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Evolutionary trajectories of two distinct avian influenza epidemics: Parallelisms and divergences

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ABSTRACT

Influenza A virus can quickly acquire genetic mutations that may be associated with increased virulence, host switching or antigenic changes. To provide new insights into the evolutionary dynamics and the adaptive strategies of distinct avian influenza lineages in response to environmental and host factors, we compared two distinct avian influenza epidemics caused by the H7N1 and H7N3 subtypes that circulated under similar epidemiological conditions, including the same domestic species reared in the same densely populated poultry area for similar periods of time.

The two strains appear to have experienced largely divergent evolution: the H7N1 viruses evolved into a highly pathogenic form, while the H7N3 did not. However, a more detailed molecular and evolutionary analysis revealed several common features: (i) the independent acquisition of 32 identical mutations throughout the entire genome; (ii) the evolution and persistence of two sole genetic groups with similar genetic characteristics; (iii) a comparable pattern of amino acid variability of the HA proteins during the low pathogenic epidemics; and (iv) similar rates of nucleotide substitutions. These findings suggest that the evolutionary trajectories of viruses with the same virulence level circulating in analogous epidemiological conditions may be similar. In addition, our deep sequencing analysis of 15 samples revealed that 17 of the 32 parallel mutations were already present at the beginning of the two epidemics, suggesting that fixation of these mutations may occur with different mechanisms, which may depend on the fitness gain provided by each mutation. This highlighted the difficulties in predicting the acquisition of mutations that can be correlated to viral adaptation to specific epidemiological conditions or to changes in virus virulence.

1. Introduction

Since the 90s, outbreaks caused by avian influenza virus of the H7 subtype have been frequently reported in domestic poultry throughout the world, causing not only important damage to the poultry industry, but also a great concern for human health, as demonstrated by the recent H7N9 epidemic in China (Chen et al., 2013). Once in poultry, this subtype can evolve into a highly pathogenic form. While the low pathogenic avian influenza (LPAI) virus causes only a mild, primarily respiratory disease in the infected domestic fowl along with production drops, the highly pathogenic avian influenza virus (HPAI) produces an extremely serious disease that can devastate the poultry population.

As shown in previous studies (Campitelli et al., 2004; Lebarbenchon and Stallknecht, 2011), the H7 viruses collected from poultry are genetically related to the viruses from wild birds, suggesting relative frequent interspecies transmissions. Similarly, the distribution of the HPAI H7 strains throughout the phylogenetic trees indicates the evolution of multiple independent highly pathogenic forms from the low pathogenic progenitors (Röhm et al., 1995; Lebarbenchon and Stallknecht, 2011; Abdelwhab et al., 2014).

Following the transmission from wild to domestic birds the virus can experience an accelerated fixation of beneficial mutations to adapt to new species and new environmental conditions. Sequence adaptations to land-based avian species, such as the acquisition of new additional glycosylation sites near the hemagglutinin (HA) receptor binding site (RBS), deletions at the

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neuraminidase (NA) stalk region or the C-terminal truncation of the non-structural protein 1 (NS1) have been observed in H7 field outbreaks in poultry (Banks et al., 2001; Campitelli et al., 2004; Iqbal et al., 2009; Dundon et al., 2006; Spackman et al., 2003; Bataille et al., 2011) as well as in experimental studies (Giannecchini et al., 2010).

Besides selective pressure applied to the virus by the host, many other changes of ecological conditions can drive the evolutionary dynamics of avian influenza viruses. Vaccination, for example, can determine an increase in the rate of mutations in the antigenic sites of the surface glycoproteins (Beato et al., 2014; Cattoli et al., 2011a,b). However, since epidemiological conditions can be different from one epidemic to the next, their influence on virus evolution can be difficult to establish. This paper aims to provide new insights into the evolutionary dynamics of different viruses experiencing similar host and ecological selective pressures.

