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Isolation and characterization of H5Nx highly pathogenic avian influenza viruses of clade 2.3.4.4 in Russia

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ABSTRACT

In 2016–2017, several subtypes of the highly pathogenic avian influenza (HPAI) virus were isolated on the territory of Russia. In the autumn of 2016, during the avian influenza virus surveillance in the territory of the Kamchatka region of Russia the HPAI A(H5N5) influenza virus was isolated. Then, during 2016–2017, multiple outbreaks among wild birds and poultry caused by HPAI A(H5N8) avian influenza virus were recorded in European part of Russia. At the end of 2017, an outbreak among poultry caused by HPAI A(H5N2) influenza virus was recorded in the European part of Russia. Phylogenetic analysis of HA of the A(H5N5), A(H5N8), A(H5N2) showed the strains belong to the clade 2.3.4.4 b. All isolated strains were antigenically closely related to candidate vaccine viruses of clade 2.3.4.4 and showed high virulence in mice. Genetic analysis revealed presence of genetic markers potentially related to high virulence in mice in all studied viruses.

1. Introduction

For the last two years there has been challenging situation regarding avian influenza in Russia. This was due to the spread of highly pathogenic avian influenza viruses of clade 2.3.4.4, which caused multiple outbreaks among wild birds and poultry in European part of Russia (World Organization for Animal Health OIE, 2016). Also several viruses of clade 2.3.4.4 were isolated during avian influenza virus surveillance in other regions of Russia.

An avian influenza virus of clade 2.3.4.4 was isolated in Russia for the first time in 2014 on the northern region of Russian Far East. At that time the strain A/wigeon/Sakha/1/2014 (H5N8) was isolated from a wild bird during avian influenza virus surveillance (Marchenko et al., 2015). Isolation of this strain showed one of the possible ways of avian influenza virus spread from endemic regions of South-East Asia to Europe and North America and determined the key role of Russian territory in the spread of avian influenza viruses (Verhagen et al., 2015; Ramey et al., 2016). In 2014 avian influenza A(H5N8) of clade 2.3.4.4 has been spreading by wild birds from Korea to Germany, Netherlands, United Kingdom and Italy and then in 2015 February to Hungary through the Russian territory (Adlhoch et al., 2014). At the same time similar viruses were spreading to North America (Lee et al., 2015). Several isolations of highly pathogenic avian influenza A(H5N2) of clade 2.3.4.4 from wild and domestic birds were reported in USA and Canada. Also the A(H5N2) subtype of avian influenza was detected in China (Ramey et al., 2016; Kaplan et al., 2016; Wu et al., 2017).

The influenza viruses of clade 2.3.4.4 have become the most widespread viruses in 2016–2017. It was designated as so-called “Fourth intercontinental wave” of highly pathogenic avian influenza spreading (Sims et al., 2017). According to OIE data outbreaks among wild birds and poultry were registered in at least 48 countries of Asia, Africa and Europe during the Fourth wave (World Organization for Animal Health OIE, 2016). The largest number of influenza A(H5N8) outbreaks among wild birds and poultry were reported in Germany, France, Hungary and Poland (Brouwer et al., 2017). During the Fourth intercontinental wave of avian influenza spread the reassortment has occurred as a result of co-circulation of highly pathogenic A(H5N8) viruses and other strains with low pathogenicity in wild birds and poultry. This reassortment led to emergence and circulation of different clade 2.3.4.4 virus subtypes including A(H5N5) and A(H5N6) (Si et al., 2017; Gu et al., 2011).

The Fourth intercontinental wave in Russia began in May 2016 from the isolation of highly pathogenic avian influenza virus A(H5N8) of clade 2.3.4.4 on the territory of Tyva Republic at Russian-Mongolian border, where this virus emerged from China (Marchenko et al., 2017). Subsequently this subtype of the virus spread to the west, where it caused outbreaks among wild birds and poultry in several regions of European part of Russia.

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2. Materials and methods

2.1. Samples collection and virus isolation

Samples from wild birds and poultry were collected during the National avian influenza virus surveillance program, which supports sample collection from 48 regions of Russia. Samples from birds included cloacal, oropharyngeal swabs and internal organs fragments. For the 2016–2017 a total of 9869 samples from wild birds and 7224 samples from poultry were collected and analyzed during avian influenza virus surveillance. As a result of avian influenza surveillance 95 avian influenza viruses including H4N6 (n = 3), H10N6 (n = 3), H13N8 (n = 2), H5N8 (n = 81), H5N2 (n = 5) and H5N5 (n = 1) influenza A subtypes were detected. In this study we report the characterization of 16A(H5Nx) avian influenza viruses isolated from outbreaks in different region of Russia. From each outbreak we have isolated several viruses. Depending on bird species involved to outbreak we select one virus from each species from each outbreak for investigation.

The Copan Universal Transport Medium (UTM-RT) System (Copan, Italy) was used for sample collection. Swabs and organ homogenates were prepared as described (Erica Spackman; World Health Organization WHO, 2002). 9-day old embryonated chicken eggs were inoculated with an aliquot of 0,1 ml of each sample, which was injected into allantoic cavity for virus isolation. Eggs were incubated for 24–48 h at 37 °C after inoculation. The allantoic fluid was tested for the presence of influenza virus by real-time RT-PCR and haemagglutination assay using 0,5% chicken red blood cells as described (Erica Spackman; World Health Organization WHO, 2002). All viruses were isolated after first passage on chicken embryos. After first passage the isolated virus was diluted from 10^{-4} to 10^{-9}. For viral propagation, an aliquot of each dilution was inoculated into allantoic cavity of 9-day old eggs as described above. The viral samples with maximum haemagglutination rate were used for titration and further research. Isolated viruses were titrated using 9-day old chicken eggs to determine EID_{50}. For this purpose aliquots of each viral sample dilutions from 10^{-5} to 10^{-10} were inoculated into allantoic cavity of six 9-day old eggs. The EID_{50} was calculated by Reed-Meunch method (Reed and Muench, 1938).

