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Research paper

A two-year monitoring period of the genetic properties of clade 2.3.2.1c H5N1 viruses in Nigeria reveals the emergence and co-circulation of distinct genotypes

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ABSTRACT

Phylogenetic analyses of the complete genomes of the highly pathogenic avian influenza (*HPAI*) 2.3.2.1c H5N1 virus strains causing outbreaks in Nigeria's poultry population from 2014 to 2016 showed evidence of distinct co-circulating genotypes and the emergence of reassortant viruses. One of these reassortants became the predominant strain by 2016, and the NA protein of this strain possessed the V96A substitution known to confer reduced susceptibility to neuraminidase inhibiting antiviral drugs. Our findings also demonstrated evolutionary relationships between Nigerian isolates and European and Middle Eastern strains of H5N1 which provides further evidence for the proposed role of migratory birds in spreading the virus, although the involvement of the live poultry trade cannot be excluded. Efforts must be directed towards improving biosecurity and gaining the cooperation of poultry farmers for more effective control of *HPAI*, in order to mitigate the emergence of *HPAI* strains in Nigeria with biological properties that are potentially even more dangerous to animals and humans.

1. Introduction

Avian influenza is caused by influenza A viruses of the *Orthomyxoviridae* family which are segmented, negative sense, single-stranded RNA viruses (McCauley et al., 2012). The genome consists of eight segments encoding at least eleven functional proteins: polymerase basic 2 (PB2) on segment 1, polymerase basic 1 (PB1) plus mitochondria-associated protein (PB1-F2) on segment 2, polymerase A (PA) plus PA-X fusion protein on segment 3, haemagglutinin (HA) glycoprotein on segment 4, nucleocapsid protein (NP) on segment 5, neuraminidase (NA) glycoprotein on segment 6, matrix protein (M1) plus the ionic channel protein (M2) on segment 7, and nonstructural protein 1 (NS1) plus nuclear export protein (NEP/NS2) on segment 8 (Cai et al., 2010; Wright et al., 2007). Influenza A viruses are further classified into subtypes based on the antigenic relationship of their surface glycoproteins, HA and NA (Munster and Fouchier, 2009). To date, 18 HA (H1 to H18) and 11 NA (N1 to N11) subtypes have been identified (Swayne and Halvorson, 2003; Fouchier et al., 2005; Watanabe et al., 2014).

The HA protein is responsible for viral attachment, and is produced as a precursor molecule, HA0, which must be cleaved to HA1 and HA2 to expose the fusion protein by host proteases. This proteolytic activation of the HA molecule is a prime determinant of the pathogenicity of avian influenza viruses which express large variation in cleavability (Klenk and Rott, 1988; Stieneke-Gröber et al., 1992; Horimoto and Kawaoka, 1994). The typical cleavage site of LPAI viruses has single basic amino acids and is limited to activation by localized trypsin-like proteases mostly found in the respiratory and intestinal tract of the host. *HPAI* viruses however possess multiple basic amino acids at the HA0 cleavage site cleavable by more ubiquitous proteases probably subtilisin-related endoproteases such as furin (Stieneke-Gröber et al., 1992). *HPAI* viruses are thus able to replicate throughout the host system, damaging multiple vital organs and tissues, resulting in severe clinical manifestations and possibly death. Another important factor influencing HA cleavability is the presence of a carbohydrate side chain (glycosylation) close to the cleavage site which interferes with protease accessibility. Loss of this molecule enhances cleavability and hence pathogenicity (Klenk et al., 2013).

HPAI outbreaks caused by the H5 or H7 subtypes have continued to adversely impact the global poultry and associated industries. Great economic losses during outbreaks result from excessive mortalities in

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affected flocks and culling of in-contact or susceptible birds as part of control measures. In 2006, the first incursion of *HPAI* H5N1 in Nigeria was reported, sequel to the emergence of the A/Goose/Guangdong/1/1996 H5N1 (GsGD) in Guangdong province of China in 1996 which circulated extensively in Asia and was subsequently disseminated to Europe and Africa (Xu et al., 1999; Joannis et al., 2006; Fusaro et al., 2009, 2010). Control measures, largely based on depopulation and decontamination employed by the Government in partnership with donor agencies, led to the eradication of the virus from the country within two years (Oladokun et al., 2012). Seven years after the last *HPAI* H5N1 outbreaks in the country, two incidences were reported simultaneously from Kano and Lagos states between late 2014 and early 2015 (OIE, 2015). Phylogenetic analysis of the HA showed that the virus belonged to the genetic clade 2.3.2.1c and clustered with H5 viruses collected in China in 2013 and with an H5N1 virus (A/Alberta/01/2014) isolated from a Canadian resident who had recently visited China (Monne et al., 2015). Clade 2.3.2.1c, which was first reported from China in whooper swans (Bi et al., 2015) has been isolated from several other wild birds species, along the Central Asian flyway and demonstrated high virulence in mice and ferrets (Bi et al., 2016; Marchenko et al., 2016; Pearce et al., 2017). The virus subsequently spread to other states of Nigeria as well as other West African countries including Burkina Faso, Ghana, Ivory Coast, Niger, Cameroon and Togo; some of which are still reporting outbreaks (OIE, 2017). Two genetic groups within clade 2.3.2.1c, namely WA1 and WA2 were identified in seven of the gene segments (Tassoni et al., 2016). Specifically, the WA1 group was described in Ghana, Burkina Faso, Nigeria and Niger, whereas the WA2 group was restricted to Niger and Ivory Coast. Subsequently, a more recent analysis of the HA gene of 14 Nigerian viruses demonstrated co-circulation of both groups in the country (Shittu et al., 2017). Previous analyses were limited in the number of samples analysed (Tassoni et al., 2016), or by analysis of only the HA (Shittu et al., 2017). Here, we analysed the complete genomes of 100 representative isolates collected between 2015 and 2016 from 16 states and the Federal Capital Territory, to provide a better understanding of the genetic characteristics and evolution of the 2.3.2.1c H5N1 viruses circulating in Nigeria.

