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Global transmission dynamics of avian influenza viruses in swine and prevalence of influenza A and other RNA viruses in saliva samples of backyard swine in KwaZulu-Natal province of South Africa

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DECLARATION

I, **Ravendra Pratap Singh Chauhan**, declare the following statements:

i. The research presented in this thesis is my original work.
ii. The experimental work and data analyses were performed by me, except otherwise stated in the published articles or manuscripts.
iii. The previously published scientific literature, when used, was rewritten in my own words with proper citations.
iv. The contents of this thesis are original and were not submitted to any other university or tertiary institution for the award of a degree or examination.
v. All the figures presented in this thesis are original and prepared by me using Adobe Illustrator.
vi. This thesis does not contain any data generated by another person or taken from another source, except otherwise stated.
vii. My contribution to each article/manuscript (chapter) is explicitly mentioned in the author contributions section of the manuscripts.
viii. This research is supported by University of KwaZulu-Natal's College of Health Sciences (CHS) postgraduate scholarship awarded to Ravendra P. Chauhan during 2018- 2020.
ix. This research is also partially supported by institutional productivity funds of Prof. Michelle L. Gordon.

Signed: _____ [redacted] _____ Ravendra Pratap Singh Chauhan (student)

Date: 16 December 2021

I endorse that the research presented in this Ph.D. thesis is the original work conducted by the student under my supervision. In the capacity of the student's supervisor, I approve the submission of this thesis.

Signed: _____ [redacted] _____ Dr. Michelle Lucille Gordon (supervisor)

Date: 16 December 2021

DEDICATION

In memory of my heavenly grandfather and father.

To my lovely daughter, Oju, and wife, Ruchika.

LIST OF PUBLICATIONS

1. **Ravendra P. Chauhan**, Michelle L. Gordon. An overview of influenza A virus genes, protein functions, and replication cycle highlighting important updates. Virus Genes, 2022. https://doi.org/10.1007/s11262-022-01904-w
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ABBREVIATIONS AND SYMBOLS

AHT	Animal health technician
AIV	Avian influenza virus
APHA	Animal and plant health
ARC	Agricultural Research Council
AREC	Animal research ethics committee
bp	Base pair
BPS	Backyard productive systems
BHQ-1	Back hole quencher-1
BSF	Backyard swine farm
cDNA	Complementary DNA
CDC	Centers for Disease Control and Prevention
CHS	College of Health Sciences
COVID-19	Coronavirus disease-19
cRNA	Complementary ribonucleic acid
cRNP	Complementary ribonucleoprotein
CSFV	Classical swine fever virus
CSV	Comma-separated values
Ct	Cycle threshold
DAFF	Department of Agriculture, Forestry and Fisheries
DALRRD	Department of Agriculture, Land Reform and Rural Development
DDBJ	DNA Data Bank of Japan
ddNTP	Dideoxynucleotide triphosphates
dNTP	Deoxynucleoside triphosphates
DNA	Deoxyribonucleic acid
dpi	Days post-infection/ Days post-inoculation
EA	European Asian
ELISA	Enzyme linked immuno sorbent assay
EMBL-EBI	European molecular biology laboratory- European bioinformatics institute
ER	Endoplasmic reticulum
FAM	Fluorescein amidite
FLI	Friedrich-Loeffler-Institut
FMDV	Foot and mouth disease virus

GAIIx	Genome Analyzer IIx
h	Hour
HA	Hemagglutinin
HEV	Hepatitis E virus
HI	Hemagglutinin inhibition
HPAIV	Highly pathogenic avian influenza virus
HRT18	Human Rectal Tumor- 18
IAV	Influenza A virus
IBV	Influenza B virus
ICV	Influenza C virus
IDT	Integrated DNA Technologies
IDV	Influenza D virus
IFN	Interferon
INSDC	International nucleotide sequence database collaboration
IRD	Influenza Research Database
Kg	Kilogram
LPAIV	Low pathogenic avian influenza virus
M	Matrix
MAA	*Maackia amurensis* agglutinin
MDCK	Madin darby canine kidney
MEGA	Molecular Evolutionary Genetics Analysis
min	Minute
ML	Maximum likelihood
ml	Milliliter
MN	Microneutralization
mRNA	Messenger ribonucleic acid
MVDL	Minnesota Veterinary Diagnostic Laboratory
n	Number
NA	Neuraminidase
NCBI	National Center for Biotechnology Information
NEP	Nuclear export protein
NES	Nuclear export signal
NiV	Nipah virus
NGS	Next-generation sequencing

NI	Neuraminidase inhibition
NP	Nucleoprotein
NPC	Nuclear pore complex
NS	Nonstructural
nt	Nucleotide
ORF	Open reading frame
PA	Polymerase acidic
PAstV	Porcine astrovirus
PCA	Principal coordinate analysis
PCR	Polymerase chain reaction
PEDV	Porcine epidemic diarrhoea virus
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PGM	Personal genome machine
PRISMA	Preferred reporting items on systematic reviews and meta-analysis
PRRSV	Porcine reproductive and respiratory syndrome virus
qRT-PCR	quantitative Real-time-polymerase chain reaction
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
rRT-PCR	Real-time reverse transcriptase- polymerase chain reaction
RT-PCR	Reverse transcriptase- polymerase chain reaction
RVA	Rotavirus A
RVB	Rotavirus B
RVC	Rotavirus C
RVH	Rotavirus H
SA	Sialic acid
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
SNA	*Sambucus nigra* agglutinin
SPF	Specific pathogen free
SVDV	Swine vesicular disease virus
TGEV	Transmissible gastroenteritis virus
TRIG	Triple reassortant internal gene
UK	United Kingdom
USA	United States of America

USD	United States Dollar
VN	Virus neutralization
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleoprotein
WGS	Whole genome sequencing
WHO	World Health Organization
ZAR	South African Rand
α	Alpha
℃	Degree Celsius
€	Euro
<	Greater than
>	Less than
µ	Micro
%	Percent

ABSTRACT

Background: The presence of α-2,3 and α-2,6 sialic acid receptors in the upper respiratory tract of swine makes them an ideal host for efficiently acquiring and disseminating avian and human origin influenza A virus (IAV) subtypes. Numerous reports have documented spillover of various avian IAV subtypes to swine in several countries. The prevalence of swine and avian IAVs in swine populations and their interactions with circulating human IAV strains may facilitate reassortment and the emergence of novel IAV strain(s) with pandemic potential, representing a severe threat to public safety. Four IAV disease pandemics have occurred and claimed millions of human lives in the past. Apart from IAV, numerous other RNA viruses such as classical swine fever virus, foot and mouth disease virus, porcine epidemic diarrhoea virus, porcine reproductive and respiratory syndrome virus, rotaviruses, transmissible gastroenteritis virus etc. may inflict severe diseases in swine and may result in significant economic losses to swine farmers, have been reported in swine populations in various countries. Of note, backyard swine farming faces a considerable challenge in biosecurity due to limited resources in most developing countries, including South Africa. To date, no study has ever reported the molecular prevalence of IAV or other RNA virus diseases in South African backyard swine populations.

Methods: First, for determining the transmission dynamics and spillover of avian origin IAV subtypes to the swine, we downloaded from the Influenza Research Database (IRD), the full-length hemagglutinin (HA) gene sequences of avian and swine IAV subtypes, reported in swine populations and the avian species globally. We performed phylogenetic, N-linked glycosylation, and principal coordinate analysis to determine the transmission patterns of avian IAVs to swine. We conducted multiple sequence alignment of a few avian and swine IAV subtypes to assess the possibility of swine-to-swine transmission. Adaptation of these avian origin IAV subtypes in swine was determined by analysing the presence of mammalian adaptation markers. For detecting the molecular prevalence of IAV in South African backyard swine, we collected oral secretion (saliva) samples (n=102) from swine at three backyard swine farms located distantly in the uMgungundlovu District of the KwaZulu-Natal province. We extracted viral RNA from the saliva samples tested, and used one-step real-time RT-PCR with a TaqMan probe to detect the IAV matrix gene. Additionally, we deep sequenced three swine saliva samples, one sample from each of the backyard farms, to determine the oral virome of the swine on these backyard swine farms. To achieve this, total

viral RNA was extracted using the NucleoMag Pathogen kit, and library preparation was performed using the Illumina Stranded Total RNA Prep. Deep sequencing was performed on an Illumina HiSeq X instrument. The fastq files containing paired-end reads were analyzed using Genome Detective v 1.135. The assembled nucleotide sequences were analyzed using nucleotide BLAST and PhyML phylogenetic tree.

Results and Discussion: From the downloaded sequences, we identified that various wild and domestic avian species had transmitted eleven of the IAV subtypes to the swine populations in several countries globally. Some of these avian IAVs had already been adapted in swine because they had acquired specific mammalian adaptation markers. Intriguingly, multiple spillover events of highly pathogenic H5N1 and low pathogenic H9N2 viruses from various avian species to swine were determined, while the spillover events of other nine avian influenza viruses were limited. While we did not detect IAV RNA in swine saliva samples obtained from the three backyard farms in the KwaZulu-Natal province of South Africa, we were able to quantify viral RNA in the saliva samples under investigation which prompted us to perform deep sequencing analysis of selected saliva samples (n=3) to explore the diversity of RNA viruses in backyard swine saliva. As a result, we identified a high diversity of swine enteric viruses in two of these saliva samples, which originated from backyard swine farm 2 (BSF2) and BSF3. In contrast, only a few RNA viruses were present in the saliva sample obtained from BSF1. These viruses belonged to 13 families of animal viruses, two families of plant viruses, two species of fungal viruses, five unclassified virus species, and a few viruses classified in the Order Picornavirales. Intriguingly, Porcine astroviruses were prevalent in these swine saliva samples; however, either partial or complete genome segments of Porcine rotavirus A, Human rotavirus B, and Rotavirus C were also identified in the saliva, which originated from BSF2, while Rotavirus C genome sequences were obtained from the saliva originated from BSF3. In addition, a near full-length Hepatitis E virus (HEV) genome was detected in the saliva sample obtained from BSF3.

Conclusion: Interactions between avian species (domestic or wild) and swine poses a significant threat to the interspecies transmission of IAV. While this study determined the events of avian to swine transmission of various avian IAV subtypes globally, the absence of active IAV infection in the present study, which comprised a limited backyard swine population of the KwaZulu-Natal province, does not exclude the presence of IAV infection in other backyard swine holdings in South Africa. While low biosecurity standards at the backyard swine farms would facilitate interspecies transmission of IAV from domestic or

wild avian species and humans to the swine, it requires continued IAV surveillance at the backyard swine farms to monitor the disease status. Of concern was the high diversity of swine enteric viruses at BSF2 and BSF3, which suggests an increased risk of diarrhoeic diseases at the backyard farms. In addition, the occurrence of rotaviruses and HEV in backyard swine makes it imperative to implement adequate biosecurity to prevent the zoonotic transmission of these viruses from swine to humans in the households to ensure public safety. Therefore, we recommend nationwide molecular surveillance to detect and track the circulation and evolution of IAV and other RNA viruses in backyard swine populations in South Africa which is critical to sustainable backyard swine farming and ensuring public health.

CHAPTER 1

GENERAL INTRODUCTION

Swine farming provides a significant contribution and shares more than one-third of the meat production globally [1, 2]. While in many countries, the large-scale commercial swine farms produce most of the pork to meet the demand of consumption in the markets, small-scale backyard swine farms co-exist for generating subsistence and food security in resource-scarce rural and semi-urban households [2]. Limited resources and the lack of biosecurity at the backyard farms make them vulnerable to the transmission of zoonotic virus pathogens, such as influenza A virus (IAV), endangering sustainable backyard swine farming. Studies conducted in various countries have reported that the lack of biosecurity at backyard swine farms provides a conducive environment for disease outbreaks resulting in significant economic losses to the swine farmers [3-10].

In South Africa, swine farming was estimated to be ZAR 19.8 billion (~ USD 1.25 billion) industry over ten years during 2001 and 2011 [11]. The primary production and supply of pork meat in South Africa are carried by the large-scale commercial piggeries located throughout the country. Unfortunately, due to economic challenges, many rural and semi-urban South African households may not be able to afford the commercially available pork meat for meeting their nutritional requirements [12]. As a result, some of these households tend to rear domestic pigs (*Sus scrofa domesticus*) in their backyards to have sustainable access to the pork meat and simultaneously generate subsistence to uplift their economic status [12, 13].

Several emerging and re-emerging virus pathogens, some of which have a potential zoonotic propensity, such as influenza A virus (IAV), have been reported in swine populations globally [14]. The IAV is the most important among zoonotically transmitted virus pathogens which have given rise to four influenza pandemics viz, 1918 Spanish flu [15], 1957 Asian flu [16], 1968 Hong Kong flu [17], and most recently, 2009 swine flu [18]. Influenza viruses belong to the virus family *Orthomyxoviridae* and have a negative-sense single-stranded RNA genome. In a recent systematic review, we reported the global prevalence of influenza virus types including IAV, influenza B virus (IBV), influenza C virus (ICV), and influenza D virus (IDV) in swine populations worldwide [14]. Swine is an important host for IAV transmission and evolution because the receptors present in the swine trachea are specific to the human and avian IAV subtypes [19]. Due to this unique swine physiology, a high probability

remains for interspecies transmission of the avian and human IAV subtypes to the swine in natural settings.

It is known that the absence of biosecurity in the backyard swine farms facilitates interactions of swine and poultry with wild birds [20, 21]. Such interactions put the animals in the backyard at high risk of IAV disease transmission [22, 23], which may trigger the disease outbreak in the backyard swine populations [12, 22, 23]. While the prevalence of IAV has been reported in backyard swine populations in several countries, including some of the countries in Africa such as Kenya [24], Nigeria [25], Ghana [26], Cameroon [27], and Egypt [28], no information on IAV disease in South African backyard swine is available. Due to the zoonotic nature of IAV transmission, it is imperative to investigate the prevalence of IAV disease in backyard swine populations. For the first time, this study describes molecular surveillance of IAV in rural areas practicing backyard swine farming in KwaZulu-Natal.

Apart from IAV, several other emerging and re-emerging RNA viruses have been reported from the swine populations in various countries. Porcine epidemic diarrhoea virus (PEDV) [29] and Transmissible gastroenteritis virus (TGEV) [30] are emerging porcine coronaviruses in family *Coronaviridae* and have single-stranded positive-sense RNA genomes [31]. These coronaviruses may cause dehydration, vomiting, and severe diarrhoea in swine, along with mortality in young piglets [32-37].

Rotaviruses are other important porcine enteric pathogens that cause diarrhoea in neonatal and young piglets [38-40]. The Rotavirus genome consists of 11 segments of double-stranded RNA. Multiple Rotavirus types, including Rotavirus A (RVA) [41-45], Rotavirus B (RVB) [42, 46], Rotavirus C (RVC) [42, 47], and Rotavirus H (RVH) [47, 48], have been reported from swine populations in various countries. In addition, rotaviruses may be zoonotically transmitted to the exposed human populations and cause diarrhoeic diseases in children [49].

Porcine astrovirus (PAstV) is another porcine enteric virus and belongs to the family *Astroviridae*. The PAstV genome consists of single-stranded positive-sense RNA and is divided into three open reading frames (ORFs) [50]. Five types of PAstVs, viz., PAstv1-PAstV5, have been reported in swine populations from various countries [51-54]. PAstVs are commonly detected in diarrhoeic swine as opposed to the healthy swine [55, 56] and have also been associated with neurologic disease in swine [53, 57]. In addition, Mamastrovirus 2 and Mamastrovirus 3 are porcine enteric viruses in the family *Astroviridae* that have been reported in swine [58].

Foot and mouth disease virus (FMDV) is a highly contagious RNA virus with a positive-sense, single-stranded RNA genome and belongs to the family *Picornaviridae* [59-61]. FMDV infection, which has been reported in swine from several countries [62-64], forms typical lesions in the swine's feet, snout, and mouth [65] and can inflict a clinically severe disease in pigs [66]. Interestingly, airborne transmission of FMDV has also been reported [67]. In addition, several other swine enteric RNA viruses of the family Picornaviridae, including Porcine enterovirus [68, 69], Porcine kobuvirus [70-72], Porcine pasivirus A [73], Porcine sapelovirus [74, 75], Teschovirus [76, 77], and Swine vesicular disease virus (SVDV) [78, 79] have been reported in swine populations in several countries. While the viruses of the family *Picornaviridae* have been associated with diarrhoeic diseases in pigs [80], Teschovirus A infections have been associated with encephalomyelitis in pigs [76, 77] and SVDV has been associated with weight loss in pigs [78].

Hepatitis E virus (HEV) is another single-stranded positive-sense RNA virus in the family *Hepeviridae* [81] and is transmitted through the fecal-oral route in swine [82]. There are eight genotypes of HEV: HEV1 to HEV8 [83]. While HEV5 and HEV6 are endemic in swine, HEV3 and HEV4 have been linked to zoonotic transmission to the exposed human population, either through direct contact or consumption of pork meat [83]. While HEV has been reported in swine populations in various countries [84-86], the infection in swine is usually subclinical, increasing the risk being an important source for zoonotic transmission to humans [87].

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus pathogen of pigs and causes significant economic losses to the swine farmers [88-92]. The PRRSV causes respiratory disease with severe pneumonia in the young piglets and reproductive failure in the breeding pigs [93, 94]. The PRRSV genome consists of a positive-sense single-stranded RNA, belongs to the family *Arteriviridae* [94]. A recent study reported a high diversity of PRRSV in Chinese swine [95].

The Classical swine fever virus (CSFV) is one of the most important re-emerging virus pathogens of swine, consists of a positive-sense single-stranded RNA, belongs to the family *Flaviviridae* [96]. The clinical signs of disease may vary as the disease progresses, ranging from high fever, gastrointestinal disorder, lethargy, and anorexia [97] to neurological signs, including incoordination, paralysis, paresis, and convulsions [96, 98]. A recent phylodynamic study suggested that CSFV may have emerged from the Tunisian sheep virus due to host-jumping from sheep to the swine [99]. The CSFV has been reported in swine in several

countries, including China [100, 101], South Korea [102], Japan [103], India [104], Israel [105], and European countries [106, 107].