2.2. Haemagglutination inhibition assay

Haemagglutination inhibition assay was carried out as described (Erica Spackman; World Health Organization WHO, 2002) using reference antigens and the ferret sera kindly furnished by Dr. R. Webby, St. Jude Children's Research Hospital (Memphis, TN), as well as ferret sera obtained for influenza H5 virus strains that previously circulated in Russia.

2.3. PCR and whole-genome NGS

Viral RNA was isolated using RIBO-sorb RNA/DNA Extraction Kit (InterLabService, Moscow, Russia) according to the manufacturer's instruction. Reverse transcription was performed using "Reverta-L kit" (Interlabservice, Russia) with random hexamer-primer. Real-time RT-PCR typing and subtyping for H5 was performed using "AmpliSens Influenza virus A/B-FL kit", "AmpliSens Influenza virus A H5N1-FL kit", "AmpliSens Influenza virus A-type-H5, H7, H9-FL kit" (Interlabservice, Russia) and RotorGene 6000 thermal cycler with the programs recommended in the manufacturer's manual of the RT-PCR kits. The NA subtyping was performed using whole genome sequencing.

Sequencing was carried out at FBRI SRC VB VECTOR Rospotrebnadzor. To determine nucleotide sequences of viral genes and genomes, viral RNA was isolated using RIBO-sorb RNA/DNA Extraction Kit (InterLabService, Moscow, Russia) according to the manufacturer's instruction. Reverse transcription reaction was carried out with Uni12 primer (Yoshihiro Kawaoka and Gabriele Neumann) using First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) according to the manufacturer's instruction. Deep sequencing of amplicons covering complete genomes was performed on an Illumina MiSeq using MiSeq reagent kit v3. (Illumina, San Diego, USA). The full-length genomes were assembled by alignment of reads to known references with bwa-0.7.15 (Li, 2013).

Phylogenetic analysis was performed using MEGA 6.0 software (http://www.megasoftware.net/) using the maximum likelihood method with 1000 bootstrap replications for HA genes of clade 2.3.4.4. For comparison, we used sequences of strains deposited in the Global Initiative on Sharing All Influenza Data (GISAID).

The analysis of amino acid sequences of the isolated strains genomes was performed using Genetic Changes Inventory (http://www.cdc.gov/flu/avianflu/h5n1/inventory.htm) and service FluSurver (http://flusurver.bii.a-star.edu.sg).

2.4. Animal experiments

To determine the virulence of each avian influenza virus isolate the female Balb/c mice weighing 12–14 g were intranasally infected. Mice were divided into 12 groups of six animals each to determine the 50% infectious and lethal doses. Animals were lightly anesthetized with CO2 before intranasal inoculation with 0,05 ml of virus diluted from 10^{-3} to 10^{-8}. Three days after inoculation six groups of mice were humanely euthanized and lung samples were taken and studied for the presence of influenza virus using 9-day old chicken embryos as described above. Other six groups were observed within 14 days to detect mortality. The 50% infectious and lethal doses were calculated by Reed-Meunch method (Reed and Muench, 1938).

To obtain sera, ferrets were intranasally infected by 6,0 $\log_{10}$EID of studied virus. Sera was collected on 21 day after infection and used for haemagglutination inhibition assay.

All applicable international, national and institutional guidelines for the care and use of animals were followed. Animal experiments were approved by the FBRI SRC VB Vector Rospotrebnadzor Animal Care and Use Committee. Animal experiments were performed in an enhanced animal biosafety level 3 facility at FBRI SRC VB Vector Rospotrebnadzor.

2.5. Susceptibility to neuraminidase inhibitors

Susceptibility of isolated strains to neuraminidase inhibitors oseltamivir and zanamivir was determined in a fluorescence-based neuraminidase inhibition assay with fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) (Biosynth AG, Switzerland). Fluorescence intensity was measured using Infinite F200 microplate reader (Tecan, Switzerland). IC50 values were determined using NAI results Excel template (HPA, London, UK) (Leang and Hurt).

3. Results

Avian influenza viruses A(H5N8) were isolated from wild birds in Altay and Kurgan regions of Russia during the routine surveillance in summer-autumn season of 2016. In November 2016 same virus subtype was isolated on the territory of Kalmykia Republic where an outbreak among backyard poultry has been detected. At the same time an outbreak on the poultry farm in Astrakhan region of Russia was registered. Subsequently avian influenza A(H5N8) become widespread in European part of Russia. From December 2016 to April 2017 outbreaks among wild birds and poultry were reported in Krasnodar and Rostov regions of Russia. An A(H5N8) outbreak, which occurred in January 2017 in Voronezh Zoo, should be especially noted. Dramatic loss of several rare species of wild birds was registered during this outbreak. In March 2017 several outbreaks among wild birds and poultry were reported in several districts of Moscow and Kaliningrad regions. In May 2017 an outbreak among poultry caused by A(H5N8) was registered in