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References

1. Pourahmad F, Pate M, Ocepek M., Borroni E, Cabibbe AM, Capitolo E, et al. *Mycobacterium angelicum* sp. nov., a non-chromogenic, slow-growing species isolated from fish and related to *Mycobacterium szulgai*. Int J Syst Evol Microbiol. 2015;65:4724–4729. https://doi.org/10.1099/ijsem.0.000642
2. Christensen H, Bisgaard M, Frederiksen W, Mutters R, Kuhnert P, Olsen JE. Is characterization of a single isolate sufficient for valid publication of a new genus or species? Proposal to modify recommendation 30b of the Bacteriological Code (1990 revision). Int J Syst Evol Microbiol. 2001;51:2221–5. https://doi.org/10.1099/00207713-51-6-2221
3. Durnez L, Suykerbuyk P, Nicolas V, Barrière P, Verheyen E, Johnson CR, et al. Terrestrial small mammals as reservoirs of *Mycobacterium ulcerans* in Benin. Appl Environ Microbiol. 2010;76:4574–7. https://doi.org/10.1128/AEM.00199-10
4. Davies E, Wieboldt J, Stanley T, Maeda Y, Smyth M, Stanley S, et al. Isolation and identification of '*Mycobacterium angelicum*' from a patient with type II respiratory failure: suggested reporting guidelines to molecular clinical laboratories. Br J Biomed Sci. 2012;69:134–6. https://doi.org/10.1080/09674845.2012.12069140
5. Robinne S, Saad J, Morsli M, Hamidou ZH, Tazerart F, Drancourt M, et al. Rapid identification of *Mycobacterium tuberculosis* complex using mass spectrometry: a proof of concept. Front Microbiol. 2022;13:753969. https://doi.org/10.3389/fmicb.2022.753969
6. Morsli M, Boudet A, Kerharo Q, Stephan R, Salipante F, Dunyach-Remy C, et al. Real-time metagenomics-based diagnosis of community-acquired meningitis: a prospective series, southern France. EBioMedicine. 2022;84:104247. https://doi.org/10.1016/j.ebiom.2022.104247
7. Lee I, Ouk Kim Y, Park SC, Chun J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol. 2016;66:1100–3. https://doi.org/10.1099/ijsem.0.000760
8. Adilijiang G, Feng S, Mi K, Deng H. Quantitative proteomics analysis of ClpS-mediated rifampicin resistance in *Mycobacterium* [in Chinese]. Sheng Wu Gong Cheng Xue Bao. 2014 Jul;30:1115–27.
9. Marsee JD, Ridings A, Yu T, Miller JM. *Mycobacterium tuberculosis* ClpC1 N-terminal domain is dispensable for adaptor protein-dependent allosteric regulation. Int J Mol Sci. 2018;19:3651. https://doi.org/10.3390/ijms19113651

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Low Susceptibility of Pigs against Experimental Infection with HPAI Virus H5N1 Clade 2.3.4.4b

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We found that nasal and alimentary experimental exposure of pigs to highly pathogenic avian influenza virus H5N1 clade 2.3.4.4b was associated with marginal viral replication, without inducing any clinical manifestation or pathological changes. Only 1 of 8 pigs seroconverted, pointing to high resistance of pigs to clade 2.3.4.4b infection.