In South Africa, some of the above swine pathogens have been reported in commercial farms, resulting in localized outbreaks. Briefly, FMDV was first reported in September 2000 on a commercial swine farm located in the Camperdown District of KwaZulu-Natal province [108]. In addition, three trade-related outbreaks of PRRSV in South African swine during 2004, 2005, and 2007 resulted in the slaughtering of 19,000 pigs and culling of 16,500 pigs, leading to a total loss of about ZAR 9.7 million (~ USD 0.7 million) [109]. The CSFV re-emerged in South African swine after 87 years in June 2005 when a disease outbreak occurred at a commercial piggery in Western Cape province, which reported a high percentage of swine mortality [110]. The CSFV outbreak led to the culling of over 335,000 pigs resulting in a significant production loss to the industry [13].

Rotavirus species, including RVA, RVB, and RVC have been reported in diarrhoeic piglets in South Africa in four studies between 1977 and 1996 [40, 111-113]. In addition, one complete genome of RVH was reported from a 10-week-old diarrhoeic piglet in South Africa in 2016 [48]. This was the only RVH genome reported in swine from South Africa. Interestingly, the year-long pilot study by Geyer *et al.* (1995) demonstrated that RVA shedding in the feces of two to four-week-old piglets persisted for up to three weeks and, therefore, provided ample opportunity for further spread of the disease to the uninfected piglets on the farm [113]. Geyer *et al.* (1996) also showed that piglets could be co-infected with RVA and RVC, which raised the question of whether the coinfection of rotaviruses could facilitate the emergence of a new reassortant rotavirus species [112].

Another important zoonotic virus pathogen, Hepatitis E virus genotype 3 (or HEV3), was recently reported in commercial and communal (free roaming) swine in the Eastern Cape and is the only study conducted in South Africa [114]. This study raised the concern of zoonotic transmission of HEV3 in the study area because the farming communities of the area routinely used swine waste as manure for enhancing the crop yield on the farms [114]. The other concern raised was that the free-roaming swine infected with HEV3 might contaminate waterbodies with feces and thus could facilitate the water-borne transmission of HEV3 in the region [114].

Recently IAV was reported on commercial swine farms in South Africa [115]. While IAV infection is a challenge for swine farming worldwide, this study provides the only data

available for IAV prevalence in South Africa and highlights the importance of the further investigation to analyze the impact of this disease on commercial and backyard swine farming.

While only limited information is available on the prevalence of some of the virus pathogens described above in South African commercial piggeries, there are no reports on the prevalence of the RNA viruses in South Africa backyard swine populations. Given the absence of biosecurity, the backyard swine farms in South Africa remain at high risk for disease transmission. Since some of these virus pathogens can be zoonotically transmitted to the exposed human populations, such as IAV, HEV, and rotaviruses, investigations to unravel the prevalence of these virus pathogens in South African backyard swine are crucial to combat zoonotic transmission events between backyard swine and exposed household members. Since swine is a known 'mixing vessel' for IAV reassortment and evolution, it poses the highest risk for zoonotic transmission and the emergence of pandemic strains. Therefore, active IAV surveillance in swine is vital for improving the available diagnostic assays and effectively generating vaccines to combat the IAV disease burden [116].

To the best of our knowledge, this is the first study to investigate the molecular prevalence of IAV and other RNA viruses in South African backyard swine populations. In this context, molecular surveillance of active IAV infection in backyard swine populations was conducted in the uMgungundlovu District of the KwaZulu-Natal province of South Africa. Additionally, we performed deep sequencing of selected backyard swine saliva samples to detect and characterize RNA virus pathogens in South African backyard swine.

1. **Research questions**
   - 1.1 What are the transmission dynamics of avian influenza viruses to porcine herds worldwide?
   - 1.2 What is the status of South African backyard swine in terms of IAV active infections in the province of KwaZulu-Natal?
   - 1.3 Are there other RNA virus pathogens present in South African backyard swine saliva samples?
2. **Aim of the study**

   To investigate the molecular prevalence of influenza A virus and other RNA viruses in South African backyard swine saliva samples in the KwaZulu-Natal province.

3. Specific objectives:

3.1 To investigate the avian to swine transmission events of various avian influenza virus subtypes globally.

3.2 To detect the IAV in saliva samples of backyard swine from selected farms in uMgungundlovu District of KwaZulu-Natal province.

3.3 To determine the subtypes of IAV using hemagglutinin and neuraminidase gene sequencing if IAV is detected in backyard swine saliva samples.

3.4 To identify the different RNA viruses present in the selected saliva samples of backyard swine using deep sequencing.

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CHAPTER 2

Virus Genes
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REVIEW PAPER

An overview of influenza A virus genes, protein functions, and replication cycle highlighting important updates

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Abstract

The recent research findings on influenza A virus (IAV) genome biology prompted us to present a comprehensive overview of IAV genes, protein functions, and replication cycle. The eight gene segments of the IAV genome encode 17 proteins, each having unique functions contributing to virus fitness in the host. The polymerase genes are essential determinants of IAV pathogenicity and virulence; however, other viral components also play crucial roles in the IAV replication, transmission, and adaptation. Specific adaptive mutations within polymerase (PB2, PB I, and PA) and glycoprotein-hemagglutinin (HA) and neuraminidase (NA) genes, may facilitate interspecies transmission and adaptation of IAV. The HA-NA interplay is essential for establishing the IAV infection; the low pH triggers the inactivation of HA-receptor binding, leading to significantly lower NA activities, indicating that the enzymatic function of NA is dependent on HA binding. While the HA and NA glycoproteins are required to initiate infection, M1, M2, NS1, and NEP proteins are essential for cytoplasmic trafficking of viral ribonucleoproteins (vRNPs) and the assembly of the IAV virions. The mechanisms that enable IAV to exploit the host cell resources to advance the infection are discussed. A comprehensive understanding of IAV genome biology is essential for developing antivirals to combat the IAV disease burden.

Keywords Influenza A virus • IAV evolution • IAV genes and proteins • IAV genome biology • IAV replication cycle • RdRp complex • Viral ribonucleoprotein

Introduction

Influenza A virus (IAV) occurs in various avian and mammalian hosts [25, 26, 28, 34, 74, 128, 143, 162). The IAV genome comprises eight gene segments of single-stranded, negative-sense RNA, ranging between 890 and 2341 nucleotides long [15, 85). Each gene segment has a viral ribonucleoprotein (vRNP) comprising an RNA-dependent RNA polymerase (RdRp) complex formed by the association of three polymerase proteins: polymerase basic 2 (PB2), polymerase basic I (PB I), and polymerase acidic (PA), with viral genomic RNA integrated to the nucleoprotein (NP) molecules [46, 136). The IAV virions have spike-like projections at the outer surface, which are composed of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) [19). The existing high antigenic variability within surface glycoproteins classifies the members of IAV into 18 HA and 11 NA subtypes [60, 138).

Events of reassortments and point mutations facilitate IAV genome evolution; as a result, novel subtypes and genotypes keep emerging, which may be capable of triggering a pandemic in an immunologically naive human population [7, 8, 95, 103). The most recent pandemic of 2009 originated from reassortments among avian, human, and swine IAV subtypes in swine in Mexico [88, 128). This highlights the implications of the circulation of IAV in a broad host range as it poses a significant threat for the emergence of another influenza pandemic. This signifies the importance of active surveillance for monitoring the antigenic diversity and IAV genome evolution. The availability of existing data on IAV genes, protein functions, and replication cycle would help clarify IAV pathogenesis and can be used to develop antivirals to combat the IAV disease burden. In this context,

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the present study emphasizes recent updates on IAV genes, protein functions, and replication cycle.

IAV genes and protein functions

The IAV virion is an enveloped spherical particle of 80-120 nm diameter [141), schematically depicted in Fig. IA, although it has also been described to form filamentous particles which are roughly equal or slightly smaller (80-100 nm) in diameter [38). While the mechanisms behind filament formation are not yet understood, it is believed that mutations in viral proteins during adaptation may result in filament formation. The IAV genome comprises eight vRNP segments composed of negative-sense single-stranded RNA. Some of the IAV gene segments encode a single polypeptide each. In contrast, others may encode more than one polypeptide (Fig. 1B), encoding a total of 17 proteins [142), each of which confers a unique function required for establishing a successful infection in the host. Each gene will be briefly discussed below, and a summary of IAV protein functions is provided in Table 1.

Polymerase basic 2 (PB2) gene

Polymerase basic 2 (PB2) is the first segment of the IAV genome and comprises 2316 nucleotides [Strain: *"A/swine/Iowa/18Tosu0505/2018(HJNJ)"*]; it encodes the largest protein of 759 amino acids. The PB2 is a component of the vRNP complex [46) and a major determinant of virulence and pathogenicity of IAV [76). PB2 is transported to the host cell nucleus independently of the PB 1 and PA subunits of the RdRp complex [78). The binding of PB2 to importin a1 mediates the transport of PB2 into the host cell nucleus [48). Localization of PB2 has also been displayed in mitochondria in !AV-infected cells [23, 76), which regulates mitochondrial viability during I AV infection to exploit the host cell resources for establishing an efficient infection [23). In addition, the PB2 possesses a specific independently folded domain with a cap-binding motif that facilitates cap

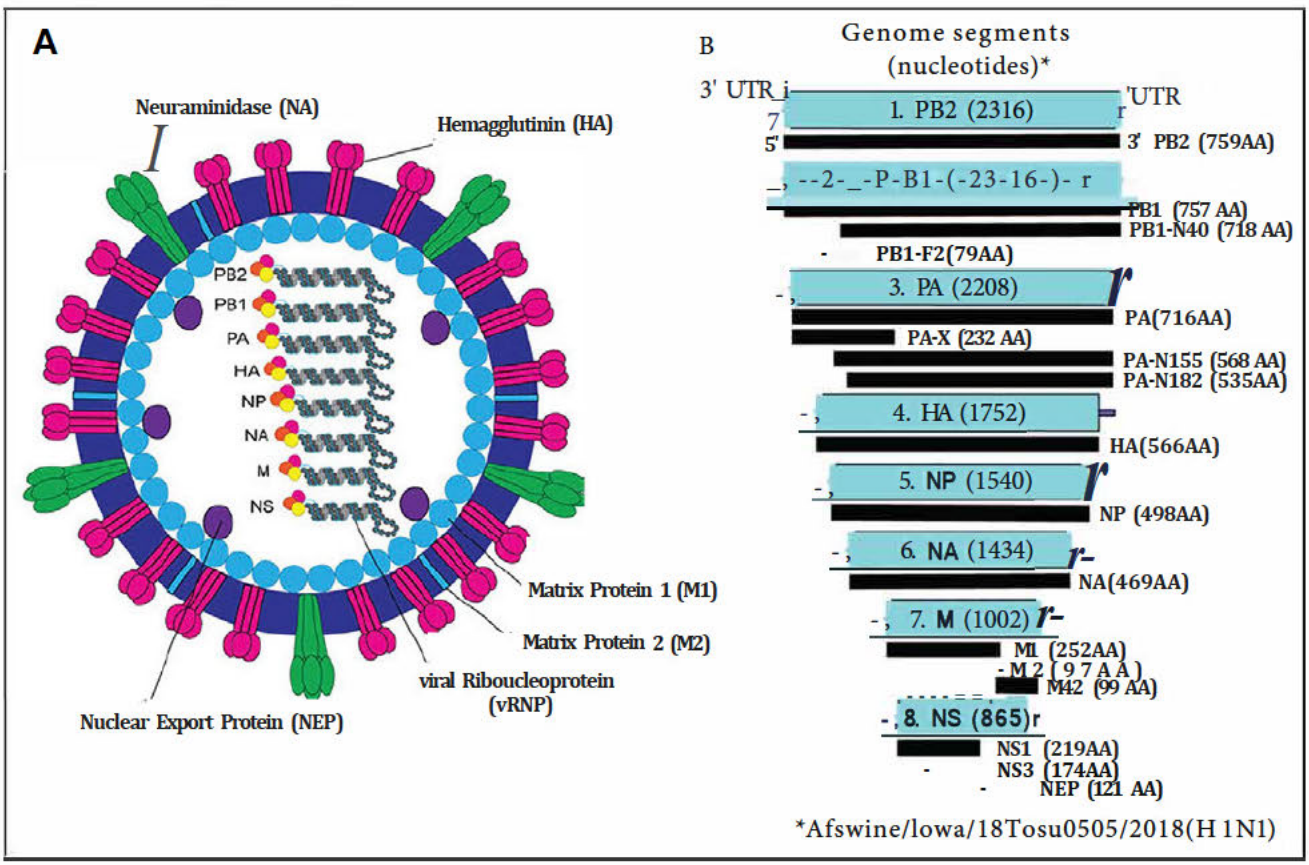

Fig. 1 A Schematic representation of I AV virion consisting of eight vRNP segments encapsidated in a host-derived lipid membrane. I AV virion is usually 80- 120 nm in diameter. The vRNP is a heterotrimer comprising the RdRp complex and viral genomic RNA on a nucleoprotein backbone. B The IAV genome segments have 5' and 3' non-coding regions. The PB2, HA, NP, and NA encode a single polypeptide each while other gene segments may encode two or more polypeptides each. The black bars represent the respective positions of I AV proteins. It should be noted that the gene size (number of nucleotides) may slightly vary among I AV strains

Table 1 Summary of major functions of IAV genome segments

Genes (# nucleotides)*	Proteins (# amino acids) *	Major functions	Citations
1. PB2 (2316)	PB2 (759)	Regulates mRNA cap recognition, regulation of mitochondrial viability	[15, 23, 52, 75, 76, 80]
2. PB1 (2316)	PB1 (757)	Catalytic subunit of RNA-dependent RNA polymerase, RNA elongation	[15, 72, 146]
	PB1-F2 (79)	Pro-apoptotic activity	[24, 82, 158]
	PB1-N40 (718)	Polymerase activity	[135, 149]
3. PA (2208)	PA (716)	Exonuclease activity supporting cap-snatching	[43, 54]
	PA-X (252)	Expression of proinflammatory cytokines, supports virus replication	[152, 157]
	PA-N155 (568)	Supports virus replication	[92, 145]
	PA-N182 (535)	Supports virus replication	[92, 145]
4. HA (1752)	HA1 (326)	Receptor binding, antigenic determinant, pathogenicity determinant	[37, 40, 109, 117, 130, 151]
	HA2 (221)	Membrane fusion	[147]
5. NP (1540)	NP (498)	Regulates nuclear import, encapsidation of the viral genome	[18, 36, 56, 97]
6. NA (1434)	NA (469)	Cleavage of sialic acid receptors, facilitates virus release, prevents virus aggregation, supports penetration through mucus layer	[31, 110]
7. M (1002)	M1 (252)	Nuclear export of vRNPs, virion assembly, matrix protein	[21, 22, 66]
	M2 (97)	Regulation of internal pH of virions, virus uncoating, virion assembly and budding	[1, 116]
	M42 (99)	Supports virus replication	[150]
8. NS (865)	NS1 (219)	Nuclear export of viral mRNAs	[33, 106, 114]
	NS2 (NEP) (121)	Nuclear export of vRNPs, enhances RdRp activity	[16, 104, 113]
	NS3 (174)	Host specificity	[121]

*Strain: *A/swine/Iowa/18Tosu0505/2018(H1N1)*

snatching through binding to the 5' cap of the host mRNA, which is required for the synthesis of viral mRNAs in the host nucleus [52].

Specific adaptive mutations, most prominently the E627K in the PB2 protein [77], have been reported to facilitate the adaptation of avian influenza viruses into mammalian hosts [10, 80, 129]. Interestingly, it has been suggested that the 1918 pandemic influenza virus might have acquired the E627K mutation during the early emergence of the pandemic [134]. This mutation was retained by the 1957 H2N2 and 1968 H3N2 pandemic viruses [120], suggesting that these viruses had a reassortant genome containing the PB2 gene of the 1918 pandemic virus. Similarly, the D701N substitution in the PB2 protein has been reported to expand the host range of H5N1 viruses to humans [41]. Intriguingly, most of the strains of avian-like H3N2 viruses circulating in European swine populations have been reported to possess 701 N in the PB2 protein [129]. Other mutations, including T271A [55], G590S, and Q591R [154] in PB2, have also been implicated in determining the IAV host range. In summary, the role of PB2 is essential for transcription, determination of pathogenicity, virulence, and the interspecies transmission of IAV.

Polymerase basic 1 (PB1) gene

Polymerase basic 1 (PB1) is the second segment of the IAV genome that comprises 2316 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and encodes the PB1 protein of 757 amino acids. The PB1 gene also encodes for a PB1-F2 protein (approximately 79 amino acids) in an alternative + 1 open-reading frame in most IAV strains [32]. Intriguingly, the PB1 gene of highly pathogenic IAV strains, for example, H5N1 viruses, encode a relatively longer PB1-F2 polypeptide comprising approximately 90 amino acids [158]. Conversely, low pathogenic strains of IAV have been reported to have a somewhat shorter polypeptide expressing PB1-F2 protein [158]. The expression of PB1-F2 can enhance the pathogenicity of IAV [24, 62] by upregulating polymerase activity [67, 81] and inducing apoptosis [32, 62]. The full-length expression of the PB1-F2 protein was attributed to the pathogenicity of IAV pandemic strains of the twentieth century [82]; however, it was suggested that the apoptotic function of PB1-F2 was strain specific [82]. The PB1-F2 protein translocates to the inner membrane space in host cell mitochondria and impairs host cell immunity [158]. The amino acid residues 46–75 located within

the C-terminal domain of PB1-F2 protein were essential for mitochondrial translocation of PB1-F2 [153].

