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Short communication

Vaccine immune pressure influences viral population complexity of avian influenza virus during infection

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ABSTRACT

Vaccines are useful tools to control influenza A virus infection in poultry, but they need to be periodically reformulated to guarantee appropriate protection from infection and to limit viral replication and circulation, which could favour the emergence of new variants. In this study, a deep sequencing approach was used to characterize and follow the evolution of the hemagglutinin of the H5N1 highly pathogenic avian influenza viral population in infected animals vaccinated with two vaccines conferring different protection levels. Results from this preliminary investigation suggested that the evolution of the viral population, as well as the abundance and heterogeneity of minority variants could be influenced by the immune pressure conferred by vaccination.

1. Introduction

Influenza A virus is a zoonotic agent with a significant impact on both public health and poultry industry. Vaccination is a useful tool used worldwide to support intervention strategies, such as stamping out and biosecurity policies, in order to keep the infection under control and prevent the transmission of avian influenza viruses in poultry (Lee and Suarez, 2005). However, as demonstrated in previous studies, the use of a vaccine strain antigenically different from the circulating viruses or application of inadequate vaccine protocols may favour the antigenic drift and cause vaccination failure (Cattoli et al., 2011b; Lee et al., 2004; Swayne, 2012). A more extensive knowledge of the mechanisms underlying intra-host evolution of avian influenza viruses circulating in vaccinated poultry populations could be of help to formulate and adopt more adequate vaccine strategies.

Previous studies conducted in partially immune pigs indicated that the variability in immune response may influence the overall diversity of swine influenza virus during infection (Diaz et al., 2015) and showed that the hemagglutinin gene displayed nucleotide mutations at the very beginning of viral infection (Diaz et al., 2013; Murcia et al., 2012). Still, to date there is no information on the intra-host evolution of highly pathogenic avian influenza (HPAI) viruses circulating in vaccinated poultry populations. The hemagglutinin (HA) is a surface glycoprotein, involved in the induction of a protective humoral and cell mediated immune response, and represents one of the major antigenic determinants of type A influenza viruses.

To provide some preliminary data on the impact of vaccination on the intra-host diversity and evolution of HPAI viruses, in this study we performed a deep sequencing analysis of swabs sampled from H5N1 HPAI experimentally infected chickens, which showed different levels of clinical and virological protection conferred by two different vaccine formulations.

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2. Material and methods

A deep sequencing analysis on the HA gene segment was performed on samples collected in a previously performed vaccination/challenge study for assessing the protective efficacy of two avian influenza vaccines against a HPAI H5N1 virus. Birds were housed into HEPA filtered poultry isolators in BSL3 animal facilities and received food and water *ad libitum*. All animals were handled in strict accordance with the Decree of the Italian Ministry of Health (n. 26 of 4 March 2014) on the protection of animals used for scientific purposes, implementing Directive 2010/63/EU.

Briefly, two groups of ten Specific Pathogen Free (SPF) day-old chicks were vaccinated twice at a 10-day interval by the subcutaneous route, using two distinct influenza inactivated experimental vaccines. The first vaccination was performed with 0.3 ml and the second vaccination was performed with 0.5 ml via the subcutaneous route in the back of the neck. Both vaccines contained each a recombinant H5-antigen which was encoded by recombinant baculoviruses. Generation of the recombinant baculoviruses and formulation of the experimental vaccines was performed as described recently (Oliveira Cavalcanti et al., 2017) with one exception, no inactivated antigen of Newcastle disease virus was added, and 512 HA units were added as H5 antigen. One recombinant baculovirus encoded for a H5-protein which is included in the VOLVAC® B.E.S.T AI + ND KV (A) belonging to clade 2.3.2. The second recombinant baculovirus (B) encoded for a consensus sequence (Patent WO 2012/054907 A2; Sequence ID 43) which was generated based on 37 amino acid sequences of Egyptian origin viruses from 2010 belonging to either clade 2.2.1 or clade 2.2.1.1.