Spread of highly pathogenic avian influenza (HPAI) virus H5N1 clade 2.3.4.4b of the goose/Guangdong (gs/GD) lineage, has exacerbated since early 2022 into a panzootic (*1*). Regional enzootic status in wild bird populations in Europe and North America, with lethal courses of HPAI virus infection in some species, produced large numbers of wild bird carcasses, easy prey for raptors and scavengers. Exposure of
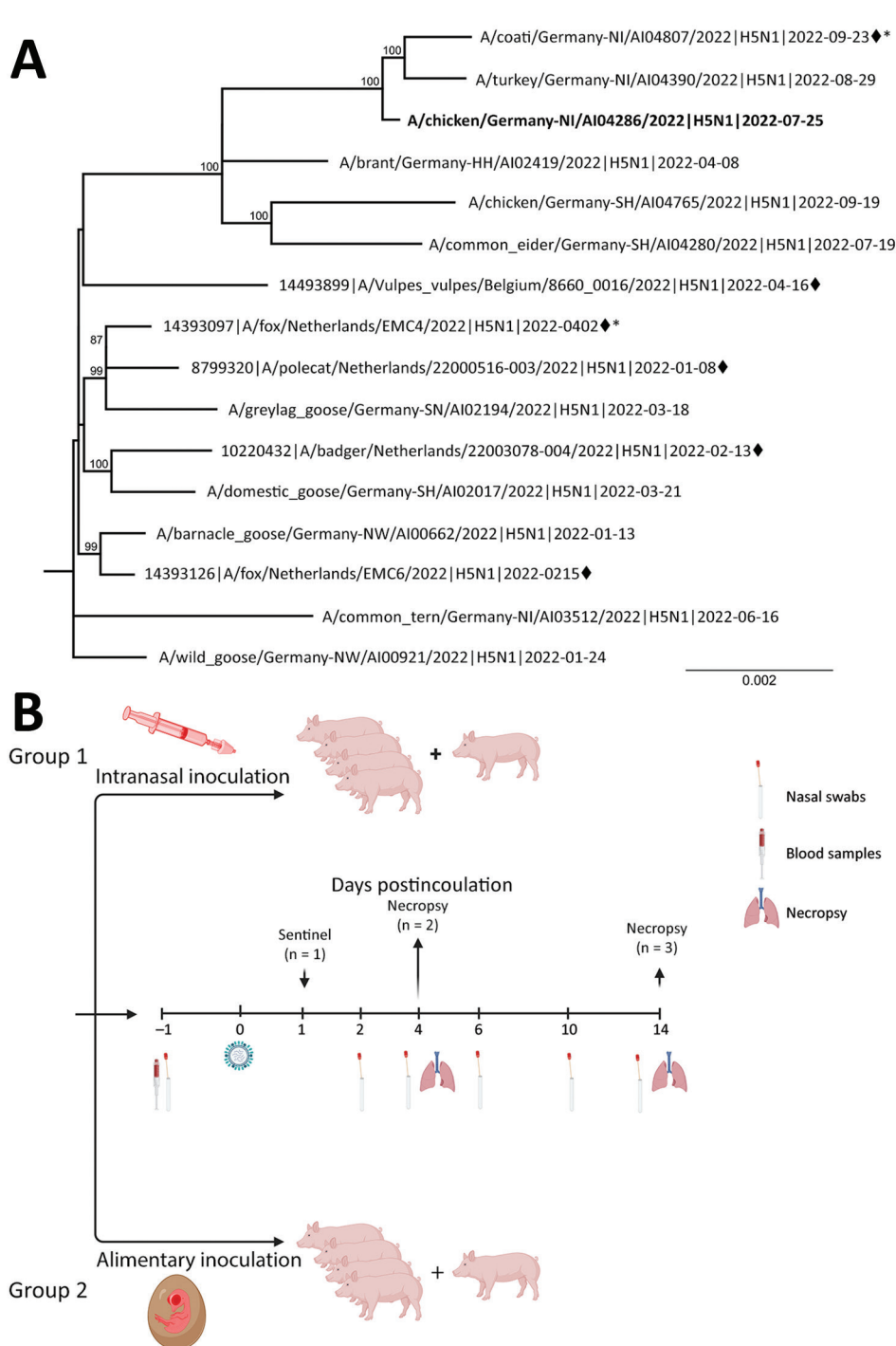
Figure. Phylogeny and experimental design for study of susceptibility of pigs against experimental infection with highly pathogenic avian influenza (HPAI) (H5N1) virus clade 2.3.4.4b. A) Maximum-likelihood phylogenetic tree (RAxML, https://cme.h-its.org/exelixis/web/software/raxml) based on 8 concatenated genome segments of selected recent HPAI H5N1 viruses from naturally infected avian hosts and from mammalian hosts (black diamonds) in Europe. Bold indicates study isolate A/chicken/Germany/AI04286/2022. Asterisks (*) indicate sequences with polymerase basic 2 E627K mutations. B) Scheme of the experimental setting of HPAI H5N1 virus infection of pigs. Four pigs, 4 months of age, were inoculated with 10^6 TCID$_{50}$ in 2 mL using mucosal atomization devices. Four pigs were each fed with 1 HPAI H5N1 virus–infected embryonated chicken egg, carrying ≈10^8–10^9 TCID$_{50}$/mL of allantoic fluid. Each pig was offered an egg, separately, in a trough and observed to complete consume it. Ten-day-old eggs were inoculated with 0.2 mL of clarified amnio-allantoic fluid of egg passage 1 and incubated for 3 days or until embryonic death was evident. Eggs were chilled until fed to pigs. Panel B created with BioRender.com and licensed by the company (agreement no. UC258UM8J3). TCID$_{50}$, 50% tissue culture infectious dose.

terrestrial carnivores and marine mammals resulted in sporadic infections, some of those terminating with fatal encephalitis (2). Frequent spill-over events, rather than consistent mammal-to-mammal transmission, were at the basis of these cases (Figure, panel A). However, recently reported HPAI outbreaks among sea lions along the Pacific coast of South America and an outbreak in a mink farm in Spain (3) may consti-

tute first examples of avian-independent transmission chains and increase public health concerns about zoonotic transmissions of this virus. Still, the total of 11 human cases globally reported for the currently dominating H5N1 2.3.4.4b lineage did not point toward increased zoonotic propensity (4).

