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Quantitative Assessment of Airborne Transmission of Human and Animal Influenza Viruses in the Ferret Model

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Abstract: The WHO has developed a tool to assess the risk of newly emerging influenza viruses with pandemic potential (TIPRA). According to TIPRA, the main parameters for assessing the risk of human-to-human transmission of a novel influenza virus are its ability to bind to human cell receptors of the upper respiratory tract (URT) and transmit in model animals. The aim of this study was to quantify airborne transmission of human and animal influenza viruses in the ferret model. The transmission of influenza viruses was studied in the ferret model in an aerobiology chamber. Airborne particles concentration and fractional composition in the aerobiology chamber were measured using an aerosol particle counter and analytical aerosol filters. Viral load in ferret nasal washings and aerosol filters was determined by titration in MDCK cells and quantitative RT-PCR. Genetic analysis of influenza viruses was performed using virus genome sequences obtained by NGS. After intranasal infection, human and animal influenza viruses replicated in the cells of nasal mucosa in ferrets. The level of virus airborne particles contamination provided by infected animals depends on the infectious dose and differs significantly between influenza virus strains. The studied avian influenza viruses show insufficient transmission in the ferret model, while human and swine influenza viruses are highly transmitted in ferrets. We propose a quantitative model of airborne transmission of influenza virus from donor to recipient ferrets. Level of influenza virus transmission in the ferret model correlates with genetic markers of virus receptor specificity and the level of virus airborne particle contamination induced by donor ferrets.

Keywords: influenza virus; ferret; airborne transmission; dose–response function

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1. Introduction

Influenza is a severe infectious disease and a serious problem for public health. The virus infects up to 15% of the world population annually and causes thousands of deaths worldwide. Influenza pandemics arise every 10–50 years; each pandemic is caused by a new virus strain that can transmit in the human population and has no immunity in humans [1].

Avian influenza viruses and mammalian influenza viruses such as swine influenza are a serious threat, since they can cause a new pandemic. Some avian influenza viruses have already crossed the interspecies barrier and can transmit from birds to humans. Despite the absence of registered cases of mass human-to-human transmission of these strains to date, there is a risk of emergence of new avian influenza viruses that can spread in the human population. Quantification of virus transmission in an animal model is an important component in assessing the pandemic risk of newly emerging avian influenza viruses [2].

Various kinetic [3,4] and stochastic [5,6] models of the epidemiological process are used to analyze the pandemic potential of new viruses. Dose–response relationship and duration of the pathogenic effect are considered input data for quantification of viral and microbial risk [7–9].

In 2016, the WHO launched the Tool for Influenza Pandemic Risk Assessment (TIPRA) and reported that virus transmission in animal models is one of the most important elements in evaluating the general risk of emergence of an influenza virus with pandemic potential [10]. Influenza A viruses are found in a wide range of host animals. The virus is often transmitted between one and different animal species, which is accompanied by genetic mutations and rearrangements [11]. These genetic changes can lead to the emergence of a virus with high human-to-human transmissibility. The emergence of a pandemic influenza virus in 2009 in addition to sporadic transmission of some influenza viruses from animals to humans highlight the necessity to monitor and assess the potential risk of occurrence of new influenza viruses that can cause pandemics. Risk assessment is aimed at determining the probability of emergence of an influenza virus with pandemic potential [12]. TIPRA recommends evaluating four viral properties for risk assessment: virus ability to bind to human cell receptors, virus genome features, ability to transmit in animal models, and sensitivity to antiviral treatment.

The basis for influenza virus transmissibility is its interaction with the host cell surface. This interaction is provided by two virus envelope proteins: hemagglutinin (HA) and neuraminidase (NA). HA binds to this cell surface receptor sialic acid and acts as one of the major factors providing species specificity of influenza virus. Avian viruses usually bind to alpha-2′3-linked sialic acid, while human viruses predominantly bind to alpha-2′6-linked sialosides [13]. NA is an enzyme cleaving sialic acids.

This cleavage facilitates virus release from the cell after infection by preventing aggregation of virions [14,15] and virus release from mucins rich in sialic acid [16,17]. The upper respiratory tract (URT) is the primary infection site in humans. The virus infects cells predominantly expressing alpha-2,6-linked sialic acids [18], while URT mucin in humans is rich in sialosides of avian type (alpha-2,3). The balance is observed between receptor-binding ability of HA and receptor cleavage activity of NA. In order to avoid inhibition by mucins, human influenza virus must have HA with low avidity to alpha-2′6-linked sialic acid and NA with high enzymatic activity against alpha-2′3-linked sialic acid. Human influenza virus must possess high ability to bind alpha-2′6-linked saccharides for effective attachment and penetration into the target cell, while effective cleavage of 2′6-linked saccharides by NA is required for virus release and prevention of virus aggregation after release from the cell. Thus, it is reasonable to assume that compatible levels of HA and NA activity are required for effective virus replication and transmission. Such studies are of strong interest for assessment of pandemic potential of influenza viruses. The role of balance of the activities of these two proteins in receptor binding was first assessed by using a new biophysical approach based on bio-layer interferometry in the real-time mode [19].

Among animal models for studying influenza virus, mice are usually unable to transmit infection from animal to animal [20–22], which makes the system unsuitable for studying virus transmissibility. Ferrets present a well-established model to study both influenza virus transmission and pathogenesis. They are susceptible to non-adapted swine and human influenza viruses and provide contact-dependent transmission from infected ferrets to an uninfected animal [23]. At the same time, highly pathogenic avian influenza viruses are non-transmissible between ferrets [24]. However, the ferret model has several practical disadvantages: they are expensive, relatively large, and thus require complex housing conditions. In addition, it is difficult to find ferrets that have not been previously infected with influenza. In this regard, guinea pig models of virus infection were developed, which are easy to handle and less expensive [25]. A more affordable and less demanding guinea pig model allows one to conduct more experiments and, thus, increases the statistical power of transmission experiments. Guinea pigs are easily infected by human and avian influenza viruses without adaptation. However, they either lack or exhibit extremely mild clinical

symptoms of influenza infection compared to ferrets and humans. Although influenza viruses can replicate at high titers in the respiratory tract of guinea pigs, they usually do not exhibit severe disease symptoms even when infected with strains pathogenic to humans and ferrets [26].

The presence of pronounced clinical disease signs, the specificity of human influenza virus infection alongside the possibility of non-contact influenza virus transmission make ferrets the most suitable model to study virus transmissibility. Natural infection has an obvious advantage, since it provides a realistic infectious dose and results in virus replication kinetics that better imitate the kinetics of natural influenza virus infection in humans [27–30].