The birds were challenged with 10^6 50% Embryo Infectious Dose (EID_{50}) of the HPAI H5N1 A/chicken/Egypt/11VIR4453-7/VRLCU/2010 virus (clade 2.2.1) (WHO/OIE/FAO H5N1 Evolution Working Group, 2012) 21 days after the booster vaccination. The virus had been isolated from pooled organs in 9- to 11-day-old SPF embryonated fowls' eggs. Antibody responses were assessed by means of hemagglutination inhibition assay (HI), 10 days from the first vaccination, 21 days from the second vaccination and 2 weeks after the challenge. Tracheal swabs (TS) and cloacal swabs (CS) were collected on days 2, 4, 6, 8 and 10 post challenge (p.c.) to evaluate viral shedding by quantitative real-time RT-PCR (qRRT-PCR) targeting the M gene (Spackman et al., 2002) and calculate the EID_{50} equivalents from the cycle threshold (Ct) values. The Egyptian HPAI H5N1 virus used for the challenge, as well as 20 TS positive by qRRT-PCR, which contain a sufficient amount of RNA (six samples from group A and fourteen samples from group B), were processed as described below. Neither of the samples obtained from CS did result in a sufficient signal from the qRRT-PCR to be included in the analysis. Total RNA was isolated using Nucleospin RNA kit (Macherey-Nagel, Düren, Germany). Viral RNA encoding the HA gene segment was retro-transcribed and amplified using SuperScript III one-step reverse transcription-PCR (RT-PCR) system with PlatinumTaq High Fidelity (Invitrogen, Carlsbad, CA) using H5 specific primers (H5-for: 5′-CRAAAG-CAGGGGTYCAATC-3′, H5-rev: 5′-GAAACAAGGGTGTTTTTAAC-3′). Sequencing libraries were prepared using Nextera XT DNA Sample preparation kit (Illumina) and processed as described by Monne et al. (2014) on an Illumina Miseq desktop sequencer. Raw data were submitted to the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession numbers SRR4244068, SRR4244069, SRR4244070, SRR4244071, SRR4244072, SRR4244073, SRR4244074, SRR4244076, SRR4244077, SRR4244078, SRR4244079, SRR4244080, SRR4244081, SRR4244082, SRR4244083, SRR4244084, SRR4244085, SRR4244086, SRR4244087, SRR4244088, SRR4244089.

FASTQC software was used to inspect quality score of raw sequence files and post processing data coming from the high-throughput sequencing pipelines. Fastq files were cleaned with Trimmomatic v0.32 (Bolger et al., 2014), using a 4-base-pair sliding-window algorithm with a quality score cut-off of 20; only reads longer than 80 nucleotides were considered and mapped to the hemagglutinin H5 reference sequence A/chicken/Egypt/11VIR4453-7/VRLCU/2010 (Gisaid accession number EPI348162) using bwa v0.7.5 (Li and Durbin, 2010). The alignment was processed with LoFreq v2.1.2 (Wilm et al., 2012) for the SNP calling; according to LoFreq usage recommendations, the alignment was first processed with Picard-tools v2.1.0 (http://broadinstitute.github.io/picard/) and GATK v3.5 (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). The frequencies of variants in the final set were computed using the diversiTools program v0.1.19 (http://josephhughes.github.io/btctools/) and confirmed by those found with LoFreq. Variants with sequence coverage less than 500X or frequency less than 1% were discarded. The numbering scheme of the amino acid residues used in this study is based on the mature sequence (signal peptide was cleaved off) of the HA of A/Vietnam/1203/2004 (H5N1) (GenBank accession number HM006759).

To explore the amino acid variability at the resulted polymorphic sites in the Egyptian H5N1 viral population, the full-length hemagglutinin sequences of H5N1 Egyptian viruses (from 2006 to 2016) were downloaded from the public database (GenBank) collapsing identical ones; a total of 345 protein sequences was obtained. The sequences were then aligned using the on-line MAFFT program (http://mafft.cbrc.jp/alignment/server/) and amino acid composition at the selected positions was carefully inspected.

From our NGS data we then calculated the Shannon Entropy (SE). SE is a measure of diversity within a viral population at a single nucleotide position or a whole sequence level. In a specific site, the highest value of SE is reached when the four nucleotides are present at a frequency of 25%, while the lowest value (zero) is obtained when only one nucleotide is present at a frequency of 100%. Low entropy may be observed in case of a significant reduction of the population size, i.e., following a bottleneck event, which at a single nucleotide level results in low or no variability. In our study Shannon Entropy was used to quantify average diversity of the HA gene of the viral populations of each sample belonging to group A and B, using the following formula:

$$E = -\frac{1}{N}\sum_{i=1}^{N}(f_{iA}\ln f_{iA} + f_{iG}\ln f_{iG} + f_{iT}\ln f_{iT} + f_{iC}\ln f_{iC})$$

where f_i is the frequency of the nucleotide A, C, G or T at position i and N is the total length of the hemagglutinin gene.

The Wilcoxon Mann–Whitney rank-sum test was used to verify whether the distribution of EID_{50} and Entropy values was the same in both vaccine groups; only six samples were collected at 4 days p.c., five from group B and one from group A, therefore they were excluded from the statistical comparison. The same test was used to assess whether the distributions of Entropy were identical between the challenge virus and each sample of groups A and B. P-value < 0.10 was considered significant. The Pearson product-moment correlation coefficient was used to verify whether the population diversity (represented by SE) and the quantity of virus (represented by EID50) were independent so as to exclude that differences of SE between group A and B were due to EID50 values.

3. Results

To provide some preliminary data on the impact of vaccination on the intra-host diversity and evolution of HPAI viruses, in this