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MICROBIOLOGY

Influenza A virus surveillance in domestic pigs in Kazakhstan 2018-2021

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ABSTRACT: This study described the results of a surveillance program monitoring circulation of influenza A viruses among domestic pigs (*Sus domesticus*) in Kazakhstan during 2018–2021. PCR data derived from 2,513 samples (nasopharyngeal swabs) collected from swine on large pig complexes and peasant farms located in different regions of Kazakhstan revealed that about 5% of samples were positive for influenza A virus RNA. This result suggested low levels of influenza A virus circulation in Kazakhstan. Subtyping of a set of samples revealed that the main strains circulating in 2018–2019 were A/H1N1 and A/H3N2. Surveillance conducted in 2020–2021 identified only A/H1N1 viruses in swine. The PCR data were confirmed by isolation of six strains: five influenza A/H1N1 viruses and one A/H3N2 virus.
Key words: swine influenza, influenza viruses, PCR screening, reassortants.

Vigilância do vírus influenza A em suínos domésticos no Cazaquistão 2018-2021

RESUMO: Este estudo descreve os resultados de um programa de vigilância que monitoriza a circulação do vírus influenza A entre suínos domésticos (*Sus domesticus*) no Cazaquistão durante 2018–2021. Os dados de PCR derivados de 2.513 amostras (zaragatoas nasofaríngeas) recolhidas de suínos em grandes complexos de suínos e explorações camponesas localizadas em diferentes regiões do Cazaquistão revelaram que cerca de 5% das amostras foram positivas para RNA do vírus influenza A. Este resultado sugere baixos níveis de circulação do vírus influenza A no Cazaquistão. A subtipagem de um conjunto de amostras revelou que as principais cepas circulantes em 2018–2019 foram A/H1N1 e A/H3N2. A vigilância realizada em 2020–2021 identificou apenas vírus A/H1N1 em suínos. Os dados de PCR foram confirmados pelo isolamento de seis cepas: cinco vírus influenza A/H1N1 e um vírus A/H3N2.
Palavras-chave: influenza suína, vírus influenza, triagem por PCR, rearranjos.

INTRODUCTION

Viruses circulating among wild and domestic animal populations are a potential risk to both animal and human health. Every year, epizootic infections cause economic damage to the agriculture and related industries. Zoonotic infections carry a high risk of pandemics, as seen in the historical examples from 1918 (H1N1), 2009–2010 (H1N1pdm2009), and the recent SARS-CoV-2 pandemic. The latter has affected more than 600 million people worldwide, of whom more than 6 million have died (KHANNA et al., 2013; WANG et al., 2020; WHO, 2022). Infection of pigs with influenza viruses has an increased risk associated with that the cells of these animals carry receptors for both humans and avian influenza A virus (IAV) of various origins; therefore, co-infection with different strains can lead to the emergence of reassortant variants (NEUMANN et al., 2009; EVERETT et al., 2020). In addition, avian IAV have been detected sporadically in pigs, including subtypes that do not usually infect these animals (TREVENNEC et al., 2011). In total, 12 subtypes of IAV have been recorded in pigs: H1N1, H1N2, H1N7, H3N1, H3N2, H3N3, H4N6, A/H4N8, H5N1, A/H6N6, H9N2, and H10N8 (ENCINAS et al., 2021; OIE, 2022; SAITO et al., 2022). It has been proven experimentally that pigs can be infected with avian IAVs of any serovariant, which can then form reassortants, and/or acquire point mutations that can lead to drug resistance or acquisition of zoonotic characteristics (KIDA et al., 1994; YAMNIKOVA et al., 2008). Influenza virus types B, C, and D have also been detected in pigs (RAN et al., 2015; KIMURA et al., 1997; CHIAPPONI et al., 2016). Studies of the variability, genetic diversity, and mechanisms

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underlying emergence of new viruses pathogenic to humans and animals makes it possible to identify novel strains in a timely manner, along with possible emergence and transmission of viruses with pandemic potential (KLIVLEYEVA et al., 2022); this information makes it possible to develop new vaccines and antiviral drugs (WEBSTER et al., 1992; TAUBENBERGER et al., 2001; BROCKWELL-STAATS et al., 2009; SHEREEN et al., 2020).