A person infected with the influenza virus contaminates the environment. This infectious material may be transmitted between people in many different ways, and the importance of different routes of infection is not yet known. The virus is shed by the infected host during events such as coughing, sneezing and talking. An "expiratory spray" is formed from particles of different sizes in which the virus is present. The virus is introduced to a new host by inhalation or contact. Contact transmission for the influenza virus is not considered dominant. Droplet transmission is often considered significant because the droplets have a high infectious potential and carry a high infectious dose; however, the droplets cannot reach the target cells in URT by inhalation and the role of the droplets is limited [31] Inhaled airborne infectious particles ($\leq$5 μm) can be shed by patients during coughing. The pandemic and epidemic influenza viruses that have circulated in humans throughout the past century were transmitted mostly via the airborne route. Of all the pathways, perhaps the most interesting is the airborne transmission of influenza; evidence to support the importance of this overall contribution is increasing but is still inconclusive [28,32]. Droplet size distribution in human coughing has shown droplet size to be coughed up between 0.6–15.9 μm and an average modal size of 8.35 μm, although the cough droplet size distribution is multimodal and has three peaks at about 1 μm, 2 μm and 8 μm [33].

In infected ferrets, seasonal A/H1N1 and A/H3N2 viruses and mammal-adapted avian A/H5N1 virus are transmitted via the air in the URT, more specifically, from the nasal respiratory epithelium, and not from the trachea, bronchus or lungs [34]. Viruses are expelled from URT via breathing, sneezing, coughing and vocal activity. In a ferret model, influenza virus transmission between animals was mediated by airborne particles larger than 1.5 μm, consistent with the quantity and size of virus-laden particles released by the donors [35]. Onward transmission by donors was most efficient before fever onset and may continue for 5 days after inoculation. Interestingly, that limited amount of virus-laden particles at submicron size were exhaled by influenza-inoculated donor ferrets, nevertheless, the researchers demonstrated that droplets smaller than 1.5 μm, artificially generated by nebulizer, efficiently infect the recipient ferrets with influenza virus.

Despite numerous studies of the influenza virus on the ferret model [27–30,36–39] there remains a knowledge gap in understanding the mechanism of transmissibility of influenza viruses and difficulties in assessing the epidemiological danger of the influenza virus based on data obtained in the ferret model. For an in-depth study of the transmissibility of the influenza virus, we have developed an aerobiological chamber. In this study on an aerobiological chamber, we have made a comparative assessment of the airborne transmission of six influenza viruses of human and animal origin in a ferret model.

2. Materials and Methods

2.1. Viruses

The pandemic influenza virus A/California/07/2009 (H1N1)pdm09 was provided by the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, USA). Avian influenza viruses A/chicken/Primorsky Krai/1771/2018 (H9N2), A/chicken/Kostroma/1718/2017 (H5N2), A/chicken/Nghe An/08VTC/2018 (H5N1), and A/chicken/Thanh Hoa/V1S5VTC /2020 (H9N2), as well as swine influenza virus A/swine/Irkutsk/155/2017 (H3N2), were

obtained from the collection of microorganisms of the State Research Center of Virology and Biotechnology "Vector". Influenza viruses were propagated in 9-day-old chicken embryos.

2.2. Animals, Animal Procedures, and Intranasal ID_{50}

Female ferrets aged 6–8 months were obtained from the breeding facilities of laboratory animals of the State Research Center of Virology and Biotechnology "Vector". Animals were kept in individually ventilated cages on a standard diet. Animal housing and all procedures and manipulations with animals were performed in accordance with the request of the State Research Center of Virology and Biotechnology "Vector" on 3 June 2022, protocol No. 2 (28 June 2022) of the Bioethics Commission of the State Research Center of Virology and Biotechnology "Vector". Serological testing was conducted to confirm the absence of virus-specific antibodies in animals before the start of the study and verify virus transmission from infected to healthy animals during the experiment. Donor ferrets were infected intranasally under anesthesia with a combination of Zoletil 100 (Delpharm Tours, France) and Xyla (Interchemie, Estonia); 0.25 mL of viral suspension was administered into each nostril. Animals were divided into four groups of 3 animals each to measure intranasal ID_{50}. Animals of each group received a serial dilution of an influenza virus strain intranasally. The disease was diagnosed in infected animals based on the presence of viral RNA in the nasal washings 4 and 6 days after infection and seroconversion status 21 days after infection. Probit analysis was used to calculate intranasal ID_{50}.

2.3. Aerobiology Chamber, Analytical Filters, and Particle Counter

To study transmissibility of influenza A viruses, an original ELC 04-60 aerobiology chamber (TIEGEL GmbH, Radeberg, Germany) was used (Figure 1). The chamber is designed for experiments in small and medium laboratory animals. The system consists of a technical air preparation module and four animal cabinet-chambers for animal exposure. The chamber is climate-controlled with the temperature range of 4–20 °C and relative humidity of 20–60%. A relative humidity of 30% and air temperature of 20 °C with decreased air pressure (−200 Pa) were maintained in the cabinet-chamber during experiments on influenza virus transmissibility in the ferret model. Aerosol samples from each cabinet-chamber were obtained through two sampling outlets to AFA-BA-3 analytical aerosol filters. Samples from the third air duct entered the aerosol particle counter (TSI AeroTrak APC 9303-01, Shoreview, Minnesota, USA), which operated in the following mode: 5 min/5 min during a 5 h animal exposure.

In order to assess the transmissibility of influenza A viruses in ferrets, animals were divided into two groups: donor group of 12 animals and recipient group of 12 animals. Donor ferrets were divided into four subgroups of 3 animals each a day before the experiment, and each subgroup was infected intranasally with an influenza virus strain at a dose of 1–10^6 FFU. Three animals of each subgroup of donor ferrets were placed in one of the four ELC 04-60 TIEGEL cabinet-chambers a day after infection. After this, a corresponding group of three naïve recipient ferrets was placed in a different cage of each cabinet-chamber. The distance between the cages was 10 cm, which excluded the direct contact between infected animals and recipients. The airflow was directed from infected donor ferrets to recipient ferrets; the flow speed was 3 cm/s. Simultaneous exposure of animals lasted 5 h daily for six days. During the exposure, air samples were captured onto particle counters and AFA-BA-3 analytical aerosol filters. Virus disease in animals was detected based on the presence of viral RNA in the nasal washings within 14 days after intranasal infection or airborne contact and seroconversion status 21 days after intranasal infection or airborne contact. The experiments were conducted one time.

Figure 1. TIEGEL ELC 04-60 dynamic aerobiology chamber.

The airborne infectious dose (AID) inhaled by the recipient ferret was calculated from viral load on two analytical aerosol filters of the chamber, expressed in FFU-equivalent, and minute respiratory volume in ferrets as:

$$AID = \frac{vF}{A} \times MV \times t, \quad (1)$$

where vF—average viral load on analytical aerosol filter, FFU-equivalent; A—volume of air passing through the analytical aerosol filter during exposure, L.; MV—ferret minute volume, L/min; t—time of exposure, min. Calculation ferret minute volume MV was conducted in accordance with [40].

