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Total number of authors: 22

Published in: Journal of Virology

Link to article, DOI: 10.1128/JVI.03046-15

Publication date: 2016

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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Spatiotemporal Analysis of the Genetic Diversity of Seal Influenza A(H10N7) Virus, Northwestern Europe

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ABSTRACT
Influenza A viruses are major pathogens for humans, domestic animals, and wildlife, and these viruses occasionally cross the species barrier. In spring 2014, increased mortality of harbor seals (Phoca vitulina), associated with infection with an influenza A(H10N7) virus, was reported in Sweden and Denmark. Within a few months, this virus spread to seals of the coastal waters of Germany and the Netherlands, causing the death of thousands of animals. Genetic analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of this seal influenza A(H10N7) virus revealed that it was most closely related to various avian influenza A(H10N7) viruses. The collection of samples from infected seals during the course of the outbreak provided a unique opportunity to follow the adaptation of the avian virus to its new seal host. Sequence data for samples collected from 41 different seals from four different countries between April 2014 and January 2015 were obtained by Sanger sequencing and next-generation sequencing to describe the molecular epidemiology of the seal influenza A(H10N7) virus. The majority of sequence variation occurred in the HA gene, and some mutations corresponded to amino acid changes not found in H10 viruses isolated from Eurasian birds. Also, sequence variation in the HA gene was greater at the beginning than at the end of the epidemic, when a number of the mutations observed earlier had been fixed. These results imply that when an avian influenza virus jumps the species barrier from birds to seals, amino acid changes in HA may occur rapidly and are important for virus adaptation to its new mammalian host.

IMPORTANCE
Influenza A viruses are major pathogens for humans, domestic animals, and wildlife. In addition to the continuous circulation of influenza A viruses among various host species, cross-species transmission of influenza A viruses occurs occasionally. Wild waterfowl and shorebirds are the main reservoir for most influenza A virus subtypes, and spillover of influenza A viruses from birds to humans or other mammalian species may result in major outbreaks. In the present study, various sequencing methods were used to elucidate the genetic changes that occurred after the introduction and subsequent spread of an avian influenza A(H10N7) virus among harbor seals of northwestern Europe by use of various samples collected during the outbreak. Such detailed knowledge of genetic changes necessary for introduction and adaptation of avian influenza A viruses to mammalian hosts is important for a rapid risk assessment of such viruses soon after they cross the species barrier.
influenza A(H10N7) virus spread south to seals off the coasts of western Denmark and Germany, which resulted in the death of between 1,500 and 2,000 seals (10). The furthest south that the seal influenza A(H10N7) virus was detected was in the Netherlands, where only dozens of seals were found dead during the outbreak.

This outbreak provided a unique opportunity to follow the evolution of an influenza A virus soon after it crossed the species barrier from birds to mammals. In order to study the genetic changes and possible adaptation of the outbreak strain in the new host, swabs and tissue samples were collected from dead seals that were found off the coasts of Sweden, Denmark, Germany, and the Netherlands, with emphasis on the timing of sampling to reflect different phases of the outbreak.

**MATERIALS AND METHODS**

**Collection of samples.** Seals that stranded between April 2014 and January 2015 off the coasts of Sweden (n = 2), Denmark (n = 4), Germany (n = 24), and the Netherlands (n = 11) were sampled. Nose swabs (NS), throat swabs (ThS), trachea swabs (TrS), trachea tissue specimens (Tr), and/or lung tissue specimens (L) were collected under aseptic conditions from harbor seal carcasses and stored at −70°C. In total, 57 samples collected from 41 different harbor seal carcasses were analyzed (see Table S1 in the supplementary material).