Possible adaption of avian influenza virus (AIV) to mammalian livestock hosts and subsequent

Table. Detection of seroconversion and of influenza A viral loads in tissues and nasal swabs of experimentally infected pigs exposed by intranasal or alimentary inoculation with HPAI virus H5N1 clade 2.3.4.4b virus*

| Infection route | Day postinoculation | Animal ID | GEq, by qRT-PCR | | | | | | | NP-ELISA seroconversion |
			Nasal swabs†	Conchae nasalis	Trachea	Lung	Ileocaecal tonsil	Colon	Brain	
Intranasal	4	1	30	–	–	–	–	–	–	–
	4	2	150	–	–	–	–	–	–	–
	14	3	30	10	–	–	–	–	–	–
	14	4	10	60	–	10	170	200	350	+
	14	Sentinel	–	–	–	–	–	–	–	–
Alimentary	4	1	–	–	5	–	–	–	–	–
	4	2	30	–	–	–	–	–	–	–
	14	3	20	–	–	–	–	–	–	–
	14	4	140	–	–	–	–	–	–	–
	14	Sentinel	–	–	–	10	–	–	–	–
Intranasal positive control‡	4	1	300,000	10,000	860,000	5,400	NA	NA	NA	–
	4	2	83,000	35,000	56,000	1,500	NA	NA	NA	–
	4	3	750,000	4,300	1,600,000	1,500	NA	NA	NA	–
	4	4	710,000	29,000	520,000	1,100	NA	NA	NA	–

*Shown are only tissues for which >1 animal has tested positive. GEq, genome copy equivalents per 0.1 mL calculated on the basis of qRT-PCR quantification of cycle values; HPAIV, highly pathogenic avian influenza; ID, identification; NA, not applicable; qRT-PCR, quantitative reverse transcription PCR; +, positive; –, negative.
†Nasal swabs positive at day 2 postinoculation for the intranasal group and day 4 postinoculation for the alimentary group.
‡Four pigs, infected with the same device and dose of a swine-adapted influenza A virus strain (subtype H1avN1, clade 1C2.1) of a former study are included for comparison (11). Pigs were sacrificed at day 4 post infection when no seroconversion was to be expected; gastrointestinal tissues and brain were not examined.

human exposure is of particular concern. In this respect, the role of pigs as a "mixing vessel" for HPAI viruses is largely unresolved. AIV can potentially be transmitted to pigs, and further reassortment with swine influenza A viruses (swIAVs) may contribute to the emergence of pandemic strains. Rare and subclinical infections of pigs with gs/GD HPAI virus have been confirmed serologically in Vietnam, Thailand, and France (5) and virologically in Indonesia (clades 2.1.1 and 2.1.3), Nigeria (clade 2.3.2.1c), China (clade 2.3.4), and Italy (clade 2.3.4.4.b) (6–8).

For our study, we purchased 4-month-old pigs (6 male, 4 female) from a conventional pig holding in Germany and exposed them nasally or by the alimentary route to high doses of the recent avian-derived HPAI virus H5N1 2.3.4.4b isolate A/chicken/Germany/AI04286/2022 (genotype Ger-10.21-N1.5). The egg-grown isolate was closely related to a mammal case but lacked any mammalian-adaptive mutations (Figure, panel A). We inoculated 2 groups of 4 pigs each intranasally or by feeding 1 infected embryonated chicken egg per animal. One sentinel pig per group was associated at day 1 postinoculation to assess the transmission by direct contact to those inoculated (Figure, panel B).

After exposure, HPAI virus H5N1 detection by real-time RT-PCR was limited to day 2 (intranasal group) and day 4 (oral group) postinoculation, with a range of 10–150 genome copy equivalents per 0.1 mL (Table). An avian-derived, swine-adapted H1avN1 strain intranasally inoculated into naïve pigs at the same dose and by the same device produced 3–4 $\log_{10}$ levels greater nasal excretion in comparison (Table) (9). It cannot be excluded that HPAI virus H5N1 detected in nasal swabs at day 2 postinoculation still represents residual inoculum. Correspondingly, except for 1 alimentary inoculated that showed tracheal viral loads at day 4 postinoculation close to the detection limit, samples from the remaining 3 pigs euthanized at day 4 postinoculation gave no indication of viral replication in respiratory or gut tissues, regardless of the inoculation route. Only in 1 intranasally inoculated animal, euthanized at day 14 postinoculation, was viral RNA detected at low levels in organ samples. In addition to samples from the respiratory and alimentary tracts, minute amounts of RNA were found also in a brain sample of that pig. The virus could not be isolated using chicken hepatoma cells and MDCK-II cells, and histologic and immunohistochemical investigations gave no evidence for inflammatory reactions or presence of viral antigen. Low viral loads in this pig impeded sequence analysis of eventual mutants. Nonetheless, this pig was the only animal that seroconverted at 14 dpi. In agreement with the virologic findings, none of the pigs showed any clinical signs or fever within 14 days of observation.

In conclusion, only 1 of 8 pigs inoculated intranasally with HPAI virus H5N1 underwent transient, low-level infection that resulted in the presence of viral RNA in several tissue specimens and seroconversion at 14 dpi. In naturally infected wild mammals, this virus was prominently detected in the brain (2).

Given the detection of viral RNA in the brain of 1 intranasally inoculated pig, it cannot be excluded that longer observation might have revealed continuing viral replication in the brain of this animal. Sialic acid α2,3-gal receptors dominate on porcine brain cells, which might have fostered replication of α2,3-adapted viruses, such as HPAI virus H5N1 (10).

Overall, we conclude that pigs are unlikely vehicles in transmitting this genotype of HPAI virus H5N1 clade 2.3.4.4b among pigs and across interfaces. However, considering the ongoing massive panzootic of this virus, a plethora of new genotypes of the circulating strain is emerging, with possibly higher permissiveness for pigs. Therefore, swine populations need to be part of HPAI virus surveillance programs, and periodic reassessment of prepandemic propensity of circulating HPAI virus H5N1 genotypes in the swine model is required.

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References

1. Alkie TN, Lopes S, Hisanaga T, Xu W, Suderman M, Koziuk J, et al. A threat from both sides: Multiple introductions of genetically distinct H5 HPAI viruses into Canada via both East Asia-Australasia/Pacific and Atlantic flyways. Virus Evol. 2022;8:veac077. https://doi.org/10.1093/ve/veac077
2. Bordes L, Vreman S, Heutink R, Roose M, Venema S, Pritz-Verschuren SBE, et al. Highly pathogenic avian influenza H5N1 virus infections in wild red foxes (*Vulpes vulpes*) show neurotropism and adaptive virus mutations. Microbiol Spectr. 2023;11:e0286722. https://doi.org/10.1128/spectrum.02867-22
3. Agüero M, Monne I, Sánchez A, Zecchin B, Fusaro A, Ruano MJ, et al. Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks, Spain, October 2022. Euro Surveill. 2023;28:5–10. https://doi.org/10.2807/1560-7917.ES.2023.28.3.2300001
4. Centers for Disease Control and Prevention. Ask the expert: highly pathogenic avian influenza A (H5N1) viruses [cited 2023 Apr 14]. https://www.cdc.gov/flu/avianflu/spotlights/2022-2023/avian-flu-highly-pathogenic.htm
5. Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, et al. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol. 2005;79:10821–5. https://doi.org/10.1128/JVI.79.16.10821-10825.2005
6. He L, Zhao G, Zhong L, Liu Q, Duan Z, Gu M, et al. Isolation and characterization of two H5N1 influenza viruses from swine in Jiangsu Province of China. Arch Virol. 2013;158:2531–41. https://doi.org/10.1007/s00705-013-1771-y
7. Meseko C, Globig A, Ijomanta J, Joannis T, Nwosuh C, Shamaki D, et al. Evidence of exposure of domestic pigs to highly pathogenic avian influenza H5N1 in Nigeria. Sci Rep. 2018;8:5900. https://doi.org/10.1038/s41598-018-24371-6
8. Nidom CA, Takano R, Yamada S, Sakai-Tagawa Y, Daulay S, Aswadi D, et al. Influenza A (H5N1) viruses from pigs, Indonesia. Emerg Infect Dis. 2010;16:1515–23. https://doi.org/10.3201/eid1610.100508
9. Graaf A, Petric PP, Sehl-Ewert J, Henritzi D, Breithaupt A, King J, et al. Cold-passaged isolates and bat-swine influenza a chimeric viruses as modified live-attenuated vaccines against influenza a viruses in pigs. Vaccine. 2022;40:6255–70. https://doi.org/10.1016/j.vaccine.2022.09.013
10. Nelli RK, Kuchipudi SV, White GA, Perez BB, Dunham SP, Chang KC. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. BMC Vet Res. 2010;6:4. https://doi.org/10.1186/1746-6148-6-4

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