2.4. Determination of Viral Load in Biological Samples in MDCK Cells Using FFU Assay

Tenfold dilutions of nasal washingss from laboratory animals were prepared and added to MDCK cell monolayer in 96-well plates. Growth medium was removed from flat-bottom plate wells with cell monolayer, cells were washed twice with supportive growth medium (DMEM, 100 μ/mL penicillin, 25 mM HEPES, 0.2% solution of bovine serum albumin, 2 μg/mL of TPCK). Next, dilutions of test samples were added, and cells were incubated for 1 h at 37 °C and 5% CO_2. The medium was then removed from wells, and cells were washed with supportive medium once. A total of 150 μL of supportive medium was added to all wells. The medium was removed after 18–20-h incubation at 37 °C and 5% CO_2, and 200 μL of 80% cold acetone (−20 °C) was added, followed by incubation for 20–30 min. Acetone was removed, and the wells were dried. Next, 50 μL of diluted 1:1000 mouse monoclonal antibodies to influenza virus nucleoprotein was added to each well. The plate was incubated at 37 °C for 1 h, wells were washed three times with PBS, and secondary rabbit anti-mouse IgG antibodies conjugated to horseradish peroxidase

(Abcam) were added at a ratio of 1:1000. After 1 h incubation, wells were washed three times with PBS, and AEC substrate (3-amino-9-ethylcarbazole, Sigma) solution was added. After 30 min incubation, the solution was removed, and the plate was washed with PBS once. Infected red-brown cells were counted, and the virus titer was calculated as the number of focus-forming units (FFU) per 1 mL of nasal washings (FFU/mL). The analysis was performed in duplicate wells.

2.5. Determination of Virus RNA Load in Biological Samples by RT-PCR

Influenza virus RNA was quantified in nasal washingss and analytical filters by quantitative RT-PCR with real-time data acquisition. The RIBO-sorb kit (InterLabService Ltd., Moscow, Russia) was used for RNA isolation. Reverse transcription was carried out using the Reverta-L kit (InterLabService Ltd., Moscow, Russia) in an incubator. The resulting influenza A cDNA fragments were amplified using the AmpliSens Influenza virus A/B-FL kit (InterLabService Ltd., Moscow, Russia). PCR and data registration were conducted on a RotorGene 6000 real-time cycler. The calibration curve of viral RNA Ct values on the concentration of influenza virus in FFU/mL was plotted to calculate the concentration of influenza virus strains in FFU-equivalents/mL. The PCR assay was performed without repeats and was considered correct when all control samples of a given test system were released.

2.6. Statistical Analysis

Statistical data processing was performed using Origin 8.1 data analysis package and Microsoft Excel. The analysis was carried out with log-transformed data due to the insignificance in this study of linear scale errors in exponential processes which, in our case, is virus reproduction.

Linear regression was used to test the relationships of measured viral particle concentrations in nasal washingss to the dose of inoculation due to the ability to test for the trend. Similarly, regression analysis was used to evaluate the calculated intranasal ID_{50} and the calculated dose–response function. Trends with R^2 values above 0.5 were considered satisfactory.

3. Results

3.1. Virus Yield in the URT Cells of Donor Ferrets

Figure 2 shows virus concentration in the nasal washings of ferrets after intranasal infection with different doses of six influenza virus strains. Pandemic influenza virus A/California/07/2009 (H1N1)pdm09 is first detected in the nasal washings of ferrets upon infection with a minimal dose of 5 FFU. Virus concentration in the nasal washings reaches the maximum value of 2.3×10^6 FFU/mL at the infectious dose of 5×10^2 FFU, while further increase in infection dose does not result in the growth in virus concentration on the mucous membrane of the URT in ferrets (Figure 2A). Avian influenza viruses A(H5N2) and A(H5N1) at low doses of intranasal infection induce virus accumulation in the nasal washings at minimal concentrations ($<4.5 \times 10^3$ FFU/mL), while virus concentration in the nasal washings does not exceed 2.9×10^4 FFU/mL for both strains at infectious dose of >1000 FFU (Figure 2B,D).

The opposite dependence of the virus concentration on the mucus membrane of the URT in ferrets on infectious dose is noted for avian influenza viruses A(H9N2) and swine influenza virus A(H3N2). Virus concentration in the nasal washings decreases with an increase in the dose of intranasal infection. Virus with a maximal concentration of $3.3 \times 10^4 - 3.9 \times 10^5$ FFU/mL is detected in the nasal washings upon infection with avian influenza A(H9N2) viruses at infectious doses of $<10^2$ FFU. Virus concentration in the washings decreases to 1.4×10^4 FFU/mL upon an increase in intranasal infection dose to 10^6 FFU (Figure 2C,E). For swine influenza A(H3N2) virus, the maximum virus concentration in the nasal washings is 1.6×10^4 FFU/mL for infectious dose of 10^2 FFU; it further decreases to 1.1×10^3 FFU/mL with an increase in infectious dose to 10^5 FFU (Figure 2F).