The purpose was to study the frequency of detection of IAV subtypes circulating in the main pig-producing regions of Kazakhstan, for understanding of the directions and rates of evolutionary variability of these viruses, to identify pathogens with the potential to cause epizootic and zoonotic infectious diseases.

MATERIALS AND METHODS

Sample collection

Biomaterials of porcine origin (nasopharyngeal swabs) were obtained in accordance with the recommendations of international organizations. Nasopharyngeal swabs were collected from animals in accordance with guidelines set down by the Committee for Research and Ethical Issues of the International Association of the Study of Pain, and all procedures were approved by the local ethics committee (Minutes No. 14 of 03/24/2022). The owners of the animals also provided consent for sample collection. Collected swabs were transported in liquid nitrogen and stored at temperatures below -70 °C (thawing and refreezing was avoided); all were examined within 1 month of collection (USDA, 2003; WOAH, 2003). Samples were collected twice a year from pig production farms in different regions of Kazakhstan. Farms housed between 1,000 and 30,000 pigs. Samples were collected from animals aged 1.5–6 months. A total of 2,513 biological samples were collected: 977 in 2018, 749 in 2019, 662 in 2020, and 125 in 2021. Figure 1 shows the percentage of samples collected in each region.

Real-time polymerase chain reaction (rtRT-PCR) with hybridization-fluorescence detection was performed to analyze biomaterials for the presence of sentinel viruses. Experiments were performed using a Rotor-Gene Q6 plex device (QIAGEN, Germany). RIBO-prep, and AmpliSense® Influenzavirus A/H1-swine-FL, AmpliSens® Influenzavirus A-type-FL, and AmpliSense® Influenzavirus A-type-H5, H7, and H9-FL kits (Federal Budgetary Scientific Institution Central Research Institute for Epidemiology of Rospotrebnadzor, Moskow, Russia) were used according to the manufacturer's instructions (AMPLISENS BIOTECHNOLOGIES). AmpliSens® Influenzavirus A-type-FL kits were used to detect HA (H1, H3) subtypes and NA (N1, N2) subtypes.

Isolation of swine IAVs

Viruses were isolated from PCR-positive samples using embryonated chicken eggs and Madin-Darby canine kidney (MDCK) cells, as described previously (KLIMOV et al., 2012).

Hemagglutination-inhibition assay

The hemagglutinin (HA) subtype of the isolated strains was determined using a cross-hemagglutination-inhibition (HAI) assay in accordance with the recommendations of the World Health Organization (WHO, 2011). Diagnostic immune sera specific for reference IAV A/Michigan/45/2015 (H1N1)pdm and A/Singapore/INFIMH-16–0019/2016 (H3N2), and for influenza A/H5N1 and H7N9 viruses (LLC "Enterprise for the production of diagnostic drugs", St. Petersburg, Russia), were used in the HAI assays, as recommended by the manufacturer.

Statistical analysis

Microsoft Office Excel and Graph Pad Prism 9 software were used for statistical data processing, as well as for tabular and graphical representation of the results (GLANTS, 1994). Percentage positivity and 95% confidence intervals were calculated for categorical variables such as detection of IAV according to year. A Chi-squared test was used to assess the significance of intra-group differences with respect to the type and subtype of viruses (again according to year). $P \leq 0.05$ was considered statistically significant.

RESULTS

Screening of samples by rtRT-PCR

The rtRT-PCR results are presented in the table 1. In total 2,513 nasopharyngeal swabs were subjected to rtRT-PCR, and IAV RNA was found in 116 (4.62%). A subtyping assay detected A/H1N1 IAV RNA in 70 swabs (2.79%), and A/H3N2 IAV in seven swabs (0.28%), collected in 2018 and 2019. The virus subtype was not identifiable in 39 PCR-positive samples (1.55%). The Chi-square test results for type A and influenza A/H1N1 viruses were significant, but those for A/H3N2 and undetermined IAVs were not. Taken together, the rtRT-PCR results for nasopharyngeal swabs indicate a predominance of