Wise et al. 2009 discovered another protein, termed PB1-N40, an N-terminal truncated form of PB1, translated by leaky ribosomal scanning and encoded by the second mRNA of PB1 [149]. The PB1-N40 is identical to the PB1 protein except for a truncated N-terminal, which is missing 39 amino acids reported as the primary interaction site with PA. As a result, PB1-N40 does not interact with PA; however, it can still interact with PB1, PB2, PB1-F2, and NP. It was observed that while PB1-N40 did not affect IAV viability, loss of expression of PB1-N40 was detrimental for IAV replication, triggering a low polymerase activity and slower replication kinetics [135]. This suggested that PB1-N40 supports IAV replication.

In the past, reassortments between avian PB1 and contemporary human influenza viruses have facilitated the emergence of the 1957 H2N2 and 1968 H3N2 influenza pandemics [64]. More recently, a naturally occurring S216G mutation in PB1 was reported to have facilitated the human adaptation of the 2009 pandemic influenza virus [72]. In addition, eight avian-origin H3N8 viruses were recently obtained from wild birds that had an S524G mutation in PB1. Interestingly, three of these viruses could transmit to guinea pigs without prior adaptation [161] which indicated a significant threat for increased transmission of these avian-origin H3N8 viruses. These findings emphasized the significance of the PB1 gene regarding interspecies transmission and adaptation of IAV.

Polymerase acidic (PA) gene

Polymerase acidic (PA) is the third segment of the IAV genome that comprises 2208 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and encodes the PA protein of 716 amino acids. The PA protein has multiple functions, including endonuclease, cap-binding, and promoter-binding activities [54]. PA, PB2, and PB1 form a heterotrimeric RdRp that binds to the viral RNA on a nucleoprotein backbone to form a viral RNP (vRNP) complex required for transcription and replication in the host cell nucleus [87, 100]. The endonuclease activity is regulated by 209 amino acid residues in the N-terminal region of the PA, a conserved domain in all influenza viruses [43], making this a potential target for anti-influenza therapeutics [160]. For example, Baloxavir marboxil, a prodrug of baloxavir acid, acts as a small molecule inhibitor of the PA protein subunit of the RdRp complex and inhibits the cap-dependent endonuclease activity of the PA protein, therefore, interferes with the IAV replication. Baloxavir marboxil was first approved in Japan and then the USA in 2018 to be taken orally as a single dose to treat acute uncomplicated influenza in people ≥ 12 years. Interestingly, Baloxavir marboxil has been more effective in reducing viral load more rapidly than other antiviral drugs, such as oseltamivir, a neuraminidase inhibitor. In addition, it was also found effective against those strains of influenza A and B viruses that were resistant to neuraminidase inhibitors such as oseltamivir [126].

In addition, the PA protein was also suggested to be implicated in IAV host switching. For example, the amino acid substitution, T522S, in PA, was implicated in the 1918 influenza pandemic and facilitated avian–to-human transmission of the 1918 avian influenza virus [134].

Jagger et al. showed that a ribosomal frameshift in PA gives rise to a second open-reading frame (ORF) expressing 252 amino acids, termed PA-X polypeptide [59]. The PA-X polypeptide expresses 191 amino acids in the N-terminal and 61 amino acids in the C-terminal of the PA protein. Intriguingly, approximately 75% of all the reported IAV genomes expressed a full-length PA-X polypeptide comprising 252 codons, while only 25% expressed a truncated version having only 232 codons (expressed only 41 C-terminal codons instead of 61) [59]. It was noteworthy that the truncated version of the PA-X polypeptides was predominantly present in swine IAV strains of the 2009 pandemic viruses along with a small percentage of swine H3N2 and swine H1N2 viruses that facilitated the emergence of the 2009 pandemic [50].

Muramoto et al. 2013 reported two N-terminal truncated proteins termed PA-N155 and PA-N182, translated from the AUG11 and AUG13 codons, respectively, in the segment 3 mRNA. They were suggested to be universally expressed in IAV strains. The evidence suggested that the mutant virus (M155L) lacking PA-N155 expression exhibited slower IAV replication kinetics in MDCK cells. In contrast, another mutant virus (M182L) lacking PA-N182 expression replicated similar to the wild-type virus. These observations suggested that even though PA-N155 and PA-N182 proteins were not essential for IAV replication, PA-N155 expression appeared important for efficient IAV replication [92].

Hemagglutinin (HA) gene

Hemagglutinin (HA) is one of the two surface glycoproteins encoded by the IAV. It comprises 1752 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and encodes 566 amino acids. Three monomers of HA (HA0) are assembled into a HA glycoprotein in the endoplasmic reticulum inside the host cell. The assembled HA homotrimers are then exported to the host cell surface through the Golgi network. The host proteases cleave the HA into two subunits: HA1 and HA2. The HA1 is a surface subunit responsible for binding to sialic acid (SA) receptors in the host, while HA2 is a transmembrane subunit that mediates the fusion of IAV and host's endosomal membranes after IAV internalization to the endosome [57].

The cleavage of HA in low pathogenic avian influenza viruses (LPAIV) which mostly possess arginine (R) at the cleavage site, occurs by trypsin in vitro [51]. Since trypsin-like proteases are restricted to the respiratory and intestinal tract of avian species, it could explain why LPAIV infection in birds is confined to the fecal–oral route. On the other hand, the cleavage of HA in highly pathogenic avian influenza viruses (HPAIV) occurs at the C-terminus of the multibasic consensus motif (R-X-R/K-R) by furin in the trans-Golgi network. Furin, a calcium-dependent serine endoprotease, is ubiquitously expressed in multiple organs and tissues, leading to systemic IAV infection, resulting in severe symptoms and fatal disease [14].

In addition, the cleavage of HA in human and swine IAV is activated by homologous proteases; for example, transmembrane protease serine S1 member 2 (TMPRSS2) and human airway trypsin-like protease (HAT) cleave HA at a monobasic cleavage site having arginine (R) [9, 105] at different times and in different cellular compartments during the IAV life cycle [14]. Cleavage of HA by HAT at the cell surface can take place at different time points either during assembly and budding of progeny virions supporting release of virus with cleaved HA or very late upon entry into a new cell during receptor binding [12]. On the other hand, cleavage of newly synthesized HA by TMPRSS2 occurs within the cell at the trans-Golgi network [13, 14, 105]. Interestingly, peptide mimetic protease inhibitors suppressed the cleavage activation of HA and resultant IAV spread in HAT- and TMPRSS2-expressing MDCK cells which suggested that these inhibitors could lead to the development of new antiviral drugs [12].

It is interesting to note that the avian IAV preferentially binds to α-2,3-linked SA and human IAV to α-2,6-linked SA. Swine are susceptible to infection with avian and human viruses because they possess both α-2,3-linked SA and α-2,6-linked SA in their respiratory tract. The abundance of SA receptors in various avian and mammalian hosts, schematically represented in Fig. 2, determines the efficiency of IAV binding and transmission [37, 40, 93, 98, 125, 139].

Several localized but severe bird flu outbreaks involving avian–to-human H5N1 transmission have been reported within family clusters, such as in Indonesia during 2006 and later in Turkey during 2015- 2016 [156], with a limited human-to-human transmission of the H5N1 virus; however, the severe respiratory illness in the exposed human subjects was reported. This happened because H5N1 viruses replicate efficiently in the lower respiratory tract of humans where α-2,3 SA receptors are more prevalent [125]. The human-to-human transmission would require an efficient H5N1 virus replication in the upper respiratory tract of humans having an abundance of α-2,6 SA receptors to facilitate the dissemination of the virus through coughing and sneezing [125].

Interestingly, a single amino acid substitution, E190D, in the HA protein appeared to have changed the binding affinity of the 1918 pandemic H1N1 influenza strain to the human SA receptors [49]. In a recent research article, we analyzed how specific amino acid substitutions in HA protein may facilitate the adaptation of avian-origin IAVs in swine [27]. Two amino acid substitutions in HA protein, Q226L, and G228S (H3 numbering), have been identified as the signature mammalian adaptation markers for avian-origin IAVs in swine [5, 27]. Amino acid substitution, T160A, was associated with swine adaptation of avian-origin H5N1, H5N2, H7N2, H7N9, and H9N2 viruses [27]. Another mutation, D225G, in the HA protein of avian H9N2 viruses was also reported to facilitate the swine adaptation of avian-origin H9N2 viruses [79]. Post-translational modification of HA by N-linked glycosylation has also been shown to influence adaptation and pathogenesis in the host [151].

The fusion of IAV virion and host endosomal membranes in the host cell cytoplasm is regulated by an irreversible conformational change in HA protein through the salt bridges, occurring at an acidic pH [109]. This was also observed during the 2009 influenza pandemic when low pH ≤ 5.5 contributed to the pathogenicity of the pandemic A(H1N1)pdm09 strain [117]. A more recent study determined that Eurasian avian-like swine H1N1 viruses required a higher acidic pH optimum for endosomal membrane fusion compared to contemporary avian H1N1 viruses of wild aquatic birds [6]. Therefore, varied host-specific fusion characteristics of HA protein may regulate interspecies transmission and adaptation of IAV subtypes in swine.

Nucleoprotein (NP) gene

Nucleoprotein (NP) is the fifth segment of the IAV genome that comprises 1540 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and encodes a nucleoprotein of 498 amino acids. It is highly conserved and is the most abundantly expressed protein in IAV-infected cells [68]. One of the major functions of NP is its vRNA-mediated interaction with the cap-binding domain of PB2 [132], which facilitates unprimed initiation of cRNA (transcription) and vRNA (replication) synthesis in the host cell nucleus [97]. The association of NP with vRNA occurs in a non-uniform manner and is required for genome packaging [11, 148] and maintaining the stability and helical structure of vRNPs [36]. In addition, the NP actively participates in the nuclear trafficking of vRNPs through nuclear export signaling (NES) [159] and nuclear localization signaling (NLS) [101] and, therefore, contributes to IAV replication. Due to its multifunctional activities and high sequence conservation among IAV strains isolated from various hosts, NP has become a high-profile antiviral drug target [58]. For example, the E339-R416 salt bridge, required for NP oligomerization and

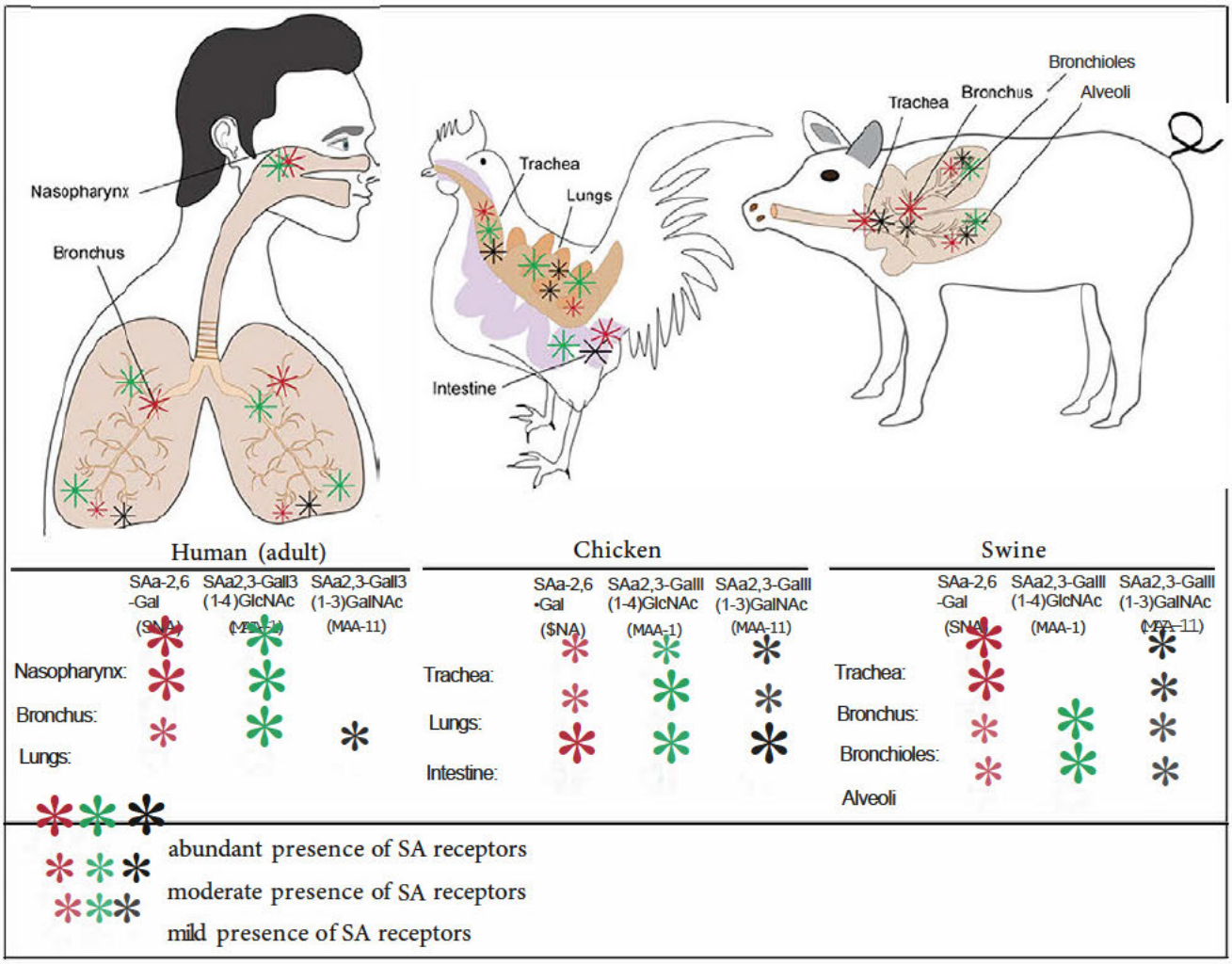

Fig. 2 A schematic representation of presence and distribution of a-2,6 (red stars) and a-2,3 SA receptors (green and black stars) in humans, chicken, and swine detected by ***Sambucus nigra*** agglutinin (SNA) and ***Maackia amurensis*** agglutinin (MAA) staining, respectively. The a-2,6 and a-2,3 (MAA-1) SA receptors are mostly present in the human nasopharynx and upper respiratory tract. In contrast, a-2,3 (MAA-II) SA receptors are present in the lower lungs of humans [98, 125]. Chicken alimentary and respiratory tract contain a-2,6, and a-2,3 (MAA-1) as well as a-2,3 (MAA-11) SA receptors [139]. The a-2,6 and a-2,3 (MAA-II) SA receptors are abundantly present in the swine trachea and bronchus, while a-2,3 (MAA-1) receptors are present in the lower lungs of swine [93]. The binding affinity of avian and human IAV to a-2,3-Jinked SA and a-2,6-linked SA, respectively, determines the host specificity of IAV. The presence and abundance of these receptors determine the host specificity and dynamics of IAV transmission. The star size represents the abundance of the corresponding receptor. The figure is based on the research data reported in the above-cited articles

essential for the vRNP activity, could become a potential antiviral drug target because E339A and/or R416A mutants inhibited IAV replication [58]. X-ray crystallography and electron microscopy showed that while wild-type NP exists as trimers, the E339A and R416A mutants exist as monomers; therefore, they could be the potential antiviral drug targets [123]. Similarly, Davis et al. 2017 characterized a conserved five amino acid region comprising Y289, K293, E294, Q308, and N309 within the NP body domain and showed that while single mutants Y289G, K293G, E294G, Q308G, and N309G only played a redundant role in viral RNA synthesis, the double mutant K293G/E294G showed the most significant defect in mRNA synthesis suggesting that this would also represent a viable antiviral target to counter IAV replication [39].

In addition, NP has also been implicated in host specificity. Zimmermann et al. 2011 suggested that human MxA protein, an interferon-induced human antiviral protein, may restrict interspecies IAV transmission from avian reservoirs to humans; however, adaptive mutations in IAV may allow them to evade MxA restriction and, therefore, would facilitate interspecies transmission. For example, they demonstrated that adaptive mutations in the NP of 2009 pandemic influenza viruses allowed them to evade MxA restriction

and, therefore, played an essential role in the host specificity of IAV [164].

Neuraminidase (NA) gene

Neuraminidase (NA) is the sixth segment of the IAV genome which comprises 1434 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and encodes the NA glycoprotein of 469 amino acids. The NA is a homotetrameric surface glycoprotein consisting of a globular head and a stalk domain that remains anchored to the membrane of the virion particle [155]. Enzymatic activity of NA cleaves terminal sialic acids, resulting in the disruption of the glycoconjugates on the surface of IAV-infected cells, allowing progeny virions to release from the infected host cells [35]. Interestingly, NA has also been implicated in complementing IAV infection in epithelial cells by removing decoy receptors present on mucins and cilia in the human respiratory system [83, 84], therefore, complementing virus entry into the host cell [31].

An interplay between HA and NA is required to allow for productive infection in the host [70, 84, 119]. Most of the IAV subtypes have an optimal NA activity at a pH between 5.5 and 6.5 [86]; however, a few IAVs have been reported as having stable NA activity at a much lower pH (between 4.0 and 5.0) [133]. The low pH triggers the inactivation of HA-receptor binding, leading to significantly lower NA activities, indicating that the enzymatic function of NA is dependent on HA binding [70]. In the event of the evolution of NA or HA that negatively impacts the attachment, replication, or transmission of the virus, specific mutations may be needed to restore the activity [84, 90]. For example, the NA glycoprotein in a human H3N2 virus acquired host receptor binding activity with a single amino acid mutation, D151G [73], has been shown to facilitate the binding affinity of NA to human α-2,6 as well as avian α-2,3 SA receptors [163].

Matrix (M) gene

Matrix (M) gene is the seventh segment of the IAV genome comprising 1002 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and may encode three polypeptides: M1, M2, and M42. M1 is the most abundant IAV protein and forms a thin layer directly underneath the lipid membrane [107]. M1 disintegrates from the virion lipid membrane leading to destabilization and disassembly of the IAV virion and the subsequent release of vRNPs into the host cell cytoplasm [22]. This is due to the conformational change at the low pH caused by histidine residues that function as pH sensors in late endosomes [127]. Another crucial function of the M1 protein is its role in the nuclear export of vRNPs [16] and IAV virion assembly. The M1 protein, due to its membrane-binding ability, facilitates interactions between lipids present in the budding virion envelope and other viral components [66, 137].