**Sample processing, PCR amplification, and Sanger sequencing.** Collected swabs were vortexed briefly in Hanks’ balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 µl/ml penicillin, 200 µg/ml streptomycin, 100 µl/ml polymyxin B sulfate, 250 µg/ml gentamicin, and 50 µl/ml nystatin (ICN Pharmaceuticals) (transport medium). Lung tissue specimens were defrosted, homogenized in transport medium by use of a Fastprep-24 tissue homogenizer (MP Biomedicals), and briefly centrifuged. Homogenization of tissues was performed under biosafety level 3 conditions. RNAs were extracted from tissue homogenate and swab supernatants by use of a High Pure RNA isolation kit (Roche), and cDNAs were prepared as described previously (11). To rule out genetic changes generated during cell culture or egg passaging culture of the viruses, mainly original materials were used, when available, for sequence analysis of the HA gene. The material that was used for sequencing for each sample is listed in Table S1 in the supplemental material. The partial HA1 gene, including the receptor binding domain (nucleotides [nt] 233 to 780), was amplified by PCR with the forward primer 5′-CACCTTACAGGACGATGGGAACAC-3′ and the reverse primer 5′-CTAACCTCGGCCCTGGTCTAG-3′, using Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies) according to the manufacturer’s protocol. In addition, the complete HA2 genes from a limited number of samples (mainly original materials) were obtained using previously described primers (12). PCR products were purified from agarose gels by use of a QiaQuick gel extraction kit (Qiagen), and consensus sequences were obtained by Sanger sequencing using a BigDye Terminator sequencing kit, version 3.0, and a model 3100 genetic analyzer (Applied Biosystems) according to the manufacturer’s instructions. In addition to the sequence analysis of the HA genes directly amplified from original material, all gene segments were amplified from influenza virus strains A/harbor seal/Germany/PV20743_L/2014 and A/harbor seal/NL/PV14-221_THS/2015, which were isolated on MDCK cells as described previously (13). A/harbor seal/Germany/PV20743_L/2014 was selected as a representative of the outbreak in Germany, while A/harbor seal/NL/PV14-221_THS/2015 was the latest influenza A(H10N7) virus detected in a harbor seal in the Netherlands (January 2015).

**Analyses of influenza A(H10N7) virus sequence data.** Consensus sequences of the gene segments of seal influenza A(H10N7) viruses A/harbor seal/Germany/S1047_14_L/2014 and A/harbor seal/NL/PV14-221_THS/2015, obtained by Sanger sequencing, were aligned with available sequence data for seal influenza A(H10N7) viruses A/Seal/Sweden/SV0546/2014 (the first isolate from this outbreak; EPI_ISL_167226), A/Seal/Sweden/SV0824/2014/H10N7 (EPI_ISL_167906), and A/harbor seal/Denmark/14-5061-1lu/2014-07 (EPI_ISL_166244) (available at the GISAID database [http://platform.gisaid.org]) by using ClustalW in MEGA6 (14). For the HA and NA genes of A/harbor seal/S1047_14_L/Germany/2014 and A/harbor seal/NL/PV14-221_THS/2015, sequences were obtained directly from original material, while sequences of the other gene segments were obtained from MDCK cell (A/harbor seal/S1047_14_L/Germany/2014) or embryonated chicken egg (A/harbor seal/NL/PV14-221_THS/2015) passaged materials.

Pairwise identities were calculated on the nucleotide and deduced amino acid levels by using MEGA6, and detection of influenza A virus sequences most closely related to the seal influenza A(H10N7) viruses was performed by BLASSTn analysis based on influenza A virus sequences available in the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and GISAID (http://platform.gisaid.org) databases. Comparison of amino acid sequences of gene segments of seal influenza A(H10N7) viruses and Eurasian wild bird influenza A viruses was performed using sequence data available in the Influenza Research Database (www.fludb.org) and the GISAID database (http://platform.gisaid.org). Amino acids of the H10 gene were numbered according to the numbering commonly used for the influenza A virus H3 gene.

**Phylogenetic analyses.** Bayesian molecular clock analysis was performed using BEAST 1.8.2 (15). The nucleotide substitution model used was HKY + G (16), with the rate heterogeneity partitioned by codon position and substitution rate and the rate heterogeneity parameters unlinked across positions. The analysis used an uncorrelated lognormal relaxed molecular clock (17) and a Bayesian SkyGrid tree prior (18). BEAST was also used to reconstruct nucleotide sequences at ancestral nodes, which were subsequently translated to amino acid sequences and used to detect amino acid changes along branches in the phylogeny. This analysis was performed on an alignment of complete HA segments from 26 selected samples, and also on a larger data set composed of these 26 sequences aligned with an additional 35 partial HA sequences (466 bp). For both BEAST analyses, the Markov chain Monte Carlo (MCMC) chain was run for 100,000,000 states, with a sampling frequency of 1 every 10,000 states. This was sufficient to ensure effective sample sizes of at least 500 for the posterior and prior probabilities, the likelihood, and all numerical model parameters. Using the program NETWORK, a phylogenetic network was constructed based on the amino acid sequences of the complete HA sequences, using the median joining method and otherwise default parameters (19; http://www.fluxus-engineering.com/).