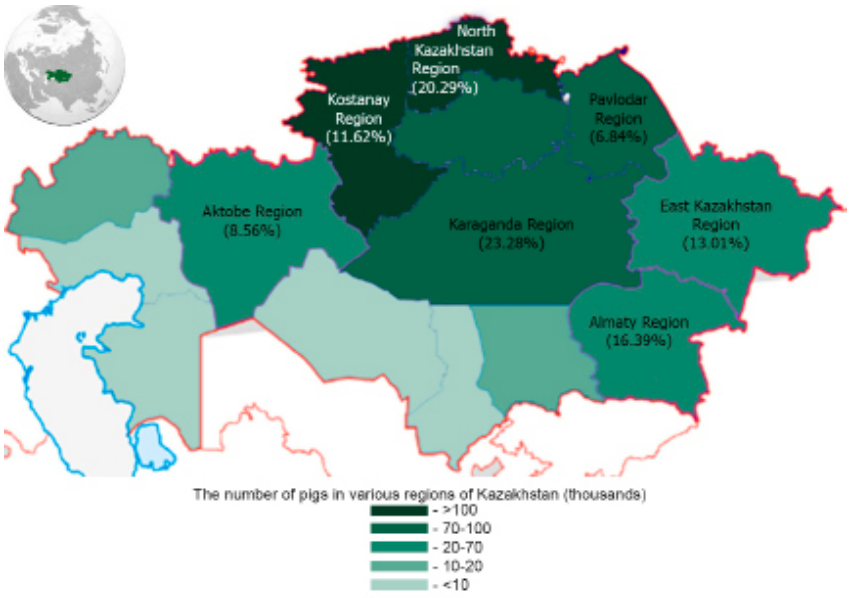

Figure 1 - Geographical distribution of samples.

A/H1N1 strains among IAV circulating in pig farms in Kazakhstan during the study period.

Isolation of swine IAV

Inoculation of the 116 PCR-positive samples into 9–10-day embryonated chicken eggs, followed by culture in MDCK cells. Identified six hemagglutinating agents, with hemagglutination titers in the range of 1:2–1:32 HA units; all samples were collected in the Pavlodar and North Kazakhstan regions, or in the Almaty region (southern Kazakhstan).

Table 1 – Results of rtRT-PCR of nasopharyngeal swabs collected from pigs.

Sample collection year	Number of analyzed samples	----------Number/percentage of PCR-positive samples (confidence intervals)----------			
		Influenza A	----------Subtype----------		
			A/H1N1	A/H3N2	Undetermined
Total	2513	116/4.62 (3.30; 5.93)	70/2.79 (1.75; 3.82)	7/0.28 (-0.05; 0.61)	39/1.55 (0.78; 2.33)
2018	977	51/5,22 (3.83; 6.61)	37/3.79 (2.59; 4.98)	6/0.61 (0.12; 1.10)	8/0.82 (0.25; 1.38)
2019	749	23/3.07 (1.99; 4.15)	6/0.80 (0.24; 1.36)	1/0.13 (-0.10; 0.360)	16/2.14 (1.23; 3.04)
2020	662	35/5.29 (3.88; 6.69)	21/3.17 (2.07; 4.27)	0	14/2.11 (1.21; 3.02)
2021	125	7/5.60 (4.16; 7.04)	6/4.80 (3.46; 6.14)	0	1/0.80 (0.24; 1.36)
P value		0.0037*	0.0343	0.2917	0.0306

*P ≤ 0.05 was considered statistically significant

Identification of the IAV subtype was performed by PCR and HAI assay of diagnostic immune sera specific for reference IAV strains. The isolates from the North Kazakhstan regions (A/swine/Petropavlovsk/01/2018, A/swine/Petropavlovsk/02/2018, A/swine/Petropavlovsk/03/2018, A/swine/Pavlodar/43/2019, and A/swine/Pavlodar/44/2019) all belonged to subtype A/H1N1, whereas the Almaty isolate (A/swine/Almaty/45/2019) was identified as A/H3N2. The whole genome sequence of influenza A/swine/Karaganda/04/2020 (H1N1) virus isolated from a clinical sample collected on the Karaganda livestock farm (Central Kazakhstan) was obtained. The results showed that the isolate belongs to clade 1A.3.2.2 lineage 1A, which includes the 2009 H1N1 pandemic strains (KLIVLEYEVA et al., 2021).

Isolation of six swine IAV strains confirms that IAV are circulating in Kazakhstan.

DISCUSSION

Pig production farms in Kazakhstan are unevenly distributed geographically. This is due to natural and climatic characteristics, as well as differences in consumption of meat products by the population. Pig production is highest in the northern part of the country (Kostanay, Pavlodar, and North Kazakhstan regions). In the western part (i.e., the Aktobe region), this industry is insufficiently developed, so there are few pig farms there.

