like internal genes.[5] All viruses were propagated in Madin-Darby canine kidney (MDCK) cells for three passages, and their titers were determined by plaque assays. All experiments with live viruses were conducted in biosafety level 3 (BSL-3) containment laboratories.

Animal experiments

Pigs (4–6 week old, $n = 5$–6) and ferrets (5 month old, male, $n = 3$) were intranasally infected with 10^6 PFU of each virus, and mice (8–9 week old, female BALB/c, $n = 10$) with a dose of 10^4 PFU. Naïve uninfected pigs ($n = 2$) were co-housed in the same cage with the inoculated ones from each group. Body weights and clinical signs were recorded daily. Virus replication was determined by titration of the virus in nasal and rectal swabs (pigs), nasal washes (ferrets), as well as from lungs and other organs (pigs and mice). Seroconversion was tested by hemagglutination inhibition (HI) assays. Histopathological and immunohistochemical analysis were performed as previously described.[7]

Statistical analysis

Statistical analysis was performed by Mean Analysis with Pasw Statistics 18 (SPSS Inc., Chicago, IL, USA). The probability of a significant difference was computed using anova (analysis of variance). Results were considered significant at $P < 0{\cdot}05$.

Results

Pathogenicity and virus replication in mice

The pathogenicity of the four viruses tested differed significantly in inoculated mice. Animals infected with 10^4 PFU of HK915 experienced the most severe body weight loss (25·1 ± 4·7%) but started to recover after 8 days post-infection (dpi). HK201 caused similar peak body weight loss (16·9 ± 4·6% on 8 dpi) in mice as did CA04 (17·3 ± 2·4%, on 7 dpi), but the onset of clinical signs and weight loss (on 4 dpi) was 1 day later than those caused by the other three viruses. The GD106-infected group suffered the least body weight loss (6·9 ± 1·9%, 5 dpi) and was the earliest to recover.

Although all four viruses were detected in the lungs with comparable virus titers on 3 dpi ($P > 0{\cdot}05$), mice inoculated with GD106 consistently showed the lowest lung index (lung weight/body weight, %) on 3, 6, and 14 dpi ($P < 0{\cdot}01$), suggesting the slightest injury and consolidation of the lungs. In concordance with the body weight change, the lung index from the HK915 group was higher than that from any other groups on 6 and 14 dpi, indicating the marked virulence of HK915 in mice. Notably, virus titer of HK201 in the nasal turbinate was lower than the other groups both on 3 and 6 dpi ($P < 0{\cdot}01$), but virus replication in the lower respiratory tract was either higher (in the trachea) or similar (in the lungs).

Pathogenicity and virus shedding in ferrets

Observations of the body weight changes caused by infection of pH1N1/09 or its genetically related swine viruses in ferrets have come to a similar conclusion as that for the mouse experiment. After nasal inoculation with 10^6 PFU of each virus, all groups of ferrets experienced transient body weight loss for 2–3 days, except for those infected with GD106, which showed no significant weight loss ($P > 0{\cdot}05$). Although ferrets from the CA04-infected group reached their peak weight loss (6·2 ± 0·8%, 2 dpi) one day earlier than those from the HK201 and HK915 groups, they began to regain body weight quickly thereafter.

HK201-infected ferrets also recovered rapidly and their body weights reached the same level as those of the GD106-infected group at 6 dpi. Comparatively, ferrets inoculated with HK915 had the most retarded body weight recovery, which did not get back to the baseline level until 11 dpi. HK201 was only detectable in the nasal wash on 2 dpi, whereas the duration of virus shedding for GD106, HK915, and CA04 was 4–6 days. By combining the data obtained from the virus titration in the mouse turbinate and ferret nasal washes, a possible conclusion can be made that HK201 may have lower transmissibility than the other three viruses.

Pathogenicity and transmissibility in domestic pigs

After inoculation or exposure by direct contact (physical contact) with the pH1N1/09 virus and its close relatives, most pigs experience no or mild symptoms, such as slight loss of appetite and inactivity. Body weight loss was only recorded in pigs inoculated with HK915 during the second week post-inoculation, but not in their contact pigs or in the other groups.

Diarrhea was observed intermittently in each of the inoculated or contact groups throughout the experiment, and viruses could be recovered in the rectal swabs, saliva, drinking water, and environmental swabs (inner cage walls accessible to the pigs) at various time points. However, virus titers in the positive rectal swabs were just slightly above the detection limit, while those from the environment sometimes could be higher. Whether these viruses can replicate in the digestive tract or were just carried-over by contaminated foods and water requires further investigation.

Although virus could be detected in the nasal swabs of all infected or contact animals, the lowest peak titer was from pigs inoculated or in contact with HK201 (0·5–1·5 log TCID50/ml lower than the other groups), suggesting unfavorable replication in the nasal cavity for this virus. Postmortem examination on 4 and 7 dpi revealed that pigs infected with HK915 had the most extensive gross lesions in the lungs, and histochemical staining of viral nucleoprotein (NP) in lung tissues on 4 dpi also suggested the best replication for HK915 in the lower respiratory tract. On 11 days post-contact (dpc), all pigs exposed to the inoculated animals developed sero-conversions (HI = 80–160) except for one from the GD106 contact group. However, on 17 dpc, its HI titer reached 40, indicating slower seroconversion.

Conclusions

This study revealed that both the 2009 pandemic H1N1 and its genetically related swine viruses could readily infect mice, ferrets, and pigs causing mild to moderate clinical symptoms. They could also transmit efficiently between pigs. When compared with the pandemic stains and its reassortant progeny (HK201), the HK915 (H1N2) virus containing the EA-like M gene in the genetic context of the TRIG virus showed consistently higher virulence in all three mammalian models tested, but it is still unknown what might happen if such a virus further reassorts to obtain the pandemic-like or EA-like NA gene. However, our findings suggest that pigs could likely maintain the prevalence of different genotypes of pandemic-related influenza viruses, and highlight the zoonotic potential of multiple strains of swine influenza virus.

Acknowledgements

This work was supported by the National Institute of Allergy and Infectious Diseases (NIAID) contract HHSN266200700005C, the Li Ka Shing Foundation, and the Area of Excellence Scheme of the University Grants Committee (grant AoE/M-12/06) of the Hong Kong SAR Government. We gratefully acknowledge our colleagues from IIII, Shantou University and SKLEID, HKU for their excellent technical assistance.

References

1 Scholtissek C, Bürger H, Kistner O *et al.* The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 1985; 147:287–294.
2 Scholtissek C. Pigs as the ''mixing vessel'' for the creation of new pandemic influenza A viruses. Med Princip Prac 1990; 2:65–71.
3 Smith GJ, Vijaykrishna D, Bahl J *et al.* Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 2009; 459:1122–1125.
4 Pereda A, Cappuccio J, Quiroga MA *et al.* Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis 2010; 16:304–307.
5 Vijaykrishna D, Poon LL, Zhu HC *et al.* Reassortment of pandemic H1N1/2009 influenza A virus in swine. Science 2010; 328:1529.
6 Gibbs AJ, Armstrong JS, Downie JC. From where did the 2009 'swine-origin' influenza A virus (H1N1) emerge? Virol J 2009; 6:207.
7 Zhu H, Wang J, Wang P *et al.* Substitution of lysine at 627 position in PB2 protein does not change virulence of the 2009 pandemic H1N1 virus in mice. Virology 2010; 401:1–5.