2. Materials and methods

2.1. Sample collection and virus isolation

Field outbreaks of a disease with case definitions consistent with *HPAI* were reported in poultry from late December 2014 in Nigeria (Fig. 1). Whole carcasses from affected farms and live bird markets were collected by the Avian Influenza Control Project (AICP) surveillance officers and shipped cold to the Regional Lab for Animal Influenzas and other Transboundary Animal Diseases, National Veterinary Research Institute, Vom, for definitive diagnosis. Liver, spleen, lung, trachea and intestine were harvested aseptically, pooled during necropsy and processed for virus isolation according to standard protocol (OIE, 2015). Briefly, 5 g of pooled tissues were homogenized in a 20% (w/v) suspension with antibiotics and isotonic phosphate-buffered saline (PBS), pH 7.0–7.4. After centrifugation at 1000*g* for 5 min, 0.2 ml supernatant of the tissue homogenate was inoculated into each allantoic cavity of five 9–11 day-old embryonating chicken eggs (ECE) obtained from a specific antibody negative flock (SAN).

Inoculated eggs were incubated in a humidified chamber at 37 °C and examined daily for embryo vitality. Eggs containing dead embryos 24 h post inoculation were chilled at 4 °C. Allantoic fluid (ALF) was harvested and tested for haemagglutination (HA) activity using 10% chicken red blood cells and all positive ALFs were bio-banked. HA positive ALFs were tested for avian influenza in a one-step RT-PCR assay targeting the matrix (M) gene (Fouchier et al., 2000) using the GeneAmp® Gold RNA PCR core kit in a 9700 thermocycler (Life Technologies, Foster City, CA, USA). M-gene positive samples were further subtyped for H5 (Slomka et al., 2007) and N1 (Huang et al., 2013). Amplified PCR products were analysed by gel electrophoresis with 1.5% agarose stained with ethidium bromide (SIGMA, Germany) and visualized using Gel Documentation system (Biostep, Germany). Samples were only declared negative if the virus failed to amplify in embryonating chicken eggs after two successive passages.

One hundred H5N1 isolates were randomly selected from a pool of HA positive samples (Supplemental Table 1), packaged according to IATA regulations and sent for further genetic characterization to the World Organization for Animal Health Reference Laboratory and the Food and Agriculture Organization of the United Nations Reference Centre for Animal Influenza at the Istituto Zooprofilattico Sperimentale delle Venezie, Italy.

2.2. Library preparation, Illumina sequencing, and data analysis

Viral RNA was extracted from the 100 H5N1-infected ALFs using a Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany). Complete influenza A virus genomes were amplified using the SuperScript III one-step reverse transcription-PCR (RT-PCR) system with PlatinumTaq high fidelity (Invitrogen, Carlsbad, CA) and one pair of primers complementary to the conserved elements of the influenza A virus promoter as described by Zhou et al. (2009). All amplified products were visualized on a 0.7% agarose gel stained with GelRed. Sequencing libraries were obtained using a Nextera DNA XT sample preparation kit (Illumina) according to manufacturer's instructions and quantified using a Qubit dsDNA high-sensitivity kit (Invitrogen). The average fragment length was determined using an Agilent high-sensitivity bioanalyzer kit. Finally, the indexed libraries were pooled in equimolar concentrations and sequenced in multiplex for 300-bp paired-end Illumina MiSeq analysis according to the manufacturer's instructions.

To obtain consensus sequences, raw sequence reads were inspected using FastQC v0.11.2 to assess the quality of data coming from the high-throughput sequencing pipelines. Raw data were filtered by removing: a) reads with > 10% of undetermined ("N") bases; b) reads with > 100 bases with Q score below 7; c) duplicated paired-end reads. Remaining reads were clipped from Illumina adaptors Nextera XT with scythe v0.991 (https://github.cohe) and trimmed with sickle v1.33 (https://github.com/najoshi/sickle). Reads shorter than 80 bases or unpaired after previous filters were discarded.

High quality reads were aligned against a reference genome using BWA v0.7.12 (Li and Durbin, 2010). Alignments were processed with SAMtools v0.1.19 (Li et al., 2009) to convert them in BAM format and sort them by position. Single Nucleotide Polymorphisms (SNPs) were called using LoFreq v2.1.2 (Wilm et al., 2012). According to LoFreq usage recommendations, each alignment was first processed with Picard-tools v2.1.0 (http://picard.sourceforge.net) and GATK v3.5 (McKenna et al., 2010; Van der Auwera et al., 2013) in order to correct potential errors, realign reads around indels and recalibrate base quality. LoFreq was then run on the correct alignments to produce a vcf file from which the consensus sequences were generated. SNPs with a frequency lower than 25% were filtered out.

Gene sequences were submitted to the GenBank database under accession numbers MF112254-MF113041.

2.3. Phylogenetic and sequence analysis

A BLAST search for each gene segment of the 100 *HPAI* H5N1 viruses was performed in the GISAID database and the most closely-related sequences were downloaded (November 2016), as well as sequences of H5N1 viruses from the 2006–8 Nigerian outbreaks. Alignments were performed using the on-line program Mafft v.7.0 (Katoh, 2013). Maximum likelihood (ML) phylogenetic trees were generated using PhyML v.3.1 (Guindon et al., 2010), incorporating a general time-reversible (GTR) model of nucleotide substitution with a gamma-distribution of among-site rate variation (with four rate

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