Since the majority of pig farms are concentrated in northern Kazakhstan, most of our samples (38.76%) were collected there, followed by the central part (23.28%), the southern part (16.39%), and the eastern part (13.01%). The smallest number (8.56%) was collected from western Kazakhstan.

Circulation of IAV among the swine population in Kazakhstan during 2018–2021

The dynamics of IAV circulation in the Kazakhstan swine population during the study period are presented in figure 2, which shows that infection of pigs with IAV varied from 3.07–5.60%, the majority of which were of the A/H1N1 subtype.

The percentage of samples containing A/H1N1 genetic material increased from 26.09% of all influenza A-positive samples in 2019, to 85.71% in 2021. At the same time, the percentage of the A/H3N2 subtype IAV fell from 11.76% in 2018 to 4.35% in 2019; no samples containing the genetic material of A/H3N2 IAV were detected in 2020 or 2021 (Figure 3).

Circulation of swine IAV in Kazakhstan and worldwide

Previously published data indicate that A/H1N1 IAV have been prevalent among swine in Kazakhstan for a long time. In 1984, three swine IAV were isolated for the first time in the country (three A/H1N1 strains isolated from material collected in eastern Kazakhstan; (LAPTEV et al., 1987). Since then, swine IAV A/H1N1 have been identified repeatedly (ONGARBAYEVA et al., 2016; SAKTAGANOV et al., 2020; KLIVLEYEVA et al., 2021). Evidence suggestst that A/H3N2 viruses are circulating alongside A/H1N1 viruses; although, A/

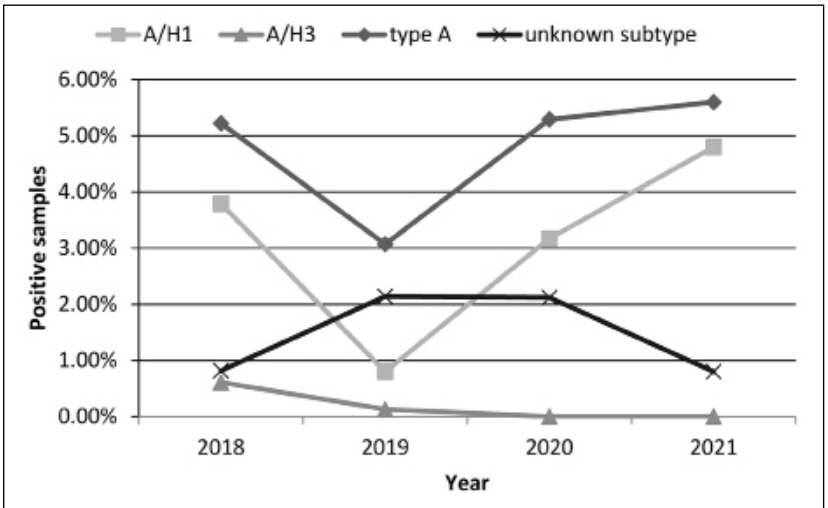

Figure 2 - Percentage of pigs positive for influenza virus in Kazakhstan from 2018–2021.

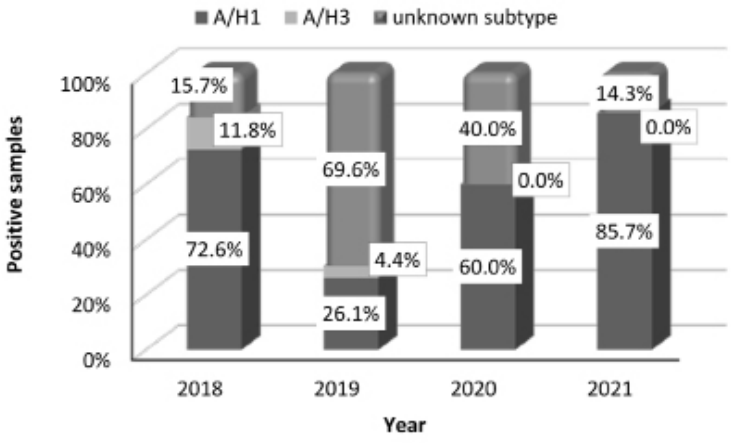

Figure 3 - Percentage of positive samples according to HA type.