The M2 protein exists as tetrameric proton channels [20] and is encoded by spliced mRNA [150]. The low endosomal pH triggers the fusion of endosomal and virion membranes upon virus entry into the host cell, followed by the opening of the M2 proton channels, causing the vRNPs to dissociate from the M1 protein for release into the host cell cytoplasm [20]. Another essential function of M2 in the IAV replication cycle is its involvement in budding the IAV virion particles; localization of M2 at the neck of the budding virions facilitates the budding [116].

Wise et al. 2012 reported a novel-spliced variant of M2, designated M42, with an antigenically distinct ectodomain [150]. While M2 protein localizes in the plasma membrane, the M42 protein localizes in the Golgi compartment. Interestingly, M42 was a feature of the 1983 influenza outbreak caused by the HPAIV strain H5N2 in North America. Sequence analysis suggested that M42 may be restricted to a significantly small minority (~0.2%) of the available IAV sequences. Information on what controls M42 expression is currently not available. Intriguingly, M42 could functionally replace M2 as it also supports efficient virus replication in tissue culture cells, inducing pathogenicity in mice [150].

Non-structural (NS) gene

Non-Structural (NS) gene is the smallest and eighth segment of the IAV genome that comprises 865 nucleotides [Strain: "*A/swine/Iowa/18Tosu0505/2018(H1N1)*"] and encodes three proteins: NS1, NS2 or nuclear export protein (NEP), and NS3. The NS1 is a multifunctional, non-structural protein and is expressed in high levels in host cells upon IAV infection [115]. NS1 suppresses host antiviral responses by inhibiting interferon (IFN) and other host antiviral proteins [65]. The NS1 predominantly localizes in the host cell nucleus upon natural IAV infection; however, a significant proportion also localizes in host cell cytoplasm post-infection [53, 96]. A more recent study monitored the cellular trafficking of NS1 protein and reported its localization in the mitochondria of transfected cells 1.5 h post-infection to protect the infected host cells from early apoptosis to produce the progeny virus particles for a prolonged duration [140]. The NS1 also facilitates the nuclear export of viral M1 mRNAs by adapting the cellular nuclear export machinery and the viral mRNA [106]. It does so by interacting with the nucleoprotein of the vRNP complex due to its RNA-binding property [114]. Interestingly, a recent study reported that activating the phosphoinositide-3-kinase (PI3K) pathway of human cells, upon binding of NS1 with p85β subunit of PI3K pathway, increased the virulence of the 1918 pandemic H1N1 virus [33]. These findings illustrated the

multifunctional roles of NS1 in the IAV replication cycle and pathogenesis.

The NS2 or NEP is encoded by the spliced mRNA and is a structural component of the IAV virion particle. NS2 is also reported to be a multifunctional protein [113]. Some of the significant functions of NS2 include its role in mediating M1 protein binding to the vRNP complex and the facilitation of the export of the vRNPs from the host cell nucleus [16, 104, 124]. A recent study determined that NS2 interacts with the N-terminal FG domain of human nucleoporin 214 (Nup214) to facilitate the nuclear export of vRNPs [122]. Interestingly, adaptive mutations in three highly conserved amino acid residues in the C-terminal domain of NS2, including M16I, Y41C, and E75G, were determined to enhance the polymerase activity of a human-derived highly pathogenic H5N1 virus at 34℃. These mutations resulted in efficient H5N1 virus replication at cooler temperatures in the human upper respiratory tract, facilitating the mammalian adaptation of H5N1 viruses [111].

Selman et al. 2012 identified a novel IAV protein-designated NS3, expressed due to activating a new donor splice site upon A374G nucleotide substitution (GAT to GGT), encoding the D125G mutation, which was associated with mice adaptation of human-origin IAV [121]. Interestingly, this mutation was associated with host jumping from various avian species to swine, human, and canine hosts and was identified in 33 natural IAV strains, including avian-to-human spillover of the 1997 H5N1 and 1999 H9N2 viruses [142]. This suggested that NS3 expression might be implicated in IAV interspecies transmission and adaptation; however, it needs further investigation [121].

Host cell entry, replication, and egress of IAV

The binding between the host SA receptor and the HA1 subunit of the IAV hemagglutinin gene leads to the cell entry of IAV mostly through clathrin-mediated endocytosis [118]; however, macropinocytosis has also been identified to be an alternative pathway of IAV entry into the host cell [42]. The low endosomal pH triggers the opening of M2 ion channels located in the IAV membrane, causing a substantial conformational change in the HA protein. This subsequently leads to the fusion of the IAV membrane with the endosomal membrane [20], resulting in the rupture of the endosomal membrane releasing the vRNPs into host cell cytoplasm [4]. The vRNPs are then transported to the host cell nucleus by an energy-dependent active process using nuclear localization signals (NLSs) via the nuclear pore complex (NPC) [131]. The recent advances in high throughput imaging and RNA labeling technologies have enabled efficient monitoring of IAV nuclear trafficking pathways in single cells, revealing that the vRNPs can be delivered in the host cell nucleus within approximately one-hour post-IAV infection [44].

In the host cell nucleus, vRNPs transcribe viral mRNAs later translated into viral proteins in the host cell cytoplasm [46]. A primer-dependent cap-snatching mechanism mediated by the RdRp complex promotes the transcription [108]. In this process, PB2 binds at the 5' caps of the nascent host transcripts followed by the cleavage by the PA 10–13 bp downstream of the PB2 binding site [112]. The carboxy-terminal domain of the PA subunit feeds vRNA to the PB1 subunit resulting in transcription, generating full-length viral mRNAs. Once full-length gene transcription is achieved, the continued association of the PB1 subunit with the 5' capped end kickstarts reiterative stuttering, which introduces polyadenylation at the 3' end of mRNA, subsequently terminates the transcription [46, 61]. A schematic overview of IAV infection cycle is depicted in Fig. 3.

The viral mRNAs are then exported to the host cell cytosol for translation. Translation of viral mRNAs for polymerase (PB2, PB1, and PA) and NP, NS1, NEP, and M1 occurs at cytosolic ribosomes. At the same time, membrane glycoproteins HA, NA, and ion channel protein M2 are translated at endoplasmic reticulum-associated ribosomes [45]. The translated viral proteins are then imported to the host cell nucleus either in the form of a heterodimer (PA and PB1) or independently (PB2 and NP) via the importin-α- importin-β pathway activated by NLSs [45, 46].

The IAV replication is distinct from transcription as it is primer independent, and the resultant RNA product is neither polyadenylated nor capped [89]. The NP, polymerase, and cRNA together form an active and stable cRNP complex which serves as a template for viral replication to synthesize the viral genome [144]. A second newly synthesized polymerase mediates the formation of nascent RNP complexes on a template RNP [89, 144]. The mechanisms responsible for the helical structure of the vRNP complex formation due to the association of polymerases, NP, and the genomic RNA are still unexplored.

The M1 [17] and NEP [94, 99] play essential roles in the nuclear export of vRNPs. Additionally, the CRM1 or 'exportin 1,' a cellular export factor, functions as a carrier for the nuclear export of vRNPs [94]. The leucine-rich nuclear export signal (NES) motifs in the NP serve as the binding site for CRM1 [47]. Upon binding, in the presence of RAN GTPase, CRM1 exports vRNPs from the host cell nucleus to the cytosol through the NPC. The RAN GTP dissociates from the vRNP complex through hydrolysis into RAN GDP, leaving the vRNPs in the cytosol for cytoplasmic transport [46]. Studies using live-cell imaging techniques have demonstrated that vRNPs are transported through dense cell cytoplasm via a microtubule network [91]. The host factor YB-1 facilitates the interaction between vRNPs and RAB-11 at the microtubule-organizing center [63], where the PB2 of

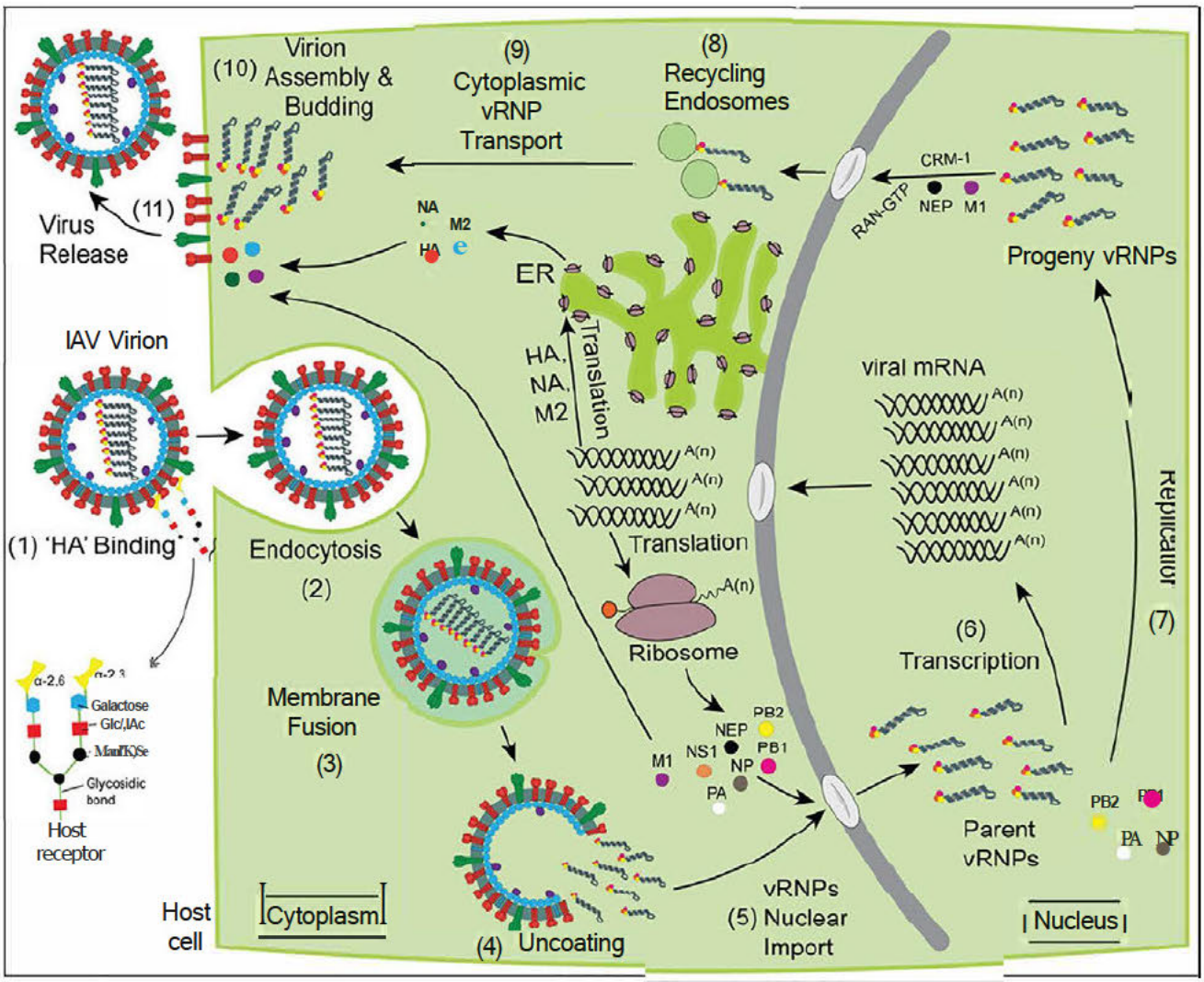

Fig. 3 A schematic representation of the IAV infection cycle in the host cell. (I) The HA glycoprotein binds to the host SA receptors, which leads to (2) virus entry into the host cell through endocytosis. (3) The low pH triggers the fusion of virion and endosomal membranes, leading to (4) the opening of M2 ion channels, which triggers uncoating the virion envelope to release the vRNPs into the host cell cytoplasm. (5) The vRNPs are transported to the host cell nucleus utilizing nuclear localization signals. (6) The carboxy-terminal domain of the PA subunit of the RdRp complex feeds vRNA to the PB I subunit resulting in transcription of full-length gene segments. The reiterative stuttering introduces polyadenylation at the 3' end of the mRNA, which terminates the transcription. The viral mRNAs are then exported to the cytoplasm for translation. Translation takes place in the cytosolic ribosomes and endoplasmic reticulum (ER)-associated ribosomes. *(I)* The NP, polymerase, and cRNA together form an active and stable cRNP complex which serves as a template for viral replication to synthesize progeny vRNPs. The M1, NEP, and NP proteins play crucial roles in the nuclear export of vRNPs through the NPC. (8) Progeny vRNPs attached to RAB-11 positive recycling endosomes are transported through the cell cytoplasm via a microtubule network. (9) The MI protein assists cytoplasmic transport of vRNPs, prohibiting their re ntry back to the nucleus and recruiting them to the plasma membrane for virion assembly and budding. (IO) The expression of viral surface glycoproteins HA and NA and M2-M I interaction is critical for assembly and budding of I AV virion particles at the host plasma membrane. (I I) The NA glycoprotein hydrolyzes the glycosidic linkages between SA receptors and sugar molecules and, thus, facilitates the release of newly assembled I AV virions from the host plasma membrane

the vRNP associates with RAB-11 [3] to facilitate cytoplasmic transport of vRNPs [2].

While the expression of HA and NA glycoproteins induces the budding of IAV progeny virions at the plasma membrane [29, 69], the M2 plays a vital role in the process [30]. Since M1 is associated with the nuclear export of vRNPs, it is believed to be associated with vRNPs during cytosolic transport to prohibit the re-import of vRNPs back to the host cell nucleus [25]. The M2-M1 interaction is critical for the assembly and budding of virion particles at the plasma membrane [25]. Due to its catalytic activity, the NA glycoprotein hydrolyzes the glycosidic linkages between SA

residues and sugar molecules. It, therefore, facilitates the release of IAV progeny virions during budding at the plasma membrane [84, 102].

In summary, establishing IAV infection, replication, and transmission is a multifactorial process involving several genes and mechanisms. The three polymerase proteins, PB2, PB1, and PA, along with nucleoprotein, are the components of the vRNP complex and the major determinants of IAV pathogenicity and virulence in the host. The nuclear import of polymerases and other viral components triggers transcription and replication in the host cell nucleus. By localizing polymerases in host cell mitochondria, IAV exploits the host cell's resources for advancing infection. While the HA and NA glycoproteins are required to initiate infection, M1, M2, NS1, and NEP proteins are essential for cytoplasmic trafficking of vRNPs and the assembly of the IAV virions. Identification of five more proteins over the last few years, including PB1-N40, PA-N155, PA-N182, M42, and NS3, suggested that our knowledge about influenza viruses is constantly evolving, particularly with the development of novel technologies. While some of these proteins have roles in IAV replication and/or host specificity, they also improved the existing understanding of IAV pathogenesis. Comprehensive knowledge of IAV protein functions and replication cycle would help identify potential targets for developing antivirals. Of note, the M2 inhibitors amantadine and rimantadine are no longer recommended by the regulatory authorities, such as the Food and Drug Administration (FDA) in the United States, due to significant drug resistance that the circulating IAV strains have acquired to this class of antivirals. Existing evidence suggests that drug resistance is rising against currently prescribed neuraminidase inhibitors, such as oseltamivir, particularly in pandemic A(H1N1) pdm09 and some of the H3N2 virus strains [71]. Therefore, exploring new antiviral drug targets such as PA and NP is imperative to combat the influenza disease burden.

Conclusion

Numerous important findings have been reported in recent years on IAV genes and protein functions, and therefore, it is essential to update our understanding of IAV genome biology. One striking observation in our study was the implication of surface glycoprotein gene NA in host receptor binding, which indicates that the full potential of NA glycoprotein in IAV pathogenesis is yet to be explored. Several underlying mechanisms responsible for the interactions between IAV proteins are yet to be explored to clarify the molecular basis of IAV genome evolution. One less known aspect is the mechanisms involved in accumulating vRNPs and other viral components at the host plasma membrane and their assembly into a virion particle. A comprehensive understanding of the IAV genes and protein functions and the mechanisms underlying the IAV replication cycle would help develop strategies to combat the IAV disease burden.

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CHAPTER 3

pathogens

MDPI

Review

A Systematic Review Analyzing the Prevalence and Circulation of Influenza Viruses in Swine Population Worldwide

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Abstract: The global anxiety and a significant threat to public health due to the current COVID-19 pandemic reiterate the need for active surveillance for the zoonotic virus diseases of pandemic potential. Influenza virus due to its wide host range and zoonotic potential poses such a significant threat to public health. Swine serve as a "mixing vessel" for influenza virus reassortment and evolution which as a result may facilitate the emergence of new strains or subtypes of zoonotic potential. In this context, the currently available scientific data hold a high significance to unravel influenza virus epidemiology and evolution. With this objective, the current systematic review summarizes the original research articles and case reports of all the four types of influenza viruses reported in swine populations worldwide. A total of 281 articles were found eligible through screening of PubMed and Google Scholar databases and hence were included in this systematic review. The highest number of research articles (n = 107) were reported from Asia, followed by Americas (n = 97), Europe (n = 55), Africa (n = 18), and Australia (n = 4). The H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses were the most common influenza A virus subtypes reported in swine in most countries across the globe, however, few strains of influenza B, C, and D viruses were also reported in certain countries. Multiple reports of the avian influenza virus strains documented in the last two decades in swine in China, the United States, Canada, South Korea, Nigeria, and Egypt provided the evidence of interspecies transmission of influenza viruses from birds to swine. Inter-species transmission of equine influenza virus H3N8 from horse to swine in China expanded the genetic diversity of swine influenza viruses. Additionally, numerous reports of the double and triple-reassortant strains which emerged due to reassortments among avian, human, and swine strains within swine further increased the genetic diversity of swine influenza viruses. These findings are alarming hence active surveillance should be in place to prevent future influenza pandemics.