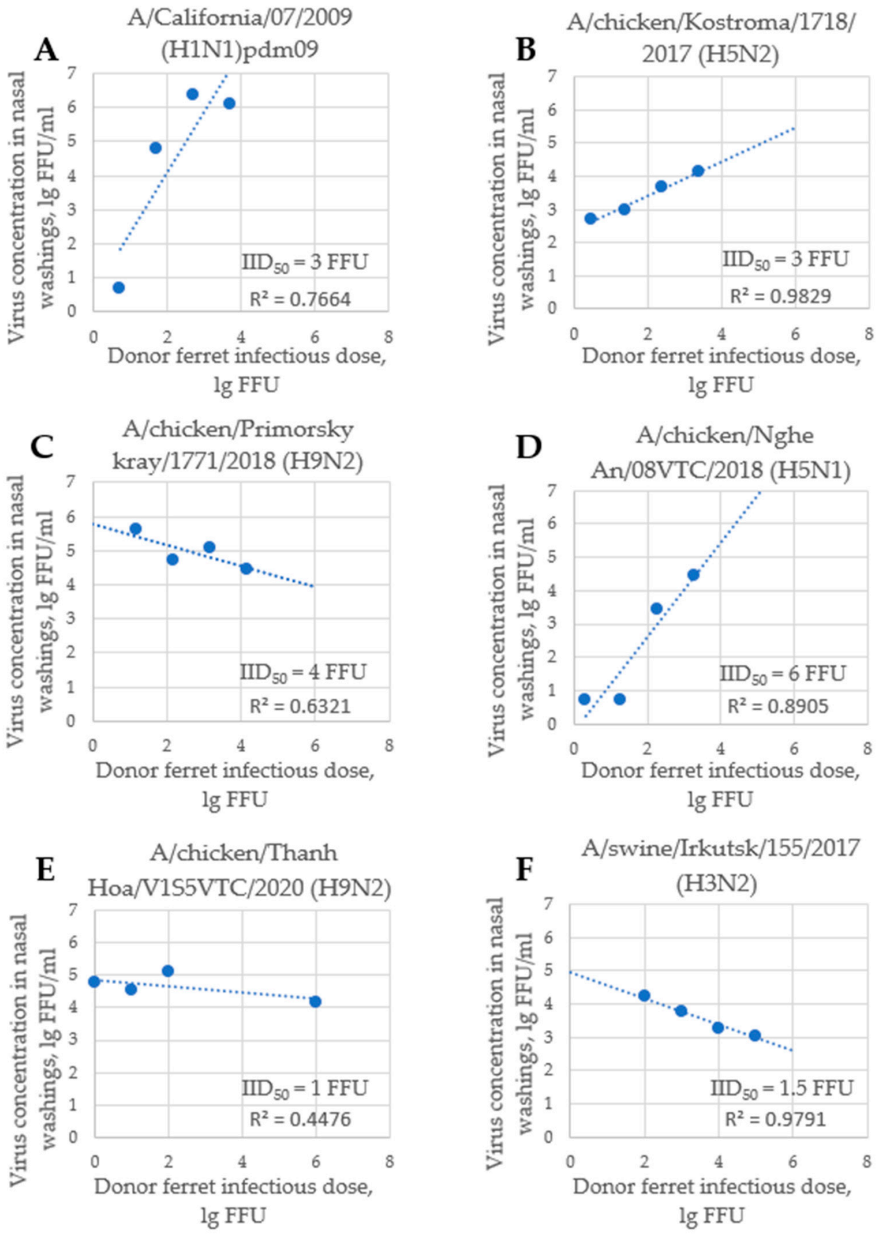

Figure 2. Effect of intranasal infectious dose (IID) on virus load in the nasal washings of donor ferrets, infected with different doses of influenza virus strains: (**A**) A/California/07/2009 (H1N1)pdm09, (**B**) A/chicken/Kostroma/1718/2017 (H5N2), (**C**) A/chicken/Primorsky Krai/1771/2018 (H9N2), (**D**) A/chicken/Nghe An/08VTC/2018 (H5N1), (**E**) A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2), (**F**) A/swine/Irkutsk/155/2017 (H3N2). The X axis—IID in donor ferrets. The Y-axis—virus titer in nasal washings of donor ferrets.

The obtained results indicate that a decrease in virus concentration on the mucus membrane of the URT is observed with an increase in intranasal infectious dose (IID) for several strains. Such a decrease in virus yield can be explained by induction of non-specific antiviral defense mechanism, for instance, via the interferon pathway. Two avian influenza viruses A/chicken/Nghe An/08VTC/2018 (H5N1) and A/chicken/Kostroma/1718/2017 (H5N2), despite the pronounced specificity to avian alpha-2′3 receptors, demonstrated the ability to infect ferrets at high infectious doses. This is apparently due to the presence of low amounts of receptors to avian influenza on the cell surface of the mucous membrane of the URT in ferrets. On the contrary, absolute predominance of human receptors type alpha-2′6 on the mucous membrane of the URT in ferrets provides high

virus yields on the mucus membrane upon infection with low doses of A/California/07/2009 (H1N1)pdm09, A/chicken/Primorsky Krai/1771/2018 (H9N2), and A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2) in ferrets. At the same time, the pandemic virus A/California/07/2009 (H1N1)pdm09 is accumulated in the URT at concentrations 10 times higher than those for A(H9N2) viruses and 100 times higher than those for A(H3N2), A(H5N2), and A(H5N1).

3.2. Genomic Characterization of Influenza Virus Strains Used in the Study

Complete genomes of 6 influenza viruses A/California/07/2009 (H1N1)pdm09, A/chicken/Primorsky Krai/1771/2018 (H9N2) (Y280 genetic line), A/chicken/Kostroma/1718/2017 (H5N2) (clade 2.3.4.4b) [41], A/chicken/Nghe An/08VTC/2018 (H5N1) (clade 2.3.2.1c), A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2) (genetic line Y280) and A/swine/Irkutsk/155/2017 (H3N2) were obtained using NGS for genetic analysis. Genetic analysis of the obtained genome sequences revealed the presence of some molecular markers of virulence and transmissibility in the HA and NS genes of the H5N1, H5N2, and H9N2 viruses. The 158D (H3 numbering) mutation in the HA gene results in the loss of the N-glycosylation site, which is often associated with a change in receptor specificity. The 145G mutation increases binding to alpha 2,6 receptors. The polybasic site in the HA gene greatly increases pathogenicity. Mutations in the NS1 205N and NS2 47A genes lead to a decrease in the antiviral response in mammals. Deletion 80-84 AA in the same NS1 gene increases virulence, and the PDZ binding domain of the NS1 gene ESEV suppresses the antiviral response (H5N1 genetic changes inventory) (Table 1).

3.3. Airborne Infection of Recipient Ferrets

AFA-BA-3 analytical aerosol filters were used to assess biological contamination of air in the aerobiological chamber. Air samples were collected onto analytical filters during the entire period of cohabitation exposure of donor and recipient ferrets. During this time, the virus trapped on the filter was inactivated, so the average virus concentration in the air of the aerobiology chamber was calculated from the viral RNA amount in the analytical filter, which was estimated by quantitative RT-PCR, and expressed in FFU-equivalents in accordance with the calibration curve. We also measured the concentration of aerosol particles in the chamber for three fractions using the particle counter: 0.3–1 μm, 1–5 μm, and >5 μm.

Ferrets create airborne pollution in the chamber. The maximum number of 16–63 $\times$ 10^3 aerosol particles of 0.3–1 μm per air liter was registered (Table 2). Influenza virus transmissibility in ferrets is known to be provided by aerosol particles of >1.5 μm [35]. The number of particles of the size 1.0–5.0 μm in the aerobiology chamber is an order of magnitude lower, their concentration is in the range of 1.7–6.4 $\times$ 10^3 particles per air liter (Table 2). Apparently, virus particles with a concentration in the air reaching the maximum value on day 4 after infection (0.012 FFU-equivalent/L) and then decreasing to 0.003 FFU/mL by day 6 after infection are distributed in this fraction. On day 4 after infection, there is an average of one viral particle (FFU-equivalent) per approximately 3.47 $\times$ 10^5 aerosol particles of >1 μm, the size corresponding to influenza virus transmissibility in ferrets. On day 6 after infection, the viral load in airborne particles decreases almost seven-fold, and one FFU-equivalent per 2.16 $\times$ 10^6 influenza transmissible airborne particles is observed.

During the period of cohabitation exposure in aerobiology chamber, recipient ferrets receive an airborne infectious dose (AID) of influenza virus from the air contaminated by donor ferrets. As shown in Figure 2, different virus concentrations were detected in the URT washings of donor ferrets infected with different IID that induced the different concentrations of airborne virus in the chamber.

Table 1. Molecular markers of virulence and transmissibility in the HA and NS genes of the studied H5N1, H5N2, and H9N2 viruses.