H3N2 viruses are more rare. (2009; KLIVLEYEVA et al., 2017; ONGARBAYEVA et al., 2022; KLIVLEYEVA et al., 2019). In the present study, the virus subtype was not identified in 39 PCR-positive samples (1.55%), possibly because they were only weakly positive.

Data regarding circulation of IAV among the swine population of Kazakhstan are similar to those worldwide (CDC, 2012; CHAUHAN et al., 2020). Since 1931, IAV with H1 and N1 surface antigens have been isolated repeatedly from pigs in different countries, including the former Soviet Union (WEBSTER et al., 1992; SHOPE et al., 1931; ISHMUKHAMETOVA et al., 2012; CHEPKWONY et al., 2021). Since 2009, A/H1N1pdm09, which emerged as a result of host adaptation and reverse zoonosis, has generated various porcine IAV reassortants with classical swine IAV, European avian-like IAV, and H3N2 viruses, among others; all have been detected in pigs worldwide (CARDINALE et al., 2012; GRAY et al., 2012; LIANG et al., 2014; HAN et al., 2012). For example, the A/H1N2 strains contain genes of the A/H1N1pdm09 variant, and the NA gene of swine A/H3N2 viruses (MORENO et al., 2011; TAKEMAE et al., 2016; PENG et al., 2016).

The H3N2 subtype was first identified in pigs in 1970. It is thought to have emerged after interspecies transmission of IAV from humans to pigs (WEBBY et al., 2000; NELSON et al., 2012; MINE et al., 2019), and it is much less common than A/H1N1. For example, in the United States, A/H1N1 viruses have spread among pigs since at least 1930 (according to the World Health Organization). A/H3N2 IAV were reported in pigs in Japan in 1993 (KATSUDA et al., 1995). Since 1998, there have been several outbreaks of respiratory disease caused H3N2 influenza viruses in swine herds in America (KARASIN et al., 2000). From 1999–2002, the European variants A/H3N2 and A/H1N1, and North American triple reassortants, were first detected in China, indicating intercontinental movement of swine viruses, possibly through importation of pork (VIJAYKRISHNA et al., 2011), or transmission of viruses between humans and pigs (TAUBENBERGER et al., 2001; BROCKWELL-STAATS et al., 2009; ADEOLA et al., 2019; RAJAO et al., 2019; RAMBO-MARTIN et al., 2020).

Therefore, various IAV variants are circulating among the swine population in Kazakhstan and worldwide, which increases the likelihood of emerging reassortants.

CONCLUSION

rtRT-PCR of biological material collected from pigs on livestock farms located in different regions of Kazakhstan during 2018–2021 revealed that IAV are circulating constantly. Infection rates in pigs during the period under study did not exceed 6%. At the same time, most of the influenza-positive samples were subtype A/H1N1 (as high as 85.71% in 2021). Circulation of IAV among the swine population was confirmed by isolation of six strains of swine IAV, five of which were A/H1N1, and another that was A/H3N2. These findings complement data obtained worldwide and in Kazakhstan. Persistence of influenza infection in pig populations, emergence of genetic reassortants, transmission of viruses to

humans, and spread throughout the population are significant risk factors for future pandemics. The main threat is emergence of zoonotic swine IAV capable of infecting humans and maintaining populations of both hosts. Understanding the mechanisms by which these viruses adapt to a new host following transmission is critical to reducing the risk. Key risk factors include movement of infected pigs, or infection of pigs with human seasonal IAV. Influenza surveillance, particularly of breeding and growing herds at high risk of persistent swine influenza infection, requires ongoing monitoring, and will be essential to protect animal health and prevent human pandemics (MOLLETT, 2023).

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they do not have financial or personal conflicts of interest that might affect their objectivity in this study.

AUTHORS' CONTRIBUTIONS

GL, NK, and TG conceived and designed experiments. AB, SB, DI, and EI performed the experiments, NO and MS carried out the lab analyses. NS, GB, and MM supervised and coordinated the animal experiments and provided clinical data. TG performed statistical analyses of experimental data. GL, NK, and TG prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

Nasopharyngeal swabs were collected from pigs in accordance with guidelines set down by the Committee for Research and Ethical Issues of the International Association of the Study of Pain and the study was approved by the local ethics commission (Minutes No. 14 of 03/24/2022). The owners of the farms also provided informed consent.

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