Keywords: swine influenza virus; influenza A virus; influenza B virus; influenza C virus; influenza D virus; avian-origin influenza virus; influenza pandemic

1. Introduction

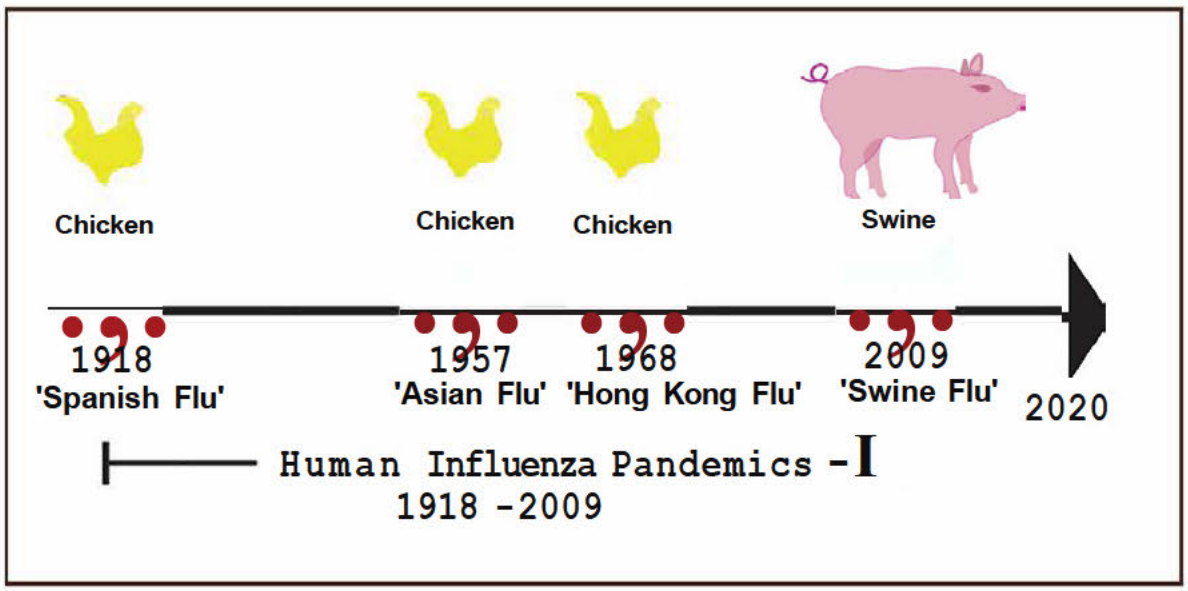
Influenza viruses are the members of Orthomyxoviridae family and have a wide host range [1-6]. Due to unique physiology, swine are considered the "mixing vessel" for influenza viruses [7]. Four types of influenza viruses have been reported in swine i.e., influenza A virus (IAV), influenza B virus (IBV), influenza C virus (ICV), and influenza D virus (IDV). The genomes of IAV and IBV have eight gene segments of single-stranded negative sense RNA while the genomes of ICV and IDV have seven gene segments [8]. Among the eight gene segments of IAV and IBV, the hemagglutinin (HA) and neurarninidase (NA) are most significant and crucial for the pathogenicity of these viruses which

determine the antigenic properties. The HA gene regulates the attachment of virus particles to the host receptor while NA gene regulates the release of progeny virus into the host cell. Co-infection of swine with two or more IAV strains may trigger the reassortment [9] which in turn, could facilitate the emergence of new influenza virus strains [10-12]. Point mutations which occur due to an error-prone RNA polymerase that lacks the ability of proof-reading and corrections during replication may also complement the genetic diversity of the influenza viruses [13]. The mechanisms of reassortment and point mutations may give rise to "antigenic shift" and "antigenic drift" within HA and NA genes, respectively, facilitating the emergence of new subtypes and lineages of influenza viruses. As a result, total 18 HA and 11 NA subtypes of IAV [14-16] and two lineages (Victoria/Band Yamagata/B) ofIBV have been reported so far in different hosts [17,18].

The host range of IAV and IBV is determined by their specificity to sialic acid receptors. The HA proteins of IAV can bind to a-2,3 and *a-2,6* sialic add receptors present in avian and human trachea, respectively [19-21]. Interestingly, swine trachea has both, a-2,3 as well as *a-2,6* sialic add receptors, due to which swine can become infected with avian and human strains of influenza viruses [22].

The genomes of ICV and IDV have a gene segment termed as "hemagglutinin-esterase-fusion" (HEF) which carries out the functions similar to that of HA and NA genes of I AV and IBV. The HEF is responsible for attachment and release of ICV and IDV virus particles into the host cell [23-25]. The particles of both virus types ICV and IDV bind to 9-O-acetylated sialic acid receptors of the host [25]. Several studies have shown that human and avian origin influenza viruses can be transmitted to swine in natural settings and thus may evolve into new strains of reassorted influenza viruses [26,27].

Historically, the first flu pandemic (Spanish flu) hit the human population in 1918 [28] and killed approximately 50 million people globally [29]. The 1918 influenza pandemic emerged as a result of reassortment in which human H1 virus acquired avian (poultry) N1 neuraminidase along with internal protein genes and evolved into what is now termed as "classical H1N1" virus [30] (Figure 1).



Fi$_{gu}$re 1. Timeline representing four human influenza pandemics between 1918 and March 2020. The first influenza pandemic known as "Spanish flu" originated in chicken in 1918. The second flu pandemic (Asian flu) and the third flu pandemic (Hong Kong flu) originated in chicken in 1957 and 1968, respectively. The most recent influenza pandemic known as "Swine flu" originated in swine in Mexico during March-May 2009.

The second flu pandemic occurred in 1957 (Asian flu) and was traced to the H2N2 virus which killed approximately two million people [31]. The third flu pandemic hit the human population in 1968 (Hong Kong flu) with an H3N2 outbreak and killed approximately two million people [31,32]. The most recent flu pandemic (Swine flu) originated in swine in Mexico during March-May 2009 [33]

and killed approximately 575,000 people worldwide [31]. The swine flu occurred due to a pandemic reassortant H1N1 virus termed as "A(H1N1)pdm09" virus [33].

Among four types of the influenza viruses, IAV is the most prevalent type and has been reported in swine in several countries. The IAV was first isolated from the nasal discharge of a swine in 1931 [34] and from the human in 1933 [35]. Strains of IAV have been reported to cause mild to severe upper respiratory tract illness in swine [36]. Strains of Victoria/B and Yamagata/B lineages of IBV were first reported in swine in the United States in 2010 [37] while the ICV in swine was first isolated in China in 1983 [38]. Both, the IBV and ICV cause mild respiratory illness in swine [39–42]. The IDV in swine was first detected in Oklahoma based swine in the United States in 2011 [5,43] which in later years has been detected in swine in China [44], Italy [45] and Luxembourg [46]. Interestingly, influenza viruses can be detected in the swine throughout the year unlike humans where seasonality affects the occurrence and progression of the disease [47,48].

An active surveillance of influenza viruses in swine is necessary for two basic reasons; to track the influenza virus evolution for improvements of the currently available diagnostic tests as well as for generating more effective vaccines for prevention and control of disease [49]. The currently available scientific data on swine influenza viruses would serve as a key to understand their evolutionary dynamics and transmission patterns. Therefore, this systematic review, for the first time, summarizes all four types of influenza viruses in the swine populations worldwide.

2. Methods

2.1. Systematic Review Protocol and Search Strategy

The guidelines and the procedures as detailed by the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [50] were followed for drafting this systematic review. Original research articles reporting influenza virus types IAV, IBV, ICV, and IDV in swine populations until February 21, 2020 were searched through PubMed and Google Scholar databases. The influenza virus sequence information was also verified using "*Influenza Virus Resource*" of NCBI (https://www.ncbi.nlm.nih.gov/genomes/FLU/) and "*Influenza Research Database*" (https://www.fludb.org/brc/home.spg?decorator=influenza). The sequence information helped in the identification of additional relevant articles reported from Indonesia, Kazakhstan and Sri Lanka.

The search terms including "Influenza outbreak in swine" OR "Influenza A virus in swine" OR "Influenza B virus in swine" OR "Influenza C virus in swine" OR "Influenza D virus in swine" OR "Influenza virus in pigs" were entered one by one in PubMed and Google Scholar databases to identify all full-text research publications or case reports which reported influenza virus types or subtypes in swine. The outcome suggesting research publications reporting influenza virus types and subtypes in swine along with the transmission of influenza viruses between human and swine, birds and swine, poultry and swine, cattle and swine as well as horse and swine were thoroughly investigated for inclusion. The search results suggesting influenza virus prevalence and/or transmission in or between species other than swine were omitted from the analysis. Occasionally, the full-text articles were also requested from the authors, if the full-text article was not available online. Two publications which could not be accessed were omitted from the analysis. Search results yielding articles in a language other than English were omitted from the analysis.

The articles were first screened through their abstracts to find out their relevance for inclusion, and, if required, the introduction and/or results and discussion sections were also screened to assess their relevance for inclusion. The relevant articles were downloaded and stored on the computer drive for further screening and refinement according to PRISMA guidelines [50]. The references of downloaded publications were also screened to identify relevant articles reporting the influenza viruses in swine, which were also downloaded to include in the analysis. An overview of the methodology can be observed in the PRISMA chart (Figure 2).

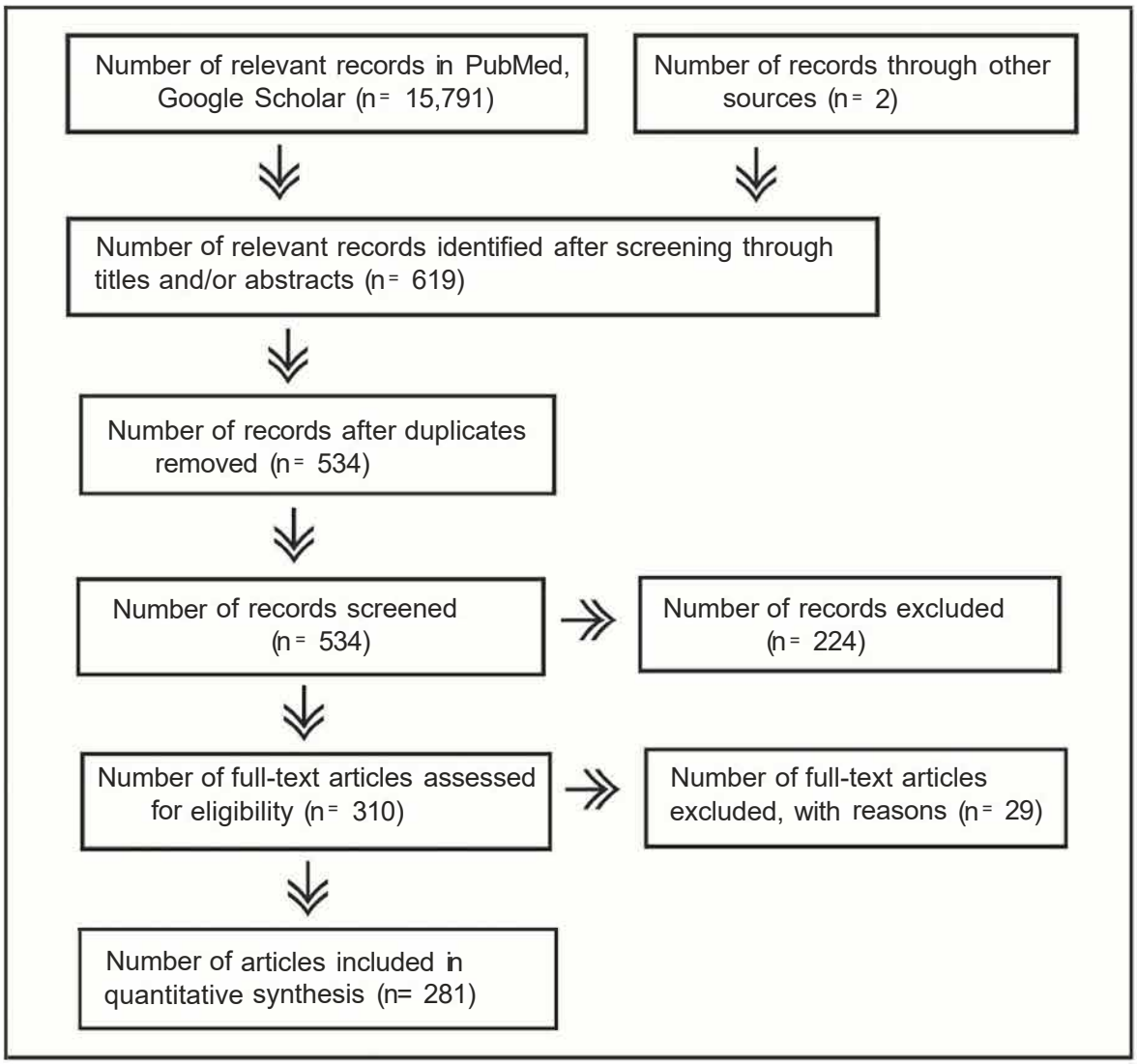


Figure 2. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) chart representing the search strategy. Total 281 articles were found eligible after applying the inclusion criteria. Original research articles reporting influenza virus types influenza A virus (IAV), influenza B virus (IBV), influenza C virus (ICV), and influenza D virus (IDV) in swine populations available until Februa1y 21, 2020 were downloaded from the PubMed and Google Scholar databases.

2.2. *Inclusion and Exclusion Criteria.*

The following criteria were applied for screening of the eligible articles:

The original research articles and case reports which documented the influenza viruses in swine in natural settings across the world were included in the analysis.

The experimental studies which did not report the natural cases were excluded from the analysis.

The reviews, letters, editorials, conference proceedings, and articles in a language other than English were not included in the analysis. Duplicate articles were also excluded from the analysis.

The eligible articles (n = 281) thus selected were included in the analysis for this systematic review.

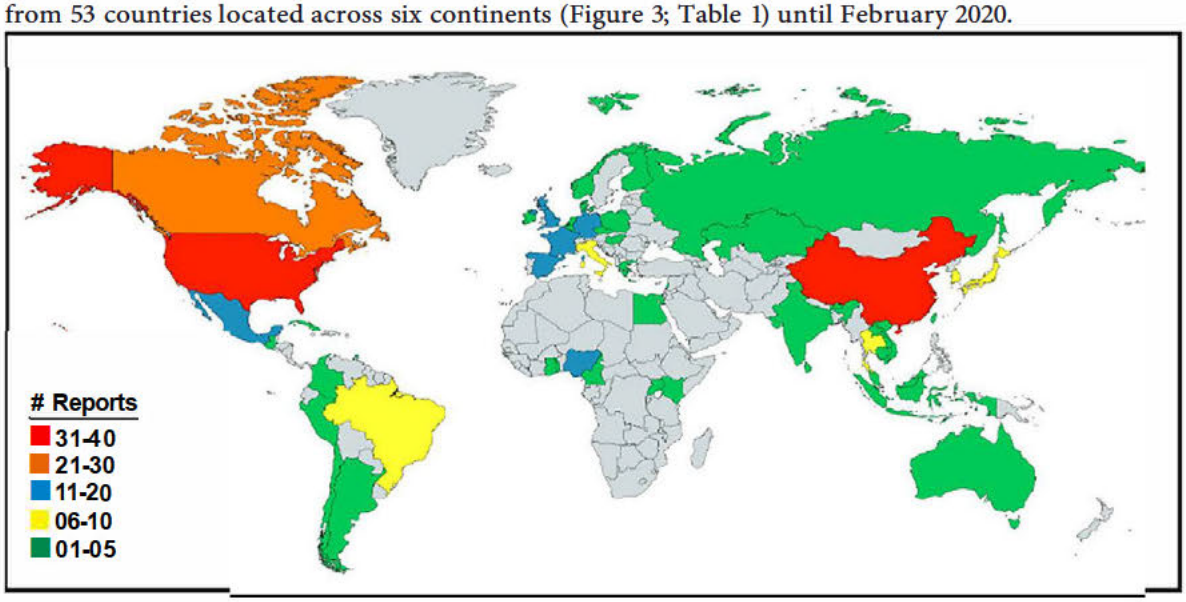
2.3. *Ethical Approvals*

This systematic review did not involve animal sampling or experimental protocols in the laboratory. The data used for writing this article were obtained from the PubMed and Google Scholar

databases. This systematic review is part of a research project which has already obtained the relevant ethical approvals from the Animal Research Ethics Committee (AREC), University of KwaZulu-Natal, Durban, South Africa; AREC Reference: AREC/041/019D. Additionally, the authors have the required permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) from the Department of Agriculture, Forestry and Fisheries (DAFF), Government of the Republic of South Africa; DAFF Reference: 12/11/1/5/4 (1425).

3.Results

The original research articles and case reports on the serological and virological prevalence of all the four genera of influenza viruses i.e., I AV, IBV, ICV and IDV were downloaded, analyzed and summarized in the region-specific manner across the world. Influenza viruses have been reported from 53 countries located across six continents (Figure 3; Table 1) until February 2020.



Fi$_{g\,u}$re 3. The world map represents the prevalence of influenza viruses i.e., IAV, IBV, ICV, and IDV in swine populations until February 2020. Highest nwnber of aiiicles were reported from the United States (*n* =40), followed by China (*n* =39), Canada (*n* =24) and other countries. The world map was created online at https://mapchart.net.

Table 1. Prevalence of Influenza viruses in swine populations worldwide.