Strain	Experiment Data: Increasing Virus Concentration with Increasing Ferret Infection Dose	HA	NS1 PDZbm Suppresses the Immune Response Prevents Early Apoptosis	NS1 Del 80-84 AA Increases Virulence	Polybasic Cleavage Site in HA, Greatly Increases Pathogenicity	Adaptation to Humans and Mammals (According to Epidemiological Data)	NS1 N200S (N205S) Together with T47A (T48A) in NS2 Are Associated with Decreased Antiviral Response in Mammals
A/chicken/Nghe An/08VTC/2018 (H5N1)	Yes (increase)	158D	Yes (ESEV)	Yes	Yes	No	NS1 S205N, NS2 A47T Do not reduce antiviral response
A/chicken/Kostroma/1718/2017 (H5N2)	Yes (increase)	158N	Yes (ESEV)	No	Yes	No	NS1 N205I, NS2 T47S Do not reduce antiviral response
A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2)	No (no change)	145G	No	No	No	No	NS1 N205S, NS2 T47A, Decreased antiviral response
A/chicken/Primorsky Krai/1771/2018 (H9N2)	No (significant dependency is not defined)	145N	No	No	No	No	NS1 N205S, NS2 T47A Decreased antiviral response
A/swine/Irkutsk/155/2017(H3N2)	No (decrease)		No	No	No	Adaptation to pigs	NS1 205N, NS2 A47T Do not reduce antiviral response
A/California/07/2009 (H1N1)pdm09	Yes (increase)		No	No	No	Strong adaptation to human	NS1 205N, NS2 A47T Do not reduce antiviral response

Table 2. Fractional composition of aerosol particles and airborne viral load (FFU-equivalent) in the air of aerobiology chamber at various time periods after intranasal infection of donor ferrets with 10 and 10^6 FFU of avian influenza A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2) virus.

Strain	Dose, FFU	Aerosol Particle Concentration Units	Concentration of Aerosol Particles									Concentration of Virus Particles, FFU-Equivalent/L		
			0.3–1.0 μm			1.0–5.0 μm			>5.0 μm					
			Day after Infection			Day after Infection			Day after Infection			Day after Infection		
			2	4	6	2	4	6	2	4	6	2	4	6
A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2)	10	Pcs/L	53,003 ± 13,512	38,260 ± 16,211	63,787 ± 32,449	5237 ± 1454	4058 ± 1842	6437 ± 3639	218 ± 84	111 ± 39	62 ± 25	ND*	0.0120	0.0030
		%	90.7 ± 23.1	90.2 ± 38.2	90.8 ± 46.2	9 ± 2.5	9.6 ± 4.3	9.2 ± 5.2	0.37 ± 0.14	0.26 ± 0.09	0.09 ± 0.03			
	10^6	Pcs/L	23,315 ± 8139	20,598 ± 10,231	16,396 ± 9955	2172 ± 834	1711 ± 903	1306 ± 900	64 ± 31	27 ± 12	26 ± 12	0.0009	0.0011	0.0010
		%	91.2 ± 31.9	92.2 ± 45.8	92.5 ± 56.1	8.5 ± 3.3	7.7 ± 4	7.4 ± 5.1	0.25 ± 0.12	0.12 ± 0.05	0.15 ± 0.07			
Unchallenged ferrets (control)	0	Pcs/L	33,761 ± 5137			3288 ± 580			644 ± 210			ND*	ND*	ND*
		%	89.6 ± 13.6			8.7 ± 1.5			1.7 ± 0.6					

ND*—not detected, below detection limit (<0.00017 FFU-equivalent/L).

Figure 3 presents the dependence of calculated dose inhaled by recipient ferrets on IID in donor ferrets.

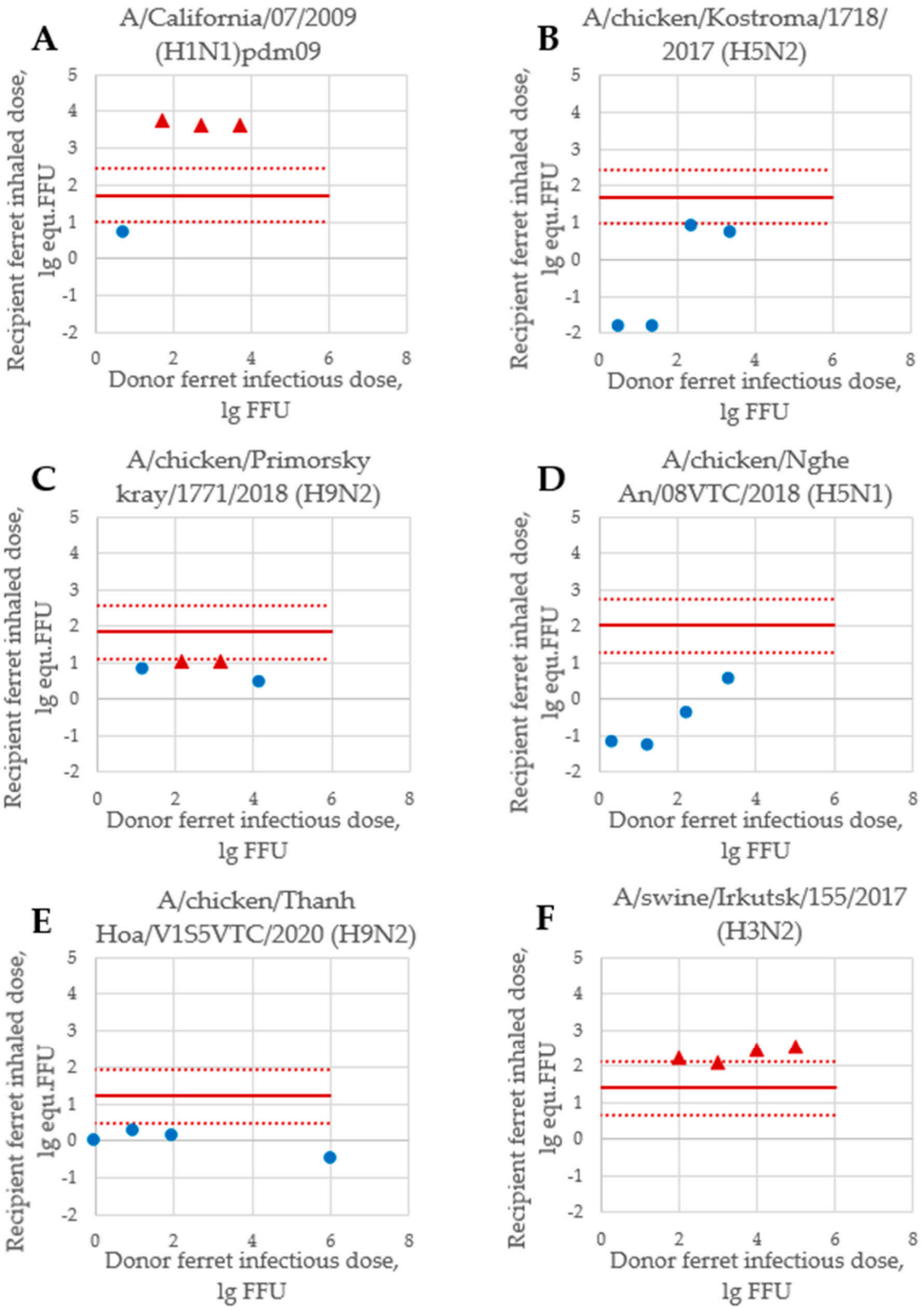

Figure 3. Effect of IID in donor ferrets on estimated AID inhaled by recipients, for donor ferrets infected with influenza virus strains: (**A**) A/California/07/2009 (H1N1)pdm09, (**B**) A/chicken/Kostroma/1718/2017 (H5N2), (**C**) A/chicken/Primorsky Krai/1771/2018 (H9N2), (**D**) A/chicken/Nghe An/08VTC/2018 (H5N1), (**E**) A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2), (**F**) A/swine/Irkutsk/155/2017 (H3N2). The X axis—IID in donor ferrets expressed in lg FFU. The Y axis—AID inhaled by recipient ferrets calculated as lg FFU-equivalents based on RT-PCR of the nasal washings from the analytical aerosol filter. Red line indicates AID_{50} for each strain. Dotted red lines indicate 95% confidence interval. Red triangle indicates AID when recipient ferrets have become infected. Blue solid dots indicate AID when recipient ferrets have not become infected.