Next-generation sequencing of partial influenza A(H10N7) virus HA genes. Sequence analysis of the available HA gene segments revealed that the region surrounding the putative receptor binding site contained the most sequence variation. To identify the presence of virus quasispecies in this region and to obtain additional virus variants present in 30 original materials collected from the seals, the partial HA1 gene (nt 233 to 780) was
Sequence similarities between the internal gene segments of these viruses than those for the HA and NA genes. The NS gene had the lowest pairwise identities of the internal gene segments, with mean values of 99.5% at the nucleotide level and 99.1% at the amino acid level. Mean pairwise identities between the HA genes of the analyzed viruses were 99.1% at the nucleotide level and 98.2% at the amino acid level, while pairwise identities between the NA genes of the analyzed viruses were 98.8% at the nucleotide level and 98.7% at the amino acid level. Additional analysis of the pairwise identities of HA and NA on the nucleotide and amino acid levels revealed that the relatively low pairwise identity of NA was caused mainly by the NA of A/Seal/Sweden/SVA0546/2014; the mean pairwise identity for the other analyzed viruses was 99.5% on both the nucleotide and amino acid levels.

By comparison of the amino acid sequences of the complete genomes of A/Seal/Sweden/SVA0546/2014 (April 2014), A/harbor seal/Germany/S1047_14_L/2014 (October 2014), and A/harbor seal/NL/PV14-221_TS/2015 (January 2015) in the database revealed that the relatively low pairwise identity of NA was caused mainly by the NA of A/Seal/Sweden/SVA0546/2014; the mean pairwise identity for the other analyzed viruses was 99.5% on both the nucleotide and amino acid levels.

### RESULTS

#### Sequence comparisons between the first seal influenza A(H10N7) virus and avian influenza A viruses

Analysis of the complete genomes of A/Seal/Sweden/SVA0546/2014 (April 2014), A/harbor seal/Germany/S1047_14_L/2014 (October 2014), and A/harbor seal/NL/PV14-221_TS/2015 (January 2015) in the database revealed that the relatively low pairwise identity of NA was caused mainly by the NA of A/Seal/Sweden/SVA0546/2014; the mean pairwise identity for the other analyzed viruses was 99.5% on both the nucleotide and amino acid levels.

#### Sequence similarities between seal influenza A(H10N7) viruses

Analysis of the mean pairwise identities on the nucleotide and deduced amino acid levels between the complete genomes of A/Seal/Sweden/SVA0546/2014 (April 2014; “early”), A/harbor seal/Germany/S1047_14_L/2014 (October 2014; “middle”), and A/harbor seal/NL/PV14-221_TS/2015 (January 2015; “late”) revealed that there were higher pairwise sequence similarities between the internal gene segments of these viruses than those for the HA and NA genes. The NS gene had the lowest pairwise identities of the internal gene segments, with mean values of 99.5% at the nucleotide level and 99.1% at the amino acid level. Mean pairwise identities between the HA genes of the analyzed viruses were 99.1% at the nucleotide level and 98.2% at the amino acid level, while pairwise identities between the NA genes of the analyzed viruses were 98.8% at the nucleotide level and 98.7% at the amino acid level. Additional analysis of the pairwise identities of HA and NA on the nucleotide and amino acid levels revealed that the relatively low pairwise identity of NA was caused mainly by the NA of A/Seal/Sweden/SVA0546/2014; the mean pairwise identity for the other analyzed viruses was 99.5% on both the nucleotide and amino acid levels.
<table>
<thead>
<tr>
<th>Influenza A virus</th>
<th>Amino acid at indicated position</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Seal/Sweden/SVA0546/2014 (April 2014)</td>
<td>V R D F GI S RRE S C D VTD H H KAR S N I D H VS E V T DI D</td>
</tr>
<tr>
<td>A/harbor seal/Germany/S1047_14_L/2014 (October 2014)</td>
<td>V R N S SI SR R E P C DV T D H H K A RSSI D H V SE V I Y I D</td>
</tr>
</tbody>
</table>

**Other seal influenza A viruses**

**Amino acid unique to outbreak strain?**

- Amino acids of seal influenza A(H10N7) viruses that were not detected in Eurasian avian influenza virus sequences are indicated in parentheses.

**TABLE 2**

Comparison of amino acid sequences of HAs of seal influenza A(H10N7) viruses at different stages of the outbreak.

<table>
<thead>
<tr>
<th>Influenza A virus</th>
<th>Amino acid at position:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Mallard/NL/1/2014</td>
<td>E A Q S G T D Q N Q N T K D P N M Q Q L E E D V D</td>
</tr>
<tr>
<td>A/Seal/Sweden/SVA0546/2014 (April 2014)</td>
<td>EV Q S G T D Q N Q N T R D S N M HL IEDDL D</td>
</tr>
<tr>
<td>A/harbor seal/Denmark/14-5061-1lu/2014-07 (July 2014)</td>
<td>KV Q N G T N QN LKT R E P S V QQ IKDDV D</td>
</tr>
<tr>
<td>A/Seal/Sweden/SVA0824/2014/H10N7 (August 2014)</td>
<td>KV K N E A D KN Q KT R E P S V QL IEDDL D</td>
</tr>
<tr>
<td>A/harbor seal/Germany/S1047_14_L/2014 (October 2014)</td>
<td>KV Q N G A D KN Q/ L KT R E P S V QQ IKDNV N</td>
</tr>
<tr>
<td>A/harbor seal/NL/PV14-221_ThS/2015 (January 2015)</td>
<td>KV Q N G A D KS LKIR E P S V QQ IKDNV N</td>
</tr>
</tbody>
</table>

**Amino acid unique to outbreak strain?**

- Amino acids of seal influenza A(H10N7) virus unique to the outbreak strains are indicated in parentheses.

\textsuperscript{a} Amino acids of seal influenza A(H10N7) viruses that were not detected in Eurasian avian influenza virus sequences are indicated in parentheses.

\textbf{Discussion}

In the present study, the molecular epidemiology of harbor seal influenza A(H10N7) virus was analyzed during the outbreak which occurred from spring 2014 until the winter of 2014 to 2015. Comparison of the “early,” “middle,” and “late” strains for seal influenza A(H10N7) viruses with each other and with the most closely related avian influenza A(H10N7) viruses showed the presence of amino acid variations between the seal influenza A(H10N7) viruses and Eurasian avian influenza A Viruses, but also a gradual accumulation of amino acid changes in the seal influenza A(H10N7) viruses. Amino acid changes were mainly detected in HA, some of which have not been detected in influenza A viruses collected from Eurasian birds. The presence of these changes suggests an adaptation of the seal influenza A(H10N7) virus to replication or transmission in the new host. Comparison of the “early” viruses revealed many changes, while comparison of the “middle” and “late” viruses revealed relatively few amino acid changes. Some of the changes detected in the “early” viruses became fixed and were also detected in the “middle” and “late” strains, suggesting that they played a role in adaptation to the new host. However, this might be biased, because the time frame between the “early” and “middle” periods was about 6 months and the time frame between the “middle” and “late” periods was only 3 months, and because more samples from the “middle” and “late” periods were analyzed.

Although the detected amino acid changes are suggestive of mammalian adaptation, the only currently known genetic marker of potential adaptation to mammals that was detected in seal influenza A(H10N7) viruses was 220L (20, 21), which suggests that the observed amino acid changes might be specific for seals and influenza A(H10N7) viruses or were due to drift. It is interesting that genetic changes were detected in the putative receptor binding site (positions 220 and 222), including a deletion variant at position 222. This deletion variant was also detected in 5% of viruses when the virus was cloned \textit{in vitro} (data not shown), which indicates that the variant was indeed present and was not a next-

Phylogenetic analyses. Phylogenetic analysis of the HA gene sequences of seal influenza A(H10N7) viruses detected in samples collected from harbor seals found dead off the coasts of Sweden, Denmark, Germany, and the Netherlands showed a ladder-like appearance suggestive of evolutionary changes accumulating over time and place was observed when “early,” “middle,” and “late” sequences were compared. Ten of these accumulative amino acid changes (82K, 204Q, 220L, 236K, 238I, 379I, 381K, 398D, 482D, and 508N) were not detected in available Eurasian avian influenza A(H10N7) virus sequences (Table 2 and Fig. 1B). In addition, a number of amino acid changes were detected in only a proportion of the analyzed HAs of influenza A(H10N7) viruses (Fig. 1B).

Next-generation sequencing analyses. Given the observed changes in the HA gene, the HA sequences were analyzed in more depth. Analyses of the reads obtained by 454 sequencing confirmed the presence of major variants as detected by Sanger sequencing (data not shown). Time-ordered analysis of deep sequencing data showed a gradual shift in the virus population, with the “middle” strain differing at only three amino acid positions, including variations at nucleotide positions 684 (S206N) and 780 (T238I) of the HA gene. In addition, variations were detected at various other positions, including variations at nucleotide positions 726 (Q220L) and 731 (G222S), which encode part of the putative receptor binding site. In addition, the complete codon of the amino acid at position 222 was not present in proportions (12 and 13%) of the reads of two samples collected from seals that were found dead in Germany (A/harbor seal/S1052_14_ThS/Germany/2014 and A/harbor seal/S1047_14_L/Germany/2014). The observed position 222 deletion variant was present only in reads with nucleotides encoding a leucine (L) instead of a glutamine (Q) at position 220. In the same samples, a nucleotide change at position 731 (G → A) which resulted in a putative serine (S) instead of a glycine (G) at position 222 was present in 5% and 7% of the reads. Also, this putative amino acid change at position 222 was present only in combination with reads that had nucleotides that encoded an I at position 220 (see Table S2 in the supplemental material).
generation sequencing artifact. Note that lowly pathogenic avian influenza viruses show moderate but not abundant attachment to the harbor seal trachea (22) and that the observed changes in HA might be associated with more abundant attachment to the trachea, as seen for seasonal human influenza A viruses in the human trachea (23). Additional studies are currently ongoing to elucidate the impact of the genetic variation at these positions.

It is interesting that pairwise identity analysis of the NA sequences from the various seal influenza A(H10N7) viruses confirmed that the NA gene segment of seal influenza virus A/Seal/Sweden/SVA0546/2014 was relatively divergent from those of the other seal influenza A(H10N7) viruses, as shown previously (8) (Fig. 3). Phylogenetic analysis revealed that the NA gene of A/Seal/Sweden/SVA0546/2014 branched with NA genes detected in wild and domestic ducks, while NA genes of other currently known seal influenza A(H10N7) viruses branched with NA genes detected recently in outbreaks of influenza A(H7N7) virus among poultry farms in the
Netherlands and the United Kingdom. This difference was observed only for the NA gene and indicated introduction of a separate NA gene rather than rapid evolution, which suggests that more than one virus has infected the harbor seal population or that the virus has moved back and forth between seals and birds. This also indicates that the various amino acid changes in the HAs of the early seal influenza A(H10N7) viruses might also reflect the diversity of viruses present in the avian reservoir, not necessarily an adaptation to seals.

In conclusion, the present study made use of a geographically and temporally structured set of primarily original samples and the availability of advanced sequencing techniques to track the genetic changes of an avian influenza H10N7 virus soon after introduction into harbor seals. The results highlight the ability of an avian influenza A virus to rapidly adapt to a mammalian host and cause an outbreak with substantial morbidity and mortality. Further in vitro and in vivo analyses are needed to elucidate the effects of these genetic changes on virus dynamics and the pathogenesis of seal influenza A(H10N7) virus infection in harbor seals. Nevertheless, the study provides another example of the genetic flexibility of influenza A viruses and their capacity for host-adaptive changes following interspecies transmission events.

ACKNOWLEDGMENTS

We thank all the people who helped with collection of samples during the H10N7 outbreaks for their commitment.

This study was financially supported by the European Commission H2020 program, under contract number 643476 (www.compare_europe.eu); by ZonMW grant 91213058; and by NIAID/NIH contract HHSN272201400008C.

FUNDING INFORMATION

This work, including the efforts of Rogier Bodewes, Saskia L. Smits, Albert Osterhaus, Ron A. M. Fouchier, Marion Koopmans, and Thijs Kuiken, was funded by European Union (643476 Compare). This work, including the efforts of Theo M. Bestebroer, Monique I. J. Spronken, Sander Herfst, and Ron A. M. Fouchier, was funded by HHS | NIH | NIH Clinical Center (NIH CC). (HHSN272201400008C). This work, including the efforts of Marleen Koopmans, was funded by ZonMw (Netherlands Organisation for Health Research and Development) (91213058).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES


7. Goldstein T, Mena I, Anthony SJ, Medina R, Robinson PW, Greig DJ, Costa DP, Lipkin WI, Garcia-Sastre A, Boyle WM. 2013. Pandemic H1N1 influenza isolated from free-ranging northern elephant seals in...