Continents	Countries	Influenza A Virus (IAV) Subtypes	Other Influenza Virus Types	References
Africa	Cameroon	H1N1, A(H1N1)pdm09	None	[51–53]
	Nigeria	H1N1, H3N2, A(H1N1)pdm09, H5N1	None	[52,54–59]
	Ghana	H3N2	None	[57]
	Egypt	H5N1, H5N2, H9N2, A(H1N1)pdm09	None	[60,61]
	Kenya	H1N1, H3N2, A(H1N1)pdm09	None	[62–64]
	Benin, Cote d'Ivoire	None	None	[65]
	Reunion island	A(H1N1)pdm09	None	[66]
	Togo	A(H1N1)pdm09	None	[67]
	Uganda	IAV	None	[68]
Asia	China	H1N1, H1N2, H3N2, A(H1N1)pdm09, H5N1, H9N2, H4N1, H4N6, H5N3, H10N5, H4N8, H6N6, H7N9, H3N8	ICV, IDV	[69–106]
	Bhutan	H1N1, A(H1N1)pdm09	None	[107]
	Cambodia	H1N1, H3N2, A(H1N1)pdm09	None	[108,109]
	Japan	H1N1, H1N2, H3N2, A(H1N1)pdm09	ICV	[110–126]
	South Korea	H1N1, H1N2, H3N2, A(H1N1)pdm09, H7N2, H5N2, H3N1	None	[127–143]
	Thailand	H1N1, H1N2, H3N2, H3N1, A(H1N1)pdm09	None	[144–159]
	Viet Nam	H1N1, H1N2, H3N2, A(H1N1)pdm09, H5N1	None	[160–163]
	India	H1N1, H2N2, H3N2, A(H1N1)pdm09	None	[164,165]
	Lebanon	H9N2	None	[166]
	Malaysia	H1N1, H3N2	None	[167]
	Laos	H3N2	None	[168]
	Russia	H1N1	None	[169]
	Taiwan	IAV	IBV	[170,171]
	Indonesia	H5N1	None	[172]
	Sri Lanka	H3N2, A(H1N1)pdm09	None	[173]
	Kazakhstan	H1N1, H3N2	None	[174]
Australia	Australia	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[175–178]

Table 1. *Cont.*

Continents	Countries	Influenza A Virus (IAV) Subtypes	Other Influenza Virus Types	References
Europe	Belgium	H1N1, H1N2, H3N2	None	[179–181]
	Denmark	H1N1, H1N2, H3N2	None	[182–184]
	United Kingdom	H1N1, H1N2, H3N2, A(H1N1)pdm09, H1N7	IBV, ICV	[185–192]
	Finland	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[193,194]
	France	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[195–199]
	Germany	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[200–203]
	Greece	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[204]
	Italy	H1N1, H1N2, H3N2, A(H1N1)pdm09	IDV	[205–217]
	Spain	H1N1, H1N2, H3N2	None	[218–221]
	Netherlands	H1N1, H1N2, H3N2	None	[222]
	Norway	A(H1N1)pdm09	None	[223–226]
	Poland	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[227–229]
	Czechoslovakia	H3N2	None	[230]
	Hungary	H1N1	None	[231]
	Czech Republic	H1N1, H1N2, H3N2	None	[232]
	Republic of Ireland	H1N1, H1N2, H3N2	None	[232]
	Luxembourg	None	IDV	[46]
	Multiple European Nations	H1N1, H1N2, H3N2	None	[233]
North America	Canada	H1N1, H1N2, H3N2, A(H1N1)pdm09, H4N6, H3N3	None	[234–258]
	USA	H1N1, H1N2, H3N1, H3N2, A(H1N1)pdm09, H4N6, H2N3	IBV, IDV	[259–303]
	Mexico	H1N1, H1N2, H3N2, A(H1N1)pdm09, H5N2	None	[304–309]
	Guatemala	H3N2, A(H1N1)pdm09	None	[310]
	Cuba	H1N1, A(H1N1)pdm09	None	[311,312]
	Trinidad & Tobago	H3N2, A(H1N1)pdm09	None	[313]
South America	Argentina	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[314–318]
	Brazil	H1N1, H1N2, H3N2, A(H1N1)pdm09	None	[319–329]
	Colombia	A(H1N1)pdm09	None	[330]
	Peru	A(H1N1)pdm09	None	[331]
	Chile	IAV, H1N2	None	[332–334]

3.1. Influenza Viruses in Swine in Africa

3.1.1. Cameroon

The first report of IAV in Cameroonian swine appeared when A(H1N1)pdm09 virus was documented during 2009–2010. The youngest infected swine was four-month old which suggested that A(H1N1)pdm09 virus can infect the young piglets [51]. Nine more swine herds in Cameroon were found infected with A(H1N1)pdm09 and H1N1 viruses during May–June 2011 [52]. A multiple-site study including free-roaming and penned swine along with domestic poultry and Columbiformes birds between December 2009 and August 2012 identified one IAV positive swine at each of the two study sites. The inter-species transmission of IAV was ruled out as all the birds were negative for the IAV [53].

3.1.2. Nigeria

The first evidence of the past IAV infection in Nigerian swine appeared in 2008 when H1N1 and H3N2 virus antibodies were detected in swine sampled at three different locations [54]. Shortly after that, in 2009, the first report of A(H1N1)pdm09 virus appeared in the Nigerian swine when one swine herd was found seropositive for A(H1N1)pdm09 virus. Interestingly, eight other swine herds were found seropositive for the H1N1 and four herds were found positive for human-like H3N2 viruses. The seroprevalence of IAV further increased as 66 swine herds were detected positive for A(H1N1)pdm09 virus and 53 herds were found seropositive for H1N1 virus in 2012 [52]. The active infection (viral RNA) of A(H1N1)pdm09 virus was first reported in the Nigerian swine between July 2010–June 2012 when 18 A(H1N1)pdm09 virus isolates were retrieved from the swine in Lagos. The zoonotic transmission of A(H1N1)pdm09 virus to the exposed human workers was ruled out as all the human samples were negative for the A(H1N1)pdm09 virus [55].

Nineteen more A(H1N1)pdm09 and five human-origin H3N2 viruses were identified in the Nigerian swine during 2013-2015 [56]. Later one more report of the human strain of H3N2 virus appeared in swine during January–February 2014 [57]. A high seroprevalence of IAV in a commercial piggery was reported in Lagos. Total 197 human and 281 swine sera samples were screened which determined that 87% human and 67% swine sera had antibodies for IAV depicting the past exposure [58] but the active infection was absent given that all nasal swabs were negative for IAV infection [58]. Lately, highly pathogenic avian influenza virus (HPAIV) strain H5N1 was detected in 22 swine samples between December 2015 and February 2016 during an ongoing H5N1 disease outbreak in Nigerian poultry [59] which indicated the inter-species transmission of H5N1 virus from poultry to swine [59].

3.1.3. Egypt

A molecular study reported the negative prevalence of avian-like H5N1 and H5N2 viruses in Egyptian swine in May 2008 [60] but the serological investigation identified H5N1 virus antibodies in seven and H5N2 virus antibodies in four swine sera samples [60] which suggested a past exposure of these swine to the viruses. The active H5N1 infection in Egyptian swine was again ruled out in October 2013 as the viral RNA could not be detected in 36 swine samples but interestingly, the antibodies against avian-like H5N1, H9N2, and A(H1N1)pdm09 viruses were detected in swine sera samples which suggested a past exposure [61]. Interestingly, 122 of the 157 swine nasal swab samples collected during 2014 and 2015 were found positive for IAV active infection using RT-PCR. As a result, HA subtyping identified 46 avian-origin H5N1, seven H9N2 and 69 A(H1N1)pdm09 viruses [61].

3.1.4. Kenya

The first report of IAV in swine in Kenya appeared in May 2010 when A(H1N1)pdm09 virus were detected in eight swine samples collected from the Asembo and Kibera counties and at a Nairobi based abattoir. The extended serological study further identified H1N1 and H3N2 viruses in swine during August 2011 to December 2012 [62]. The active IAV infection was reported in four household members

having acute respiratory illness while the backyard swine were negative for the IAV and IBV in Kiambu county during September 2013–August 2014. On the contrary, the serology identified the IAV antibodies in 230 swine sera samples suggesting a past exposure of IAV [63]. The A(H1N1)pdm09 virus was again reported in five swine samples collected from a slaughterhouse in Kenya during September 2013–September 2014. Interestingly, all the 288 human subjects including the slaughterhouse workers or the traders and farmers who had visited the slaughterhouse were negative for IAV, hence ruled out the zoonotic transmission [64].

3.1.5. Other African Countries

Swine in Benin and Cote d'Ivoire reported no prevalence of IAV during 2009–2010 [65] while A(H1N1)pdm09 virus was detected in swine in Reunion Island during 2009–2011 [66] and in Togo during October 2012–January 2014 [67]. The human strain of H3N2 virus was detected in swine in Ghana during January–February 2014 [57]. One more report documented IAV in swine in two districts of Uganda in 2015 [68].

Overall, influenza viruses have been reported in swine from eight African countries including Cameroon, Nigeria, Egypt, Kenya, Reunion island, Togo, Ghana, and Uganda (Figure 4A). The A(H1N1)pdm09 virus, which originated in Mexican swine in 2009, has been reported in all except Ghana and Uganda. Interestingly, the HPAIV strain of H5N1 has been reported in swine in Nigeria and Egypt while HPAIV strain H5N2 and low pathogenic avian influenza virus (LPAIV) strain H9N2 have also been reported in the Egyptian swine (Table 1).

3.2. Influenza Viruses in Swine in Asia

3.2.1. China

China is considered the epicenter of influenza viruses [69]. The first seroprevalence of IAV in Chinese swine was documented during 1977–1982 when antibodies for 38 H1N1, 22 H3N2, 12 H4N6, 12 H5N3, and seven H9N2 viruses was detected in swine sera obtained from apparently healthy swine [70]. The first ever report of ICV in swine was documented from the apparently healthy swine in Beijing when 15 ICV isolates were retrieved during January–December 1981 [38]. Three isolates of reassortant H1N2 virus were identified after an influenza-like illness triggered abortions and mortalities in sows on a swine farm in November 2004 [71]. The same year, LPAIV strain H9N2 was isolated from the sick or dead swine in China which was the first ever isolate of H9N2 virus retrieved from a swine [72].

First human-origin H1N1 and four human-origin H3N2 virus isolates in Chinese swine were retrieved during 2005–2006 [73]. Further, two isolates of swine H3N2 viruses, four isolates of avian-origin HPAIV strain H5N1 and two isolates of H1N1 viruses were detected in swine nasal swab and lung tissue samples collected from swine in central provinces of China during 2004–2006 [74]. Surprisingly, two isolates of equine influenza virus H3N8 were also detected in swine during December 2005 and January 2006 [74]. Another report of avian-origin H9N2 virus in Chinese swine was documented during 2006–2007 when four H9N2 virus isolates with closely related nucleotide sequences were retrieved from swine [75]. Each of the two different investigations reported 19 H1N1, one H1N2 and nine H3N2 virus isolates from Chinese swine during 2006–2009 [76,77]; the H1N2 virus and all nine isolates of H3N2 viruses were either double or triple-reassortant viruses [76].

The first report of HPAIV strain H5N1 in swine was documented during October 2008–May 2009 when two H5N1 virus isolates were retrieved from apparently healthy swine [78]. The third report of avian-origin H9N2 virus in Chinese swine appeared when 144 apparently healthy swine across four provinces viz., Yunnan, Guangdong, Fujian and Zhejiang were found H9N2 positive over a four-year period during March 2008–March 2012. The frequent interactions of birds to the swine at the study sites was reported which was suspected to be the most likely source of infection [79]. Further, a novel strain of avian-origin H4N1 virus was isolated from a Chinese swine in 2009 [80].

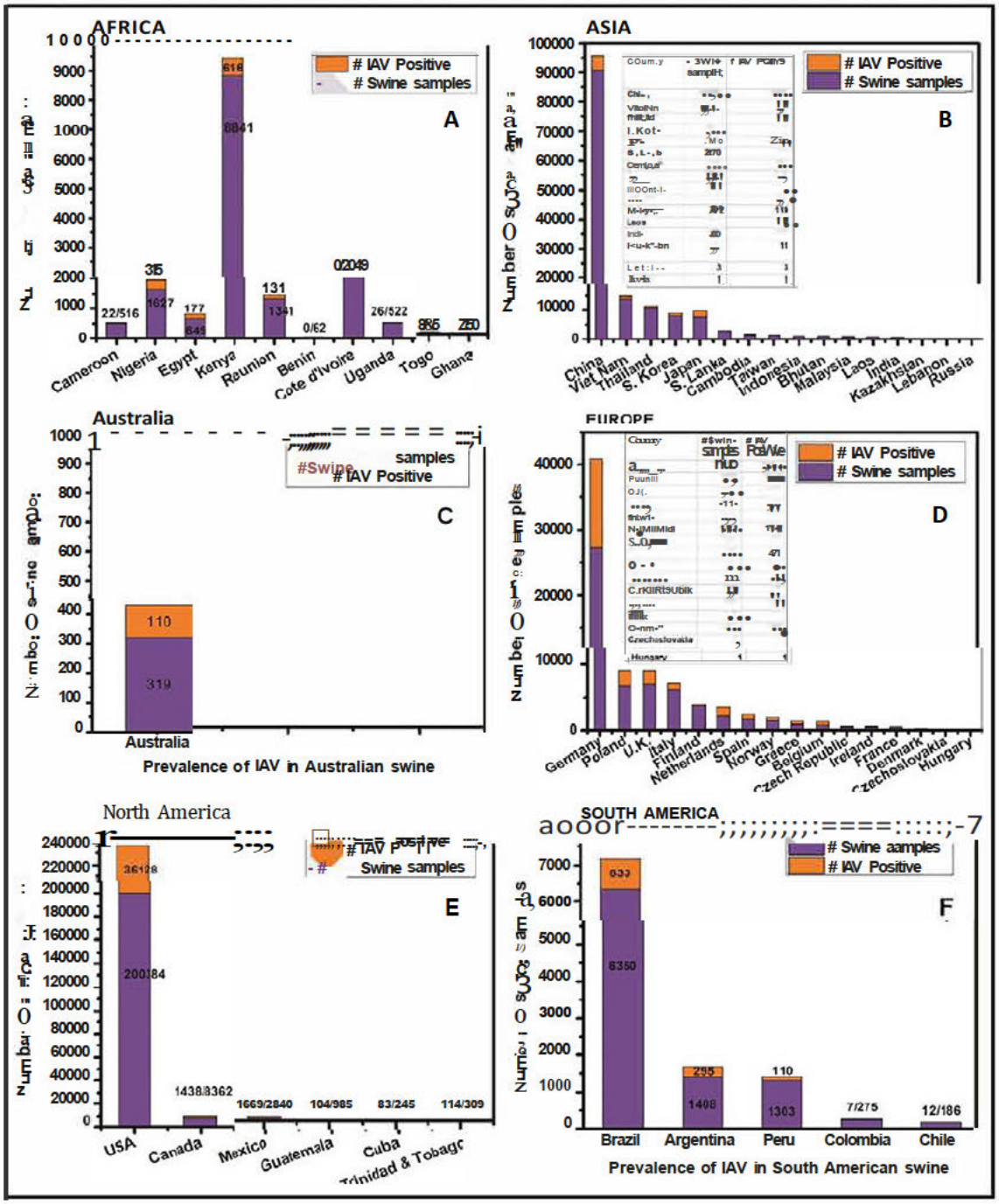


Figure 4. Country-specific prevalence of IAV in swine populations worldwide. (A) IAV prevalence in African swine population with highest prevalence reported from Kenya. (B) IAV prevalence in Asian swine where most cases were reported from China. (C) IAV prevalence in Australian swine. (D) IAV prevalence in European swine population. Germany reported the highest number of cases in Europe. (E) IAV prevalence in North American swine with most of the cases in the United States. (F) IAV prevalence in South American swine with most cases reported from Brazil The variations in the prevalence of influenza viruses in swine populations among countries may be because of certain factors including (a) swine populations differ greatly among countries; (b) surveillance efforts differ greatly among countries; (c) the non-English publications were excluded from the analysis. These graphs do not represent the severity of disease as the swine populations and the objectives of surveillances may vary among countries.

Several classical and avian-like H1N1, Eurasian avian-like H1N1, triple-reassortant H1N1, H1N2, H3N2 and A(H1N1)pdm09 viruses were reported in Chinese swine between 2009 and 2016 [81–91]. A triple-reassortant H1N1 virus having the internal genes of avian, human, and swine lineages of influenza viruses was reported from a two-month old piglet on a Guangdong based swine farm in January 2010 [92]. Three reassortant H3N2 virus isolates having internal genes of A(H1N1)pdm09 virus were reported in swine between November 2010 and June 2011 [93].

A three-year old boy was diagnosed with European origin avian-like H1N1 virus on a family swine farm in a rural area of the Jiangsu province in December 2010 which speculated a zoonotic transmission from swine to the boy [94]. The first report of H10N5 avian-origin influenza virus in a domestic swine in Hubei province further extended the diversity of swine influenza viruses and provided another evidence of interspecies transmission of avian influenza virus to the swine under natural conditions [95]. Several other avian-origin H3N2, H4N8, H6N6, H7N9, H5N1, and H9N2 virus antibodies were detected in swine in China during April 2010–June 2014 [86,96–98].

Another interspecies transmission of avian-like H1N1 virus in southern China was observed when 219 swine and 61 swine farm workers were identified to be infected with avian-like H1N1 swine influenza virus between March 2011 and March 2013 [99]. Further a zoonotic transmission of H9N2 virus was identified at a Shandong based swine farm during May 2013–April 2014 when H9N2 virus antibodies were detected in 84 swine and four farm workers. The wild birds visiting swine feeding sites at the swine farm were speculated to serve as the carrier for H9N2 virus [100]. Zoonotic transmission of H1N1 virus was reported on a swine farm in Shandong province between March 2015 and February 2016 among the swine exposed human workers having influenza-like illness. As a result, five of the 32 (15.6%) nasal swab samples were found IAV positive; a married couple exposed to swine were found infected with H1N1 virus [88].

The IAV infection was also documented in 44 wild boars in Jilin province of China between April 2015 and February 2016 [101]. The first report of the IDV prevalence in Chinese swine documented 21 IDV positive swine in the Guangdong province in 2016 [44]. The swine IDV sequences shared high similarity (99–100%) with IDV sequences reported earlier from the bovine species in China [102] which indicated the transmission of IDV from bovine to swine in China.