Between 1999 and 2004 the densely poultry populated area of Northern Italy experienced two distinct H7 epidemics: one in 1999–2001 caused by an H7N1 virus, the other in 2002–2004 originated by an H7N3 virus. The H7N1 epidemic (1999–2001) was caused by a LPAI strain, which mutated into a highly pathogenic form after circulating in the industrial poultry population for approximately 9 months (from the end of March to December 1999) and causing 199 outbreaks. The HPAI strain provoked the death or culling of over 16 million poultry, as well as substantial economic losses to the industry before its eradication in April 2000. Four months later, the LPAI H7N1 re-emerged, affecting other 78 flocks. To reduce the economic impact of this second wave of LPAI viruses, a DIVA (Differentiating Infected from Vaccinated Animals) vaccination campaign was initiated in November 2000 (Capua and Marangon, 2007; Mulatti et al., 2010). The second epidemic started in October 2002 and was caused by an H7N3 LPAI strain, which, according to previous phylogenetic analyses, was probably introduced from the wild bird reservoir into the domestic poultry (Campitelli et al., 2004). To contain the rapid spread of the infection, from January 2003 a DIVA vaccination campaign was carried out in layers, capons and meat turkeys. The virus managed to circulate for 1 year (until October 2003) and to infect a total of 388 poultry holdings. Similarly to the H7N1 strain, the LPAI H7N3 subtype re-emerged 1 year later, in September 2004. However, thanks to the ongoing vaccination program, this time it caused only 28 new outbreaks (Capua and Marangon, 2007).

Using a Bayesian phylogenetic approach, in our previous study (Monne et al., 2014) we compared the evolutionary dynamics of the H7N1 HPAI viruses with those of low pathogenicity collected during the 1999–2001 epidemic and provided evidence of the origin of the HPAI strain from the LPAI viruses. Starting from these results, here we compared the evolutionary trajectories of two distinct naturally occurring epidemics (the 1999–2001 H7N1 and the 2002–2004 H7N3), which had affected the same domestic species (mainly turkeys and chickens) reared in the same geographic area (Veneto, Lombardia and Emilia Romagna regions) for similar periods of time (about 2 years).

2. Materials and methods

2.1. Viruses included in this study

In this study, we generated the complete genome sequences of 35 H7N3 avian influenza A viruses collected from poultry in Northern Italy from October 2002 to December 2004. In addition, we sequenced the partial genomes of five samples from which whole genome sequences could not be obtained. Sequences of 37 H7N3 viruses from the 2002–2004 epidemic publicly available in the Influenza Virus Resource at GenBank were also included in the analysis.

These data were compared to the HA sequences of 144 samples and to the complete genome of 109 isolates collected during the 1999–2001 LPAI/HPAI H7N1 epidemic, sequenced and analyzed in our previous study (Monne et al., 2014).

Epidemiological information (collection date and province of collection) for all the H7N3 viruses included in this study is available in the Supplementary material (Table S1).

2.2. Sanger sequencing

Viral RNA was extracted from the infected allantoic fluid of specific-pathogen-free fowls' eggs using the Nucleospin RNA kit (Macherey–Nagel, Duren, Germany) and reverse transcribed with the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). PCR amplifications were performed by using specific primers (sequences are available on request). Amplicons were subsequently purified with ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The products of the sequencing reactions were cleaned-up using the PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD) and analyzed on a 16-capillary ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

2.3. Library preparation, Illumina sequencing and data analysis

To assess virus population diversity, next-generation sequencing (NGS) was performed on all the H7N3 clinical samples available in our repository (eight tracheas and one pool of organs). These nine samples were collected during the first 3 months of the epidemic. Unfortunately, no clinical samples collected after January 2003 were available. Full sample details are described in Table S1. These newly generated sequences were analyzed together with the NGS data generated by Monne et al. (2014) for six LPAI H7N1 clinical samples.

Viral RNA was extracted directly from the infected clinical samples using the Nucleospin RNA kit (Macherey–Nagel, Duren, Germany) and processed as described by Monne et al. (2014). In summary, the complete influenza A genomes were amplified with the SuperScript III One-Step RT-PCR system with Platinum®Taq High Fidelity (Invitrogen, Carlsbad, CA) (Zhou et al., 2009). Sequencing libraries were obtained using Nextera DNA XT Sample preparation kit (Illumina). Finally the indexed libraries were pooled in equimolar concentrations and sequenced in multiplex for 250 bp paired-end on Illumina MiSeq, according to the manufacturer's instructions.

Raw sequence reads were inspected using FASTQC to assess the quality of data. Fastq files were cleaned with PRINSEQ and Trim Galore to remove low quality bases at the 5′ and 3′-end of each read and to exclude reads with a Phred quality score below 30 and shorter than 80 nucleotides. Reads were aligned to A/turkey/Italy/8535/2002 (H7N3) reference sequences using Stampy (Lunter and Goodson, 2011). The BAM alignment files were parsed using the diversiTools program (http://josephhughes.github.io/btctools/) to determine the average base-calling error probability and to identify the frequency of polymorphisms at each site relative to the reference used for the alignment. Only polymorphisms with a frequency above 2% were considered.

2.4. Phylogenetic and molecular analyses

Sequences of the HA gene and the gene segments coding for the six internal proteins of the H7N1 and H7N3 viruses were aligned and compared with the most related sequences available in

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