FRIEDRICH-LOEFFLER-INSTITUT
since 1910
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

Virulence determinants of clade 2.3.4.4 H5N8 viruses in Pekin ducks

David Scheibner[1], Angele Breithaupt[2], Christine Luttermann[3], Diana Palme[1], Maryna Kuryshko[1] Claudia Blaurock[1], Thomas C. Mettenleiter[4] and Elsayed M. Abdelwhab[1]

[1]Institute of Molecular Virology and Cell Biology, [2]Department of Experimental Animal Facilities and Biorisk Management, [3]Institute for Immunology, [4]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany

Abstract

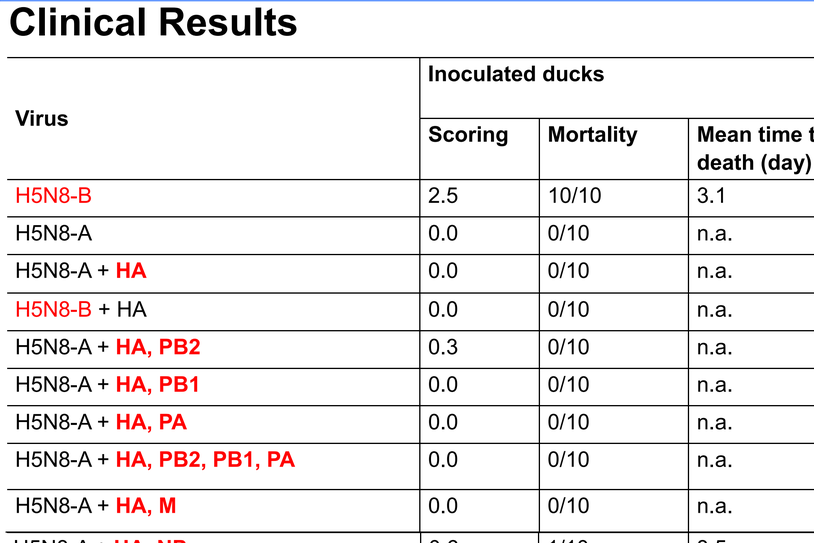
Avian influenza viruses (AIV) are a common threat to poultry and human health and cause periodically huge socioeconomic losses worldwide. Depending on the two major surface glycoproteins, 16 hemagglutinin (HA) and 9 neuraminidase (NA) distinct subtypes can be distinguished. All HA/NA subtypes are circulating in wild waterfowl, the natural reservoirs. In domestic birds, AIV are either low pathogenic (LPAIV) causing mild or no clinical signs or highly pathogenic (HP) causing up to 100% mortality in few days. In chickens, LPAIV H5 and H7 viruses can evolve to HPAIV due to acquisition of polybasic amino acids in the HA cleavage site (HACS). Conversely, ducks rarely succumb to morbidity and mortality after HPAIV infections and the HACS alone is not the main virulence determinant. Since 2014, H5N8 clade 2.3.4.4 caused high mortality in domestic and wild birds worldwide in three major waves in 2014, 2016 and 2020/2021. Although all H5N8 viruses possessed polybasic HACS and were highly virulent in chickens, viruses in 2016-2021 only were high pathogenic in Pekin ducks. In this study, the virulence determinants leading to differences in virus phenotypes between German H5N8 clade 2.3.4.4 viruses in 2014 and 2016 were analyzed. Sequence analysis of H5N8 viruses from 2014 and 2016 revealed several mutations in all eight gene segments. Using reverse genetics, single, double and multiple H5N8-2016 segments were swapped with those from H5N8-2014 and recombinant viruses were rescued and characterized. The minimal genetic constellation for the exhibition of high virulence in Pekin ducks was determined. *In vivo*, the H5N8-2016 HA alone did not increase the virulence of H5N8-2014 in Pekin ducks. We further tested double reassortants of H5N8-2014 carrying HA in addition to single segments from H5N8-2016. Virulence of H5N8-2014 increased dramatically after reassortment of H5N8-2016 HA in combination with NP, NS and/or NA. The high mortality caused by these viruses was associated with the degree of endothelial tropism and systemic spread in different tissues. *In vitro,* the replication of the H5N8-2014 virus in primary duck cells was increased by swapping the HA and NP from H5N8-2016. The latter exhibited increased HA receptor binding affinity and NA activity compared to the H5N8-2014 virus. Taken together, in this study we determined the multifactorial genetic constellation and underlying mechanism for the high virulence of H5N8 clade 2.3.4.4 viruses in Pekin ducks, which improves our current understanding for the evolution of HPAIV in different avian species.

Clinical Results

Virus	Inoculated ducks			Contact ducks
	Scoring	Mortality	Mean time to death (day)	Mortality
H5N8-B	2.5	10/10	3.1	5/5
H5N8-A	0.0	0/10	n.a.	0/5
H5N8-A + **HA**	0.0	0/10	n.a.	0/5
H5N8-B + HA	0.0	0/10	n.a.	0/5
H5N8-A + **HA, PB2**	0.3	0/10	n.a.	0/5
H5N8-A + **HA, PB1**	0.0	0/10	n.a.	0/5
H5N8-A + **HA, PA**	0.0	0/10	n.a.	0/5
H5N8-A + **HA, PB2, PB1, PA**	0.0	0/10	n.a.	0/5
H5N8-A + **HA, M**	0.0	0/10	n.a.	0/5
H5N8-A + **HA, NP**	0.6	1/10	8.5	2/5
H5N8–A + **HA, NA**	0.3	1/10	4.0	1/5
H5N8–A + **HA, NS**	0.3	1/10	5.0	1/5
H5N8–A + **HA, NP, NA**	1.4	3/7	5.3	3/5
H5N8–A + **HA, NP, NS**	2.0	10/10	5.1	5/5
H5N8–A + **HA, NP, NS, NA**	2.2	10/10	4.1	5/5

Pekin ducks were inoculated oculonasally and observed for 10 days. They were scored daily depending on their clinical signs (0=healthy, 1= one or mild clinical signs, 2=more than one or severe clinical signs, 3= dead) HA of clade B alone was not enough to increase virulence of H5N8 A, conversely H5N8 B carrying H5N8-A-HA was avirulent. Thus, double reassortants were tested and NP, NA and NS in combination with HA from clade B increased virulence of H5N8 A. Minimum constellation for high virulence of H5N8 A in Pekin ducks was HA, NP and NS while NA also contributed particularly to widespread tissue tropism.

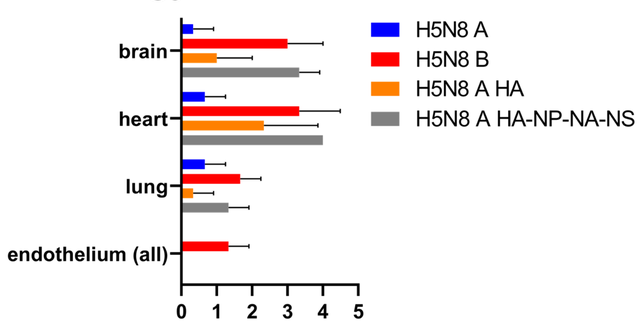
Virus Shedding



Viral shedding was investigated using RT-qPCR in oropharyngeal and cloacal swabs at day 4 post inoculation.

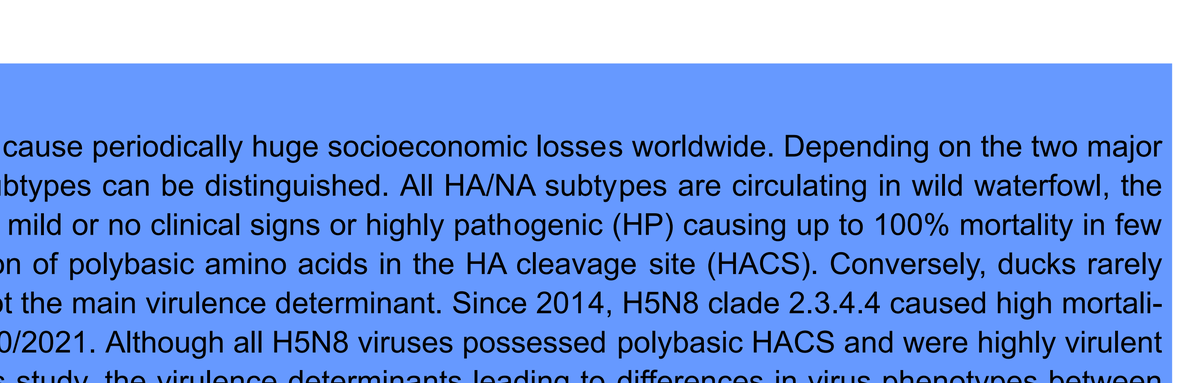
Particularly in the oropharyngeal swabs, the highest virus loads were detected for the H5N8 B virus. In the cloacal swab samples H5N8 B and the H5N8_HA,NP,NA,NS showed comparable high viral loads.

Pathology



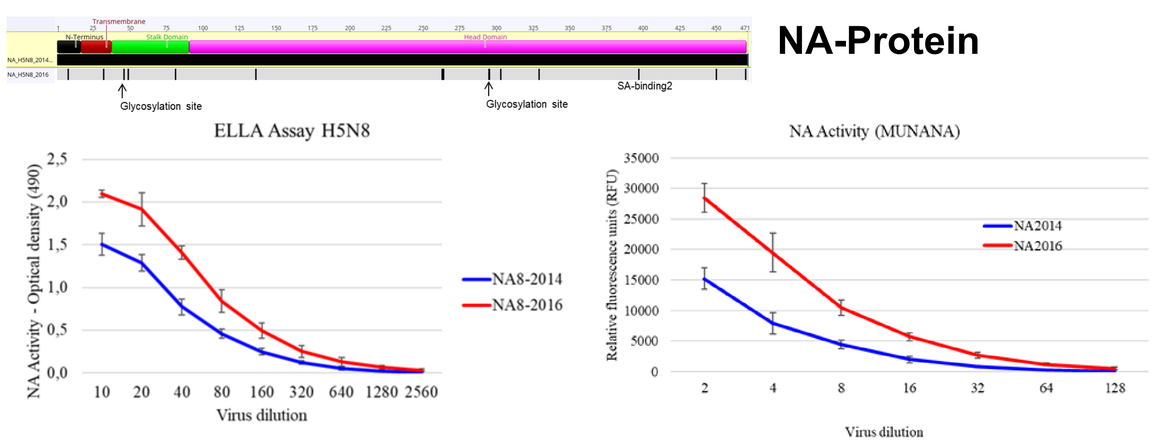
On day 4 after inoculation, distribution of the NP antigen was detected using immunohistochemistry. Comparing tropism to the parenchyma of brain, heart and lungs H5N8 B and H5N8 A-HA,NP,NA,NS showed similar distribution. Interestingly, only the H5N8 B WT was detected in the endothelium.

HA-Protein



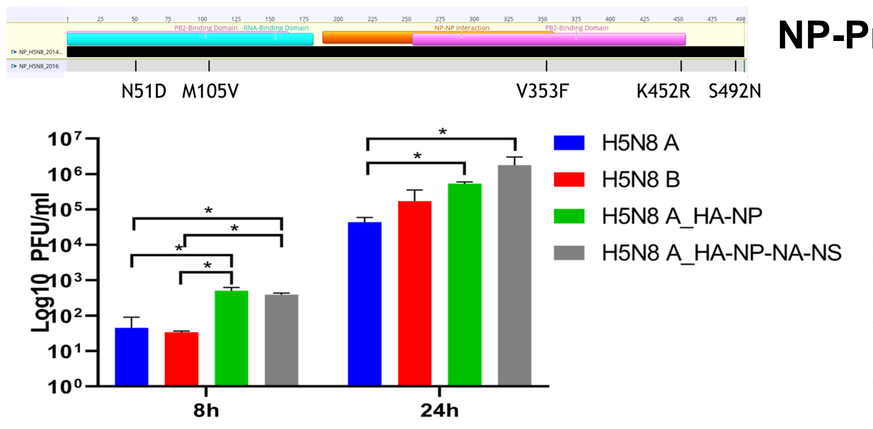
19 aa differences were found in the HA. Since some of these mutations are located in the receptor binding domain the affinity to avian-like α-2.3 SA receptors was determined. H5N8 B showed higher binding affinity than HA A. Moreover, Cleavability on primary duck cells was investigated. Both HAs were efficiently cleaved after 8 and 24 h.

NA-Protein



There are 14 mutations in the NA, distributed in all domains. The NA-activity of H5N8 B was higher than that of H5N8 A.

NP-Protein



Only five mutations were found in the NP protein. H5N8-B NP increased replication of H5N8-A in primary duck cells after 8 and 24 hpi. The role of NP on polymerase activity and interaction with the host immune response needs further investigation.

Summary

- **H5N8 clade 2.3.4.4 B was more virulent than clade A in Pekin ducks**
- **Virulence determinants are in the HA, NP and NS and to a lesser extent in the NA**
- **Mutations in the HA and NA increased receptor binding affinity and sialidase activity, respectively. Mutations in the NP was important for efficient virus replication in primary duck cells**