3.2.2. Hong Kong and Tibet

Hong Kong is a special administrative region while Tibet is an autonomous administrative region under the control of People's Republic of China. The H1N1 and H3N2 virus isolates were successfully retrieved from apparently healthy swine in Hong Kong during July 1993–June 1994 [103]. Further, classical swine H1N1, H3N2 and avian-like H9N2 viruses were identified in Hong Kong based swine between March 1998–June 2000; two independent introductions of the avian-like H9N2 viruses were ascertained from avian species to the swine [27,104,105].

The first information of IAV seroprevalence in Tibetan swine appeared during April–December 2010 when antibodies against H1N1 and H3N2 viruses were detected in swine sera collected from Tibet [106].

3.2.3. Bhutan

The first report of H1N1 seroprevalence in swine in Bhutan appeared when H1N1 virus was detected in backyard as well as breeding swine during October 2011 and February 2012 [107].

3.2.4. Cambodia

The H1N1 virus was reported in swine over a five year-period between 2006–2010 while the A(H1N1)pdm09 and H3N2 viruses were identified only in 2010 [108]. Later three triple-assortant H3N2 viruses were isolated and sequenced from the backyard swine between May 2011 and July 2012 [109].

3.2.5. Japan

The antibodies against A/Hong Kong(H3N2) virus termed as "*A/Swine/Wadayama/5/69*" were first detected in Japanese swine in 1969 [110,111]. The H3N2 virus seroprevalence in Japanese swine was further documented in Sendai City during 1977 to 1980 [112]; the transmission between human and swine was also suggested [112]. The first active IAV infection was reported when two reassortant H1N2 virus isolates were retrieved from the Japanese swine having influenza-like disease in 1978. The isolated H1N2 virus was believed to be a recombinant of H1N1 and H3N2 viruses [113]. Further 340 swine were diagnosed with H1N1 antibodies in Toyama Prefecture between 1978–1982. A lower seroprevalence was observed during the summer months while the seroprevalence was relatively higher during the winter season [114]. Again, one more H1N2 virus was isolated and characterized from the sows in Ehime Prefecture in September 1980 [115]. Intriguingly, 18 H1N1, H1N2 and H3N2 viruses were detected in swine imported from the United States, however, all the imported swine from the Europe were negative for the IAV infection. This was the first report of the IAV infection in the imported swine [116].

The ICV seroprevalence (19%) in Japanese swine was first reported in the Hyogo Prefecture during July 1981–June 1982 [117] but swine in Yamagata Prefecture were found seronegative for the ICV between August 1979 and March 1986 which suggested a localized transmission of ICV in swine within Hyogo Prefecture [118].

Several other reassortant H1N2 virus isolates were reported in Japanese swine after 1991 [119]. One novel reassortant H1N2 virus appeared to have emerged from the A(H1N1)pdm09 virus was reported in swine in Gunma Prefecture while two other H1N2 viruses appeared to have emerged from the Japanese H1N2 viruses with internal genes from A(H1N1)pdm09 virus. One more H1N2 virus was detected in swine which was closely related to the Japanese H1N2 virus [120].

The immunohistochemistry identified lesions in the lungs of the sick swine infected with reassortant H1N2 virus [121]. Additionally, several H1N1 and H3N2 viruses have also been reported in Japanese swine between 1990 and 2017 [122,123]. Interestingly, six H1N1 virus isolates were identified with reassorted genes from A(H1N1)pdm09 virus while one H1N1 isolate appeared to have H1 gene from Japanese swine influenza virus with internal genes of A(H1N1)pdm09 virus. Further, one H3N2 virus isolate was determined to have genes of Japanese swine influenza and A(H1N1)pdm09 viruses [124]. These results reflected the occurrence of the reassortment events between Japanese swine influenza and A(H1N1)pdm09 viruses.

IAV seroprevalence has lately been reported in wild boars (*Sus scrofa leucomystax*) in Japan. Three wild boars in the Yamaguchi Prefecture were found seropositive for A(H1N1)pdm09 virus while nine wild boars in Tochigi Prefecture were seropositive for the swine H1N1 virus. But, the active IAV infection could not be identified in these wild boars as all the nasal swab samples were negative for IAV and IBV [125]. In a more recent investigation, fifteen wild boars were found seropositive for A(H1N1)pdm09 virus in Kagoshima Prefecture between November 2014–December 2017 while two of these fifteen wild boars had antibodies against H1N2 and H3N2 viruses as well [126]. This reflected a past exposure of the Japanese wild boars to the IAV strains.

3.2.6. South Korea

The first active IAV infection in the Korean swine was identified in December 1998 when three H3N2 virus isolates were recovered from the swine experiencing an acute influenza-like respiratory disease. The close relatedness of these Korean swine H3N2 isolates with human-origin H3N2 viruses reported from Korea between 1987–1999 suggested the events of reverse zoonosis [127]. One unique H7N2 virus isolate was detected in swine which had seven gene segments originated from Hong Kong avian-origin H7N2 virus isolated in 1978 and the NS gene originated from Hong Kong H5N3 virus isolated in 1977. Additionally, four typical swine influenza H1N1 viruses were identified in swine [128].

Several H1N1, H1N2, and H3N2 viruses were detected in symptomatic South Korean swine after 2000 [129–134]. The IAV localization in the swine lung tissues was confirmed by immunohistochemistry [130]. Total 35 avian-origin H5N2 viruses of Eurasian lineage were identified in swine in different South Korean provinces during 2004–2008 which suggested cross-species transmission of H5N2 virus [135].

Three H1N1 virus isolates closely related to US isolates of H1N1 were obtained from 45-day-old piglets in Korea in January 2005. The other swine farms in the proximity of this index farm were negative for the H1N1 virus [136]. Further, one H1N1, two H1N2, and one H3N2 subtypes of IAV identical to the American strains based on their HA and NA gene sequences were obtained from swine nasal swab, lung, and thoracic fluid samples during 2005–2006 which suggested that there was no probability of arising of these IAV strains in Korea through recombination [137].

Two novel isolates of swine H3N1 virus with high genomic similarity to each other were retrieved from two different swine farms in Korea during March–April 2006 which would be due to a common origin of these isolates. These viruses had human-like H3 gene while other gene segments originated from swine influenza viruses within Korea. High reactivity of the 52 swine sera samples to H3N1 virus antibodies suggested a previous exposure and probability of the swine to swine transmission of H3N1 virus [138].

The human to swine transmission of A(H1N1)pdm09 virus was reported in Chungbuk province where 42 A(H1N1)pdm09 virus isolates were recovered from swine lung tissues [139]. The reassortment between A(H1N1)pdm09 and swine H1N2 viruses emerged into a novel reassortant H1N2 virus in swine [140]. A triple-reassortant H3N2 virus was identified in swine during December 2011–May 2012 which indicated the IAV reassortment was taking place in Korean swine [141]. A swine fever eradication campaign identified nine A(H1N1)pdm09, two classical H1N1 and one H1N2 viruses in wild boars which were hunted and killed in South Korea during 2012 [142]. More recently, a complete genome sequence of H1N1 virus was reported from a domestic swine in Korea in 2016 [143].

3.2.7. Thailand

The occurrence of IAV in Thai swine was first reported during November–December 1978. Active H3N2 infection was detected in one swine while several other swine had H3N2 antibodies [144]. Two H1N1 virus isolates from Thai swine were first recovered in January 1988 [145]. Several studies reported H1N1, A(H1N1)pdm09, H1N2, and H3N2 viruses in swine exhibiting respiratory disease symptoms between 2000 to 2014. Intriguingly, one swine sample was found co-infected with four IAV subtypes including H1N1, H1N2, H3N1, and H3N2 viruses [146–152].

The first evidence of H5N1 seroprevalence in Thai swine was documented in 2004 when eight H5N1 positive swine sera samples were identified [153]. Later ten H1N1 and two H3N2 virus isolates were retrieved from piglets aged between 4 to 12 weeks during 2008–2009 [154]. Interestingly, most of the virus isolates retrieved in this study were obtained from 4 to 8 week-old piglets which was in agreement of a previous report stating that swine influenza viruses can be successfully retrieved from piglets less than ten weeks of age [155].

A zoonotic transmission of IAV was reported at a Thai swine farm where all the swine were found positive for either H1N1 or H1N2 virus. Interestingly, two farm owners, 46 swine handlers, four veterinarians, five farm cleaners and two farm office workers also reported IAV seroprevalence. This study claimed that there was transmission of swine influenza viruses from swine to human however the possibility of human to swine transmission was ruled out [156].

After a respiratory disease outbreak in nursery piglets, 15 nasal swabs were found positive for A(H1N1)pdm09 virus between December 2009 and March 2010. Fifteen sera samples of the farm workers along with three sera from dogs and one serum obtained from a cat were negative for IAV, hence the interspecies transmission of IAV was ruled out [157].

The first report of active infection with reassortant H1N1 virus in Thai swine appeared in February 2010 but the follow up screenings conducted after two and three months, respectively confirmed the cessation of the active infection as the viral RNA was not detected anymore [158].

The reshuffling and reassortment of IAV internal genes were reported in Thai swine in February 2012. The HA and NA genes of H1N1 virus isolates clustered with the Eurasian swine-like IAV lineage while the H3N2 viruses diverged and formed a separate group. All the internal genes of H1N1 and H3N2 virus isolates appeared to be derived from A(H1N1)pdm09 viruses which confirmed the events of reassortments [159].

3.2.8. Vietnam

The events of reverse zoonoses were suggested after the detection of A(H1N1)pdm09 virus seroprevalence in Vietnamese swine during October 2009–March 2010 [160]. One more evidence of reverse zoonosis was identified during February–March 2010 after six triple-reassortant H3N2 viruses having a novel cluster of the Triple Reassortant Internal Gene (TRIG) cassette were isolated. The HA and NA genes of these reassortant H3N2 isolates originated from human H3N2 viruses reported between 2004–2006 while the other six internal genes had a high similarity with the Korean and American isolates [161].

Two more studies reported the H1N1, A(H1N1)pdm09, HIN2, and H3N2 virus isolates during February 2010–December 2013 from clinically healthy swine with no influenza disease symptoms [162,163]. Additionally, the antibodies for A(H1N1)pdm09 and H3N2 viruses were detected in swine which suggested a past exposure of swine to these viruses [163].

3.2.9. India

A high seroprevalence of H1N1, H2N2 and H3N2 viruses was detected in human and swine sera in Calcutta, India during 1982–1990 [164]. The first active infection of IAV in Indian swine appeared in 2009 when A(H1N1)pdm09 virus isolates were reported from a swine farm located in Uttar Pradesh. Interestingly, the retrieved A(H1N1)pdm09 virus sequences were similar to the North American and Korean viruses which might be either because of trade or long-distance transmission [165].

3.2.10. Lebanon

After an influenza outbreak on Lebanese poultry farms in 2005 the farmers fed the carcasses of the dead flocks to the swine. Intriguingly, a following investigation found that three swine were seropositive for the H9N2 virus while approximately one-third of the poultry farm workers were seropositive either for H1 or H9 viruses [166]. These results revealed the interspecies transmission of IAV among poultry, farm workers and swine.

3.2.11. Malaysia

The seroprevalence of H1N1 and H3N2 viruses in four to six-month-old Malaysian swine at 41 swine farms was reported during May–August 2005. Co-infections of H1N1 and H3N2 were detected in 29 swine samples [167].

3.2.12. Laos

The seroprevalence of H3N2 virus in swine samples obtained from the slaughterhouses in Laos was reported between May 2008 to January 2009 [168].

3.2.13. Russia

A full-length genome sequence of a reassortant H1N1 virus was reported from a Russian swine in 2016. The HA and NA genes of this virus isolate shared 90% identity with the H1N1 viruses that were reported from humans in the USA in the 1980s [169].

3.2.14. Taiwan

The human to swine transmission of IAV was speculated after IAV antibodies were detected in 147 Taiwanese swine during June 1969–May 1970. The results were further confirmed with virus isolation which retrieved 13 IAV isolates [170]. More recently, IBV of Victoria/B lineage was detected in swine nasal swab samples collected from apparently healthy swine at three swine farms in 2014 [171].

3.2.15. Indonesia

An active IAV infection in 52 swine within four provinces in Indonesia was identified during 2005–2009. Interestingly, 39 H5N1 virus isolates were successfully retrieved and sequenced [172].

3.2.16. Sri Lanka

The first report of influenza in Sri Lankan swine was documented during 2004–2005 after one human-like H3N2 virus was identified. Later, A(H1N1)pdm09 virus isolates were identified in swine during 2009–2012. A spillover of these viruses from human to swine was speculated [173].

3.2.17. Kazakhstan

One recent investigation in Kazakhstan during 2017–2018 identified nine H1N1 and eight H3N2 viruses in human while seven H1N1 and four H3N2 viruses were identified in swine. Interestingly, 10 of the human samples were also positive for IBV infection while the swine samples were negative for IBV [174].

In summary, the influenza viruses have been reported in swine in 16 Asian countries including China, Japan, Thailand, South Korea, Viet Nam, Cambodia, Taiwan, India, Bhutan, Russia, Laos, Malaysia, Lebanon, Indonesia, Kazakhstan, and Sri Lanka (Figure 4B). Apart from the most common IAV strains of H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses, several avian-origin H5N1, H5N3, H4N1, H4N6, H4N8, H6N6, H7N9, H9N2, and H10N5 influenza viruses were also reported in Chinese swine. Horse to swine transmission of equine influenza virus H3N8 was reported in China. Additionally, avian-origin H7N2, H5N2 viruses were identified in South Korean swine while H5N1 was reported in Indonesian swine. Interestingly, after the swine were fed upon dead poultry carcasses in Lebanon the H9N2 virus was detected in Lebanese swine. The IBV was reported in Asian swine only in Taiwan while strains of ICV were reported in swine in China and Japan while IDV was recently reported in Chinese swine (Table 1).

3.3. Influenza Viruses in Swine in Australia

Swine influenza was first reported in Australian swine only in 2009 after a swine farm owner reported coughing symptoms among swine. Simultaneously, some of the human workers on the farm also developed influenza like symptoms and hence stayed out of the farm until recovery. Later, the farm owner also developed similar symptoms following which he was tested for A(H1N1)pdm09 virus which resulted positive. As a result, a representative number of swine showing coughing symptoms and loss in appetite were sampled for molecular diagnostics and serology which confirmed that 12 swine were positive for H1N1 virus [175].

Second report of IAV in Australian swine appeared on a Queensland farm in August 2009 when a veterinarian observed elevated temperature, coughing and loss of appetite in swine. Simultaneously, two of the staff members on the farm exhibited influenza-like symptoms and hence were sampled for diagnostic testing using nasal swabs. Interestingly, both the staff members and four of the swine were found positive for the A(H1N1)pdm09 virus. Sequencing identified that the HA gene of A(H1N1)pdm09 virus retrieved from a staff member was identical to the virus retrieved from the swine which suggested transmission of A(H1N1)pdm09 virus between swine and human [176].

Third report of IAV in Australian swine appeared when a respiratory disease outbreak in swine and the farm workers occurred in Perth, Western Australia during 2012 which identified 43 IAV

positive swine. Sanger sequencing of HA and NA genes identified six novel HIN2, three novel H3N2, one A(H1N1)pdm09 and two seasonal H3N2 viruses in swine. On the contrary, only one out of eight human workers were found positive for seasonal H3N2 virus. This study could not conclude the event of zoonotic transmission of IAV between swine and human workers at the farm [177].

The fourth report of IAV was documented when 14 IAV positive swine were identified at a commercial swine farm in Western Australia during July–September 2012 and later during September–November 2016. Additionally, 17 swine were determined to be IAV positive in southern Queensland. The complete genomes of 10 IAV isolates retrieved in Western Australia and Queensland were successfully sequenced which identified seven H1N2, two human-like H3N2 and one H1N1 virus [178].

Overall, four reports of IAV outbreaks in swine in New South Wales, Queensland and Western Australia were available (Figure 4C). The H1N1, H1N2, H3N2 and A(H1N1)pdm09 subtypes have been reported from Australian swine with relatively low prevalence.

3.4. Influenza Viruses in Swine in Europe

3.4.1. Belgium

The H1N1 virus was identified in swine lung tissues or trachea of two of the deceased sows after an influenza-like disease erupted at two swine farms in January 1979. Interestingly, it was also reported that the identical virus was detected in wild ducks in Germany [179]. Since it was already established that H1N1 from wild ducks can successfully infect swine if inoculated via intranasal route [179] hence this observation suggested the transmission of H1N1 from wild ducks to the swine [180]. A second investigation isolated three avian-like H1N1, two H1N2 and twelve human-like H3N2 viruses from eight commercial swine farms in March 1999 [181].

3.4.2. Denmark

Denmark has been running a passive surveillance program for IAV detection in swine since 2011. The H1N2 virus having the H1 gene which evolved from H1N1 avian-like viruses and N2 gene which evolved from human H3N2 viruses was reported in swine during 2011–2013 [182]. This was an example of how IAV can evolve through reassortment and may emerge into a new IAV strain.

The other investigation included swine sampling at different time intervals to assess the persistence of IAV shedding in Danish swine which detected one avian-like H1N1 and 107 reassortant H1N2 viruses. This study observed that most of the swine were shedding IAV right before achieving six weeks of age. Surprisingly, a piglet as young as just three days was found infected with IAV [183].

Two H3N2 isolates having H3 genes from seasonal human influenza along with internal genes that originated from A(H1N1)pdm09 virus and NA genes from contemporary N2 swine influenza viruses that have been in circulation in Denmark were retrieved from young piglets at two locations during 2011–2014 [184]. H3N2 virus was also detected from piglets having respiratory illness and from sows with reproductive problems in commercial piggeries in 2014 [184].

3.4.3. United Kingdom

The H3N2 virus antibodies were first detected in English swine in 1973 which revealed the past exposure of swine to H3N2 virus [185]. Later, the antibodies for H1N1 and H3N2 viruses were detected in swine at a slaughterhouse in England during 1991–1992 [186]. Interestingly, this serological investigation also reported the antibodies for IBV in eight and for ICV in 198 swine [186].