Figure 3 shows that recipient ferrets received AID of 3.4×10^2–5.5×10^3 FFU-equivalents of A/swine/Irkutsk/155/2017 (H3N2) and A/California/07/2009 (H1N1)pdm09 influenza viruses, which is significantly higher than AID_{50} for this strains (25–52 FFU-equ). Recipient ferrets inhaled about 10 FFU-equivalents of A/chicken/Primorsky Krai/1771/2018 (H9N2) influenza virus, which is close to lower value of AID_{50} confidence interval at 95% for this strain (13 FFU).

Other influenza virus strains induced chamber contamination to a much lesser extent: recipient ferrets inhaled no more than 8 FFU-equivalents during cohabitation with donor ferrets.

Figure 4 presents the dependence of the probability of infection in recipient ferrets kept in the downward airflow to infectious donor ferrets in the aerobiology chamber on the influenza virus dose inhaled by recipient ferrets from the air contaminated by donor ferrets. The inhaled dose is presented as IID_{50}, which makes it possible to combine the results for all studied strains in one graph.

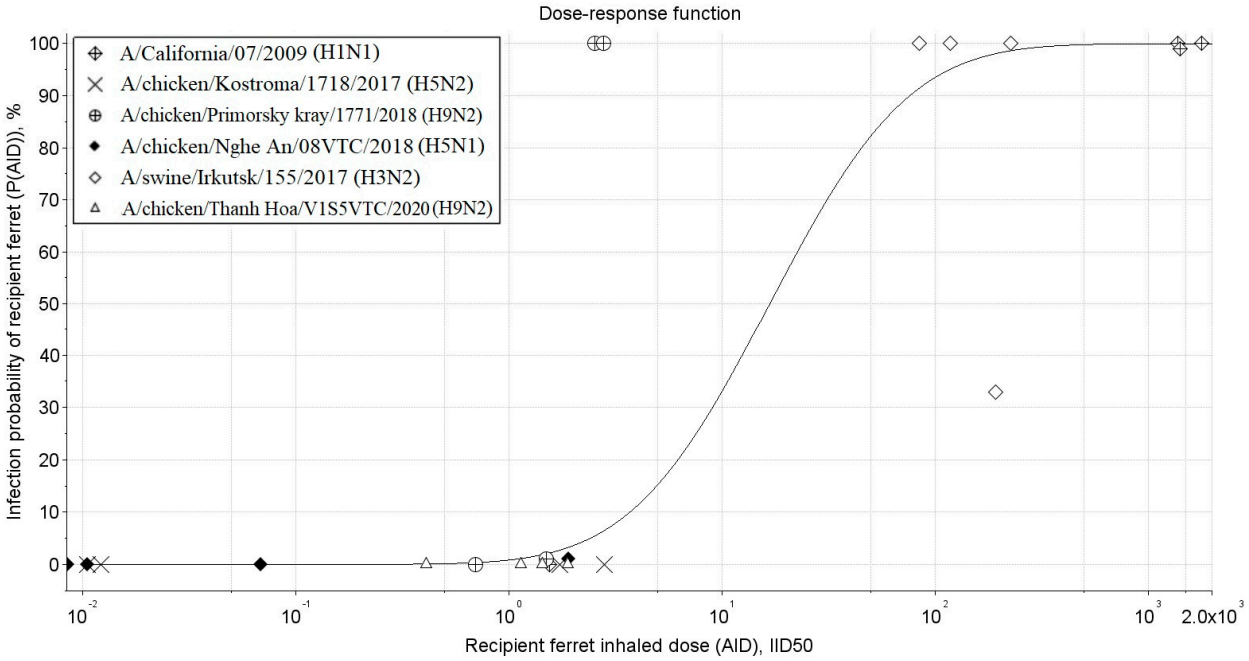

Figure 4. Effect of AID received from the air contaminated by donor ferrets on the probability of infection P(AID) in recipient ferrets. The X axis—AID inhaled by recipient ferrets expressed in IID_{50} units. The Y axis—proportion of infected recipient ferrets expressed as %. Experimental points for different influenza virus strains were approximated using a sigmoid function with the following parameters: AID_{50} = 16.75 IID_{50} and σ = 1.18. Curve parameters were calculated based on the experimental points using Origin 8.1 data analysis package.

The dose–response curve was reconstructed using Origin 8.1 software package by approximating the experimental data using the Levenberg–Marquardt algorithm and the function:

$$P(\mathrm{AID}) = 1 - \prod_{n=1}^{t}\left(1 - \frac{1}{2}\left(1 + erf\left(\frac{log(\mathrm{AID}_n) - \log(\mathrm{AID}_{50})}{\sqrt{2\sigma^2}}\right)\right)\right), \quad (2)$$

where «t» is the time from the start of donors' impact on recipients, with AID_{50} and σ as parameters. The values of inhaled AID were normalized by the value of IID_{50}, which were measured for each of six strains in intranasally infected ferrets.

The obtained correlation is characterized by the following parameters: AID_{50} = 16.75 IID_{50} (3.14–89.33, p = 0.95) and σ = 1.18 $\pm$ 0.43. Thus, we managed to evaluate the dose providing infection in 50% recipient ferrets by airborne transmission from donor ferrets. This dose means that more than 50% of recipient ferrets are infected after inhaling >16.75 IID_{50}.

Thus, transmissibility of influenza virus in the ferret model is highly probable in the case when donor ferrets can maintain a significant virus level in the surrounding air, which provides an inhaled dose of influenza virus sufficient enough to infect recipient ferrets.

4. Discussion

We proposed a ferret model in the aerobiology chamber study and quantified transmission of human and animal influenza viruses using this model to study influenza virus transmissibility. Ferrets are a well-established model for both transmission and pathogenesis of influenza viruses because their clinical symptoms are similar to those of humans [23,24,27,28,42–47].

Despite numerous studies on influenza virus transmissibility in the ferret model, there are no clearly established conditions determining the level of viral load in the environment that is required for donor ferrets to provide virus transmission to recipient ferrets. Animal experiments provide reasonable estimates for human susceptibility in dose–response studies [48]. A dose–response function connects a number of pathogens with a probability of infection. Various mathematical dose–response models are used to describe the probability of infection of the body [49]. So, from the hypothesis of independent action for a homogeneous population, a one-parameter exponential dose-effect relationship P(ID) = 1–exp(–pID), is valid [50]. Here, P(ID) is the probability of infecting an organism when a certain number of virions enters it, having a Poisson distribution with an average value equal to ID, the parameter p is the probability of infecting the host with one virion. The probability of infecting the host with a single virion, p, is related to the 50% infectious dose ID_{50} of the virus to the host by a simple ratio $p = \ln 2/ID_{50}$, where ID_{50} is expressed as the number of virions. The exponential one-parameter dose–response model is useful for microbiology, but researchers widely use the popular two-parameter Poisson beta function model $P(ID) = 1 - [1 + (ID/ID_{50})(2^{1/\alpha} - 1)]^{-\alpha}$ with parameters ID_{50} and α [51]. The two-parameter Poisson beta function is the best known of the algebraic dose–response models with sigmoid curves. It fits well with another two-parameter lognormal distribution model $P(ID) = 1/2[1 + \mathrm{erf}(\lg(ID/ID_{50})/2^{1/2}\sigma_{lg})]$ with parameters ID_{50} and σ_{lg}, where ID_{50} is an infecting dose corresponding to a 50% response, σ_{lg} is a standard deviation for a normally distributed logarithm of ID, "erf" is an error function. The lognormal distribution for representing dose–response data is indistinguishable from the Poisson beta function for the most practical purposes. Mathematically, it can be shown that if the probability of infection (PN) is the product of the set of underlying probabilities of several events (Pis), i.e., $P(N) = \prod P(i)$, and if each of these underlying P(i) is uniformly distributed within its defined range, then the resulting probability of infection (PN) has a lognormal distribution [52,53]. The parameters of the lognormal distribution model are intuitive clear for researchers and can be measured experimentally. For this reason, we have chosen a lognormal distribution model to describe our experimental results in ferrets.

Our study demonstrates the relationship between influenza airborne and intranasal ID_{50} in the ferret model which, in turn, substantially determines airborne transmissibility.

Influenza virus transmission between ferrets is highly probable when donor ferrets can maintain the virus level in the environment that provides the inhaled dose of influenza virus of >16.75 IID_{50} in recipient ferrets for the studied influenza virus strains. Lower level of the virus in the environment, even a single virus, can also induce airborne transmission, but at very small probability.

Airborne particle contamination is provided by donor ferrets due to airborne excretion of influenza virus from the infected animals. The significant difference between IID_{50} and AID_{50} value may be explained by the different route of the virus entering into the nasal cavity. The IID_{50} value was measured for intranasally infected ferrets, whereas AID_{50} is obtained in ferrets infected via airborne route. In this case, only part of the inhaled AID may be sedimented in nasal mucosa, another part of the inhaled virus is eliminated during exhalation, and the sedimented virus dose in the nose is smaller than the inhaled virus dose [54].

The pattern of influenza virus accumulation on the mucous membrane of the URT in ferrets is unique for each specific strain. A comparative study of intranasal infection in ferrets with six influenza A virus strains showed that three strains demonstrate a decrease in viral concentration on the mucous membrane of the URT with an increase in the dose of intranasal infection. The latter three strains increase the virus yield on the mucosa of the URT with an increasing infectious dose. These three viruses include 2009 pandemic influenza strain A/California/07/2009 (H1N1)pdm09 and avian influenza A(H5) strains A/chicken/Kostroma/1718/2017 (H5N2) and A/chicken/Nghe An/08VTC/2018 (H5N1). The remaining three strains, which caused avian influenza virus outbreaks in poultry farms, namely A/chicken/Primorsky Krai/1771/2018 (H9N2) and A/chicken/Thanh Hoa/V1S5VTC/2020 (H9N2), as well as swine influenza A/swine/Irkutsk/155/2017 (H3N2) virus, circulating in a pig farm in the Irkutsk region, demonstrated either weak or strong negative correlation between virus concentration on the mucous membrane of the URT in ferrets and an intranasal infectious dose with an increase in intranasal infectious dose. Non-specific antiviral response was hypothesized to explain a decrease in viral replication upon an increase in infectious dose for the three studied strains of subtypes (H9N2) and A(H3N2). The opposite pattern for other strains, namely an increase in viral yield with an increase in infectious dose, primarily for A(H5) strains, is apparently due to inhibition of the host antiviral response to infection.

This study revealed significant differences in the reproduction of influenza viruses on the mucosa of the URT of ferrets for different strains. Genetic analysis revealed amino acid substitutions, which were correlated with the characteristics of a nonspecific antiviral reaction of the organism. The two studied strains A/chicken/Kostroma/1718/2017 (H5N2) and A/chicken/Nghe An/08VTC/2018 (H5N1) with a positive correlation between intranasal infectious dose and virus yield on the mucous membrane of the URT differ in the presence of the C-terminal amino acid region ESEV in the PDZ-binding motif of NS1, which prevents early apoptosis and inhibits expression of interferon-stimulated genes thus providing a high level of virus replication [55,56]. Furthermore, hemagglutinin in these two viruses differs in the presence of the polybasic site R-X-R/K-R-R-K-R-G at 323–330. This region is susceptible to proteolytic cleavage, found in many A(H5N1) viruses, and associated with a pronounced increase in pathogenicity [57,58].

Two influenza A(H9N2) virus strains do not possess the above genetic markers associated with a high level of viral replication. However, proteins NS1 and NS2 contain two related substitutions (N200S in NS1 and T47A in NS2), which provide decreased antiviral response in the host [36]. This is manifested in a weak correlation between virus accumulation in the URT in ferrets and low negative dependence on the infectious dose.

Swine influenza virus A/swine/Irkutsk/155/2017 (H3N2) is the only strain lacking genetic markers of host's antiviral response inhibition and showing the maximum negative correlation between viral yield and infectious dose.

In this study, the ability to transmit H1N1pdm09, H3N2 viruses isolated from pigs, H5N1, H5N2 and 2 H9N2 viruses isolated from birds was studied in ferrets. Airborne transmissibility in ferrets has been shown for seasonal influenza viruses H1N1pdm09 A/California/07/2009 and H3N2 A/swine/Irkutsk/155/2017. Ferrets are animal models for human influenza infection and have been shown to be transmissible with seasonal influenza viruses [37]. The H3N2 A/swine/Irkutsk/155/2017 virus is reassortant with the HA, NA genes from the H3N2 seasonal influenza virus and internal genes from the seasonal

influenza virus of another H1N1pdm09 subtype. Reassortant H9N2 viruses with internal genes from seasonal influenza viruses have previously been shown to be able to replicate and be transmitted in ferrets [59]. Since the introduction of A/goose/Guangdong/1/96 (H5N1) in China in 1996, influenza viruses of this subtype have spread throughout the world. As a result of reassortment with low pathogenic influenza viruses, there has been a further increase in genetic diversity and the emergence of viruses of different subtypes, which can be designated as H5Nx [60]. It has been established that most studied H5Nx viruses of clade 2.3.2.1. and 2.3.4.4. are not transmitted between mammals [61]. However, some of them have a limited ability to be transmissible by contact in ferrets and other animal models [62]. As a result of the work, it was found that the studied viruses A/chicken/Nghe An/08VTC/2018 (H5N1) and A/chicken/Kostroma/1718/2017 (H5N2) are not transmitted between ferrets by airborne particles. Limited available information about genetic markers of transmissibility often does not allow predicting or evaluating transmissibility and, in some cases, human and avian H5Nx viruses with the same known markers may differ in transmissibility [61]. The main markers of transmissibility are markers that determine the receptor specificity of the virus, the stability of the HA protein when the virus exits the endosome into the cell, as well as markers associated with virus replication. Basically, these markers are located in different positions of the receptor-binding site of the HA protein and the PB2 protein [28,63]. The influence of markers in HA at positions 158 (position associated with the glycosylation site), 224, 226, 318 (H3 numbering) on transmissibility was established in a ferret model study of an artificial H5N2 reassortant containing HA from H5N2 and NA and internal genes from the H3N2 seasonal influenza virus [63]. Obtained reassortant virus with HA from avian influenza and internal genes from seasonal influenza virus became transmissible via airborne particles. Another study showed that the G228S mutation in HA of H5Nx viruses, in addition to those listed, affects the transmissibility of the virus in ferrets by direct contact [64]. The profile of the studied viruses A/chicken/Nghe An/08VTC/2018 (H5N1) and A/chicken/Kostroma/1718/2017 (H5N2) differed between them by one amino acid at position 158 of the HA gene and was as follows: HA 110H, 158D/N, 160A, 224N, 226Q, 228G, 318T, PB2 627E, which corresponded to the "avian" type. At the same time, the remaining genes of the studied H5Nx viruses may contain markers that have not yet been identified that affect transmissibility.

The published literature also shows limited direct contact transmission in ferrets for H9N2 subtype viruses [65]. For the A/chicken/Primorsky Krai/1771/2018 virus studied in this work, airborne transmission was shown, which was established by the presence of specific antibodies in the blood on day 21 after exposure. For the A/chicken/Thanh Hoa/V1S5VTC/2020 virus, no ferret-to-ferret transmission has been shown. The lack of isolation of the virus by the recipient ferret may indicate a low adaptability of this virus to mammals. The main markers of transmissibility of H9N2 viruses are markers that determine receptor specificity. Significance of transmissibility markers is strongly dependent on their position in the genome on their genetic environment. Thus, the decisive role of the Q226L [H3 numbering] mutation was previously demonstrated [65], but modern viruses of the H9N2 subtype almost all contain the Q226L mutation. Then, many mutations were found on another viruses, including the key ones at positions 190, 226, and 227, which determine receptor specificity and avidity of binding to receptors [66]. Further monitoring of H9N2 viruses made it possible to establish that mutations T205A, D208E, V216L, V245I in viruses circulating in 2002-2005 and mutations S119R, D145G, Q156R, A160D, T212I, Q227M, R246K in viruses, circulating in 2011-2017, increased affinity for either human-type receptors or both human- and avian-type receptors [67]. According to GISAID EpiFlu, almost all H9N2 viruses isolated from human infections in 2018-2020 have these mutations. At the same time, some of these mutations were absent in avian H9N2 virus genomes. Genetic analysis of the studied influenza viruses A/chicken/Primorsky Krai/1771/2018 and A/chicken/Thanh Hoa/V1S5VTC/2020 showed that almost all of the mutations associated with increased affinity to human type receptors were present in the HA gene, except for markers in positions 119, 145 and 160, which had different amino

acids (119Q, 145N, 160N). It is possible that absence of these markers of host specificity and potential absence of other markers not yet identified make the viruses less adapted for transmission in mammals, in particular, ferrets, compared to bird-to-human transmission. The studied viruses differ from each other by the substitution at position 145N/G in HA, and this difference may be the reason for the observed difference in transmissibility.

Virus replication on the mucous membrane of the URT in ferrets leads to viral contamination of the chamber air. We showed that donor ferrets infected intranasally with the pandemic influenza A/California/07/2009 (H1N1)pdm09 virus create airborne contamination in the aerobiology chamber at the level providing infection in recipient ferrets with the respiratory dose of 4.2×10^3–5.5×10^3 FFU-equivalents, which is 100 times higher than AID_{50} for this strain. Similarly, donor ferrets intranasally infected with swine influenza virus A/swine/Irkutsk/155/2017 (H3N2) create viral contamination inside the aerobiological chamber at such a level that recipient ferrets receive a respiratory dose of 1.3×10^2–3.4×10^2 FFU-equivalents, which is more than 10 times the value of the AID_{50} for this strain. Part of the donor ferrets intranasally infected with avian influenza virus A/chicken/Primorsky Krai/1771/2018 (H9N2) create viral contamination inside the aerobiological chamber at such a level that recipient ferrets receive a respiratory dose about 10 FFU-equivalents, which is close to lower value of AID_{50} 95% confidence interval.

The other studied influenza viruses induced chamber contamination to a significantly lesser extent, and recipient ferrets inhaled <8 FFU-equivalents during cohabitation with donor ferrets.

Despite the fact that virus was detected in the nasal washings of ferrets after intranasal infection with each of the six strains, only three strains demonstrated the ability of donor ferrets to contaminate the chamber environment at the level, when the airborne virus concentration was sufficient to infect recipient ferrets.

5. Conclusions

Our results obtained in the ferret model cannot be directly extrapolated to humans. However, they allow for ranking viruses based on their transmissibility between mammals. The obtained results make it possible to develop an evidence base of phenotypic and genetic markers of transmissibility of new animal influenza viruses to carry out timely anti-epidemic measures by constantly monitoring viruses in the environment in order to preserve the life and health of the human population.

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Abbreviations

AEC	3-amino-9-ethylcarbazole
AID	Airborne Infectious Dose
AID50	50% Airborne Infectious Dose
cDNA	Complementary Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
ELC	EuroLabClime
FFU	Focus-Forming Units
GISAID	Global Initiative on Sharing All Influenza Data
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ID_{50}	50% Infectious Dose
IgG	Immunoglobulin G
IID	Intranasal Infectious Dose
IID_{50}	50% Intranasal Infectious Dose
MDCK	Madin-Darby Canine Kidney
NGS	Next-Generation Sequencing
NS	Nonstructural Protein
PBS	Phosphate-Buffered Saline
RNA	RiboNucleic Acid
RT-PCR	Reverse Transcription, Polymerase Chain Reaction
TIPRA	Tool for Influenza Pandemic Risk Assessment
TPCK	Tosyl Phenylalanyl Chloromethyl Ketone
URT	The Upper Respiratory Tract
WHO	World Health Organization

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