A molecular investigation identified a novel H1N7 virus in swine in England which had six of its RNA segments closely related to those of human viruses while two RNA segments were identical to those of equine viruses which concluded that the H1N7 strain may have evolved due to reassortment between human H1 and equine H7N7 viruses [187,188].

The first report of A(H1N1)pdm09 virus in English swine appeared in September 2009 when histology and immunofluorescence assays followed by molecular diagnostics and sequencing confirmed four A(H1N1)pdm09 virus infected swine in the Northern Ireland [189]. After this, 17 more A(H1N1)pdm09 virus isolates were reported in swine in England during September 2009–October 2010 which revealed that A(H1N1)pdm09 virus was in circulation in English swine during the 2009 flu pandemic [190]. The same year, four H1N2 virus isolates were reported in English swine which had six internal genes of A(H1N1)pdm09 virus along with HA and NA genes of H1N2 virus hence were identified as the novel reassortant H1N2 strains [191]. In a more recent study, two more IAV positive swine were identified in the United Kingdom in 2016 [192].

3.4.4. Finland

However the first report of seroprevalence of H1N1 virus in Finnish swine appeared in 2008 during an investigation which detected H1N1 virus antibodies in swine at seven swine farms which further increased to 24 swine farms in 2009 [193] but the first isolate of avian-like swine H1N1 virus (indicative of active infection) was detected from the lung tissues of a swine in February 2009. Later, the first A(H1N1)pdm09 virus in Finnish swine was detected in November 2009 [193]. Three more swine were identified with IAV antibodies during May 2011–January 2014 which was due to a past exposure to IAV [194].

3.4.5. France

The H1N1 viruses in turkey and swine were identified after the swine influenza outbreak hit the turkey population in Brittany, France in February 1983 which suggested that IAV transmission happened from swine to turkey [195]. Later two strains of H1N2 virus were isolated from six swine exhibiting influenza-like illness in Brittany during 1987–1988 [196]. Another investigation reported H1N1, H1N2, and H3N2 viruses in swine herds experiencing respiratory disease in Brittany region [197].

A negative prevalence of IAV was reported in wild boars in Camargue during September 2009–November 2010 given that all the 315 nasal swabs obtained from either hunted or trapped wild boars along with all the sera samples were negative for IAV [198].

A more recent investigation reported the zoonotic transmission of A(H1N1)pdm09 virus from swine to a farmer in January 2018. This farmer along with a veterinarian collected nasal swab samples from three pregnant sows exhibiting influenza-like illness on the swine farm and submitted to a local diagnostic laboratory for analysis which, as a result, were found IAV positive. Few days later, the farmer and the veterinarian both developed the influenza-like symptoms. The farmer was later diagnosed with A(H1N1)pdm09 virus [199].

3.4.6. Germany

Sixty-five IAV positive wild boars were identified across five German states during 1997–2006. Cloning and sequencing identified H1N1 and H3N2 viruses in these wild boars [200]. Later thirteen H1N1, three reassorted A(H1N1)pdm09 and four H1N2 viruses were detected in swine during 2009–2010. Interestingly, the A(H1N1)pdm09 virus isolates had high similarity with the A(H1N1)pdm09 viruses reported earlier in humans within Germany which suggested a reverse zoonotic transmission of the A(H1N1)pdm09 virus [201].

A nationwide sero-surveillance identified 12,585 swine with H1N1, 9,566 swine with human-like H1N2, 12,220 swine with human-like H3N2 and 11,086 swine with A(H1N1)pdm09 virus antibodies during June 2009–December 2012 which reflected a high seroprevalence of influenza viruses in German swine population [202].

Later 273 IAV positive swine exhibiting influenza-like illness were detected between January 2010–December 2013. Subtyping successfully distinguished 198 of 273 samples into H1N1, H1N2, H3N2 and A(H1N1)pdm09 viruses. The H1N1 virus was the most widely occurring in German swine while A(H1N1)pdm09 virus had the lowest prevalence [203].

3.4.7. Greece

The H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses were detected in swine sera samples collected from apparently healthy swine at 42 swine farms during 2002–2004 and from 46 swine farms during 2010–2012 [204].

3.4.8. Italy

The seropositivity of Italian swine to H3N2 virus was first reported during December 1976–November 1977 when 24 swine were detected with H3N2 antibodies [205]. The first report of H1N1 active infection in Italian swine appeared during an influenza disease outbreak between 1977 to 1986 which identified 63 H1N1 viruses [206]. Further, four H3N2 viruses were detected in swine nasal swabs originated from three swine farms and an abattoir during 1981–1982 [207].

Later 47 H1N1 and 37 H3N2 viruses were detected in swine during 1992–1995. Interestingly, four human sera samples were also positive for H1N1 and 77 samples were positive for H3N2 viruses which might be due to the transmission between human and swine [208]. Further IAV seroprevalence was detected in the age group of three-month to four-year old swine during 2002–2004 [209].

The first report of A(H1N1)pdm09 virus in Italian swine appeared after a respiratory disease outbreak in piggeries in Lombardia region of Northern Italy in November 2009. Piglets experienced diarrhea and weight loss while the sows experienced reduction in reproduction rate [210]. Two more A(H1N1)pdm09 virus isolates were reported in female swine in Sicily in December 2009 [211] while five isolates of A(H1N1)pdm09 virus were identified in swine at three different locations during 2011–2012 [212].

A novel strain of reassorted H1N2 virus having 99–100% identity through six gene segments with A(H1N1)pdm09 virus along with HA and NA genes similar to H1N2 virus was reported in swine in Mantua Province [213]. Reassorted H1N2 viruses were again detected in 34 piglets during 2013–2014 [214].

Seroprevalence of Italian wild boars with one H1N1, ten H1N2, and one H3N2 viruses at two different locations was reported during 2012. On the contrary, active infection was found only in three wild boars whose nasal swabs were positive for the IAV [215]. One more investigation reported active infection of IAV in 12 wild boars while 78 wild boars had IAV antibodies during July–December 2012 [216]. Further molecular and serological investigations detected avian-like H1N1 viruses in Italian wild boars [216].

The first complete genome sequence of IDV in Italian swine was retrieved from a symptomatic sow in 2015 which was identified to be closely related to the IDV sequence reported in Oklahoma swine in 2011 [217]. Further IDV prevalence in Italian swine was reported when 14, three and four swine were found positive for IDV antibodies in Veneto, Emilia Romagna and Lombardia regions, respectively during June 2015–May 2016. As a result, swine clinical samples collected during 2013–2014 were investigated retrospectively for IDV prevalence but were reported negative. An extended serological investigation detected IDV antibodies in 364 swine sera samples collected during 2015. These findings suggested that IDV was in circulation in Italian swine population only after 2014 [45].

3.4.9. Spain

Isolation and characterization of 12 H3N2, nine H1N1 and one H1N2 viruses reported the prevalence of influenza viruses for the first time in Spanish swine herds experiencing the respiratory illness and pneumonia during November 2001–April 2004 [218]. More strains of H1N1, H1N2 and H3N2 viruses were isolated, sequenced and characterized in Spanish swine during 2006–2011 [219–221]. Interestingly, five H1N1, three H1N2, and four H3N2 virus isolates retrieved between January 2010 and August 2011 had significant similarities with other European isolates which was an evidence of continent-wide transmission of these swine influenza viruses [220].

3.4.10. Luxembourg

A molecular investigation reported a negative prevalence of IDV in swine in Luxembourg during 2009 but later successfully detected three IDV positive swine during 2014–2015. Further, the serological investigation confirmed that swine in Luxembourg were free from IDV during 2012 but interestingly, IDV antibodies were detected in 17 swine samples collected during 2014–2015. These observations suggested that IDV was not in circulation in swine in Luxembourg during 2009–2012 but became prevalent at a low frequency later during 2014–2015 [46] which was almost the same time IDV was reported in Italian swine populations [45].

3.4.11. The Netherlands

A serological investigation of swine in the Netherlands identified 601 H1N1, 584 H1N2, and 229 H3N2 virus antibodies in 29 swine herds during January–May 1999 [222] with no further evidence of IAV in swine in the country after that.

3.4.12. Norway

After the 18 swine which were experiencing influenza-like illness were found infected with A(H1N1)pdm09 virus on a Norwegian swine farm in October 2009 the surveillance was expanded to the 39 nearby swine farms which determined that 23 of these farms were positive for the A(H1N1)pdm09 virus. Intriguingly, one human subject at the index farm who had influenza-like symptoms was also found positive for A(H1N1)pdm09 virus. This study suggested that the symptoms first appeared in the human subject at the index farm and later the disease got transmitted to the swine. Hence the findings of this study suggested the reverse zoonosis of the influenza virus from human to pig [223].

Further molecular and serological investigations identified 48 more swine herds that were positive for IAV during September 2009–October 2010 [224]. A more comprehensive nation-wide surveillance in Norwegian swine identified 16 A(H1N1)pdm09 virus positive swine herds during 2009 which later increased to 190 swine herds in 2010 [225]. Later 194 more swine were found infected with A(H1N1)pdm09 virus in Norway between April and July 2011 and reported that the IAV infected swine took longer to weigh 100 kg body mass [226].

3.4.13. Poland

The first active IAV infection in swine in Poland was reported in 2010 when 21 oral fluid samples collected from three swine farms detected IAV [227]. Soon after, five avian-like H1N1 viruses were reported from the swine lung tissues during 2011–2013 [228]. Later a serological surveillance identified 1212 H1N1, 851 H1N2, 1012 H3N2, and 572 A(H1N1)pdm09 virus antibodies in swine during March 2011–February 2015 [229]. Surprisingly, 34 of these swine had antibodies against all four IAV subtypes i.e., H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses [229] suggesting the past co-infections.

3.4.14. Czechoslovakia

The human-like H3N2 virus was isolated from a swine in Czechoslovakia during 1969–1972 [230]; however, no other reports ever appeared from the country in later years.

3.4.15. Hungary

Complete genome of an H1N1 virus was reported from a Hungarian swine having fever and conjunctivitis in May 2011 [231]. This was the only report of H1N1 virus in the swine in Hungary.

3.4.16. Multi-National Surveillances in European Countries

A large-scale investigation across seven European countries reported a high seroprevalence (>62%) of IAV antibodies in swine populations of Belgium, Germany, Spain, Italy while a relatively lower (<21.25%) seroprevalence was observed in swine populations of Czech Republic, Poland and Ireland

during 2002–2003. Antibodies against H1N1, H1N2, and H3N2 viruses were reported in swine from the European countries under surveillance except Poland where swine had antibodies against only H1N1 virus [232].

A virological surveillance across five European countries including Belgium, United Kingdom, Italy, France and Spain reported 169 IAV positive swine during 2006–2008. The H1N1, H1N2, and H3N2 viruses were detected in swine from Belgium, Italy, and Spain while the samples from United Kingdom and France were found infected with H1N1 and H1N2 viruses [233].

Briefly, the virological and/or serological prevalence of influenza viruses in European countries (Figure 4D) identified the strains of H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses in swine populations of the United Kingdom, Ireland, Italy, Germany, France, Norway, Finland, Denmark, Belgium, Spain, Poland, Greece, Hungary, Netherlands, Czech Republic, and Czechoslovakia while the swine in Luxembourg and Italy were found infected with IDV.

3.5. Influenza Viruses in Swine in North America

3.5.1. Canada

Shortly after a respiratory disease outbreak in swine in Manitoba, an autopsy was done on a dead swine on March 1, 1967. The histopathology confirmed the bronchitis in the deceased swine and a strain of IAV designated as "*S/Manitoba/647/67*" was characterized using IAV antisera [234]. The first report of H1N1 virus in Canadian swine appeared in Quebec during 1980s–1990s when five genotypes of H1N1 virus were identified [235]. Since then several studies have reported H1N1, H1N2 and H3N2 viruses in Canadian swine [236–243].

Another study reported nine isolates of swine influenza viruses with an antigenic variant from the sick swine having proliferative pneumonia in Quebec, Canada during 1990–1991 [244]. In a retrospective diagnosis, only one formalin-fixed paraffin embedded swine lung tissue collected during 1991 was found IAV positive with immunohistochemistry. This investigation suggested that immunohistochemistry can be useful in retrospective diagnosis of the swine influenza virus [245].

The broncho-intestinal pneumonia in lung tissues of dead swine was reported on a swine farm which exhibited disease symptoms including coughing, weight loss, and labored breathing. Interestingly, before the onset of the disease symptoms, this farm conducted a routine serological surveillance of influenza virus which identified H1N1 virus in only one of the twelve swine samples [246].

Following this surveillance, a three-month old swine from the same farm was found positive for avian influenza virus H4N6. The complete genome of this H4N6 virus was reported in 1999. This was the first ever report of an avian-origin H4N6 virus in swine. The proximity of the swine farm to a natural lake where several wild bird species including waterfowls which were reported to visit frequently might be the reason behind the introduction of this avian influenza virus strain to the swine [246]. Later three avian-origin H3N3 influenza virus isolates were recovered from swine in eastern Ontario exhibiting weight loss and coughing during October 2001. On a nearby farm located approximately 30 kms away, another H3N3 virus isolate was recovered from the swine. There was no recorded movement of the swine between these two farms. Since these were avian-origin H3N3 viruses hence the role of birds in transmission cannot be ruled out. Later, on a third farm, where an influenza like disease had been affecting mainly the nursery piglets, an H1N1 virus was recovered in May 2002 [247].

Reassortant H1N1 and H1N2 viruses were detected in swine nasal swab or lung tissue samples obtained from three-week old piglets and sows exhibiting typical influenza-like symptoms in Ontario during 2003–2004 [248]. First triple-reassortant (avian/classical swine/human triple-reassortant) H3N2 viruses from four swine and one human nasal samples were identified in Ontario during 2005. The phylogenetic analysis determined that all the virus sequences were 100% identical to each other which apparently emerged from triple-reassortant H3N2 viruses reported in US based swine

in 1988 [249]. One more report of triple-reassortant H3N2 (trH3N2) viruses appeared on the swine farms located in Saint-Hyacinthe, Assomption and Saint-Foy during early 2009. The trH3N2 viruses identified in this study were determined to be closely related to North American/Canadian trH3N2 viruses reported earlier [250]. Later A(H1N1)pdm09 and H1N1 viruses having internal genes of triple reassortant H3N2 virus were reported in swine in four provinces including Manitoba, Alberta, Saskatchewan and Quebec during 2009 [251].

The first evidence of A(H1N1)pdm09 virus in Canadian swine appeared in 2009 after the human workers at a swine farm developed influenza-like illness. The investigation identified that two farm workers along with 56 swine were positive for the A(H1N1)pdm09 virus. Transmission of A(H1N1)pdm09 virus from human to swine was suggested [252]. The same year 17 more swine were detected with A(H1N1)pdm09 virus after a respiratory disease outbreak hit the Alberta swine farms [253].

A reverse zoonotic transmission of A(H1N1)pdm09 virus to swine from a human subject who visited Mexico and returned to the swine farm was reported in April 2009. As a result, ten swine having severe disease were sacrificed for necropsy which identified lesions in the bronchioles corresponding to the influenza virus disease. Virus isolation and sequencing identified the A(H1N1)pdm09 virus. Additionally, A(H1N1)pdm09 virus was identified in two more human subjects who were exposed to the swine hence indicated the occurrence of zoonoses on the swine farm [254].

Later during summer 2009, ten more A(H1N1)pdm09 viruses from five swine herds in Manitoba were reported. Virus shedding was observed up to 20 days post-infection after the appearance of the clinical symptoms in swine [255]. This observation was in agreement of a previous report which documented the experimental infection of swine in the laboratory and determined that virus shedding occurs until 11th day after appearance of the clinical symptoms [256]. Another investigation reported nine A(H1N1)pdm09 and four H3N2 viruses after an influenza-like disease outbreak on a Quebec based swine farm in December 2010 [257].

The effect of microclimatic conditions on the transmission dynamics of swine IAV in the barns was studied which observed that high relative humidity in the environment during summer months suppresses the aerosol transmission of the droplets which in turn decreases the transmission of IAV [240]. The high relative humidity in the environment would facilitate the generation of larger droplets which do not tend to shrink easily and hence are less likely to be aerosol transmitted to a longer distance as they fall on the ground quickly after their formation [240,258]. As a result, a lower transmission of IAV is observed usually during the summer months. On the contrary, the IAV transmission increases during winter months when relative humidity is relatively lower [258].

3.5.2. United States

The IAV was first isolated from the nasal discharge of a swine in the United States in 1931 [34] and from the human in 1933 [35]. The first report of human-origin IAV in swine appeared in the United States on 24 May 1937 after an unexpected result was observed when the serum sample of a sick swine obtained from a State Prison Farm located in New Jersey neutralized the antibodies of human influenza virus. A series of investigations made a strikingly new observation that swine had suffered from a human strain of influenza virus [259].

Serological investigations conducted during 1950s suggested that the weight loss and mortalities among swine were due to swine influenza viruses [260,261]. Swine influenza viruses were isolated from febrile swine at nine occasions during 1965–1968 in Wisconsin and Nebraska [262]. Additionally, swine influenza antibodies were also detected in swine sera samples collected from six farms [262]. A virological surveillance conducted in Memphis, Tennessee and Madison, Wisconsin during May 1976 to June 1977 successfully isolated 478 influenza viruses from swine nasal swabs collected at abattoirs; approximately 300 of which were characterized to be swine H1N1 viruses. Additionally, the serological surveillance identified that 21% of the 9400 swine sera samples had swine H1N1 virus antibodies [263]. A small percentage (1.4%) of swine sera samples were found positive for the swine

H3N2 viruses which was further confirmed by virus isolation [263]. Interestingly, this study identified inter-species transmission of swine influenza viruses between human and swine [263].

A novel swine-origin H1N1 virus termed as "*A/New Jersey/76 (Hsw1N1)*" was detected at Fort Dix Army training camp in New Jersey in January 1976. The outbreak was localized and was limited to Fort Dix only. As a result, 230 soldiers were found infected with this novel virus; 13 of which had severe respiratory disease with one death due to viral pneumonia [264–266]. Since this novel swine-origin H1N1 virus quickly disappeared from Fort Dix hence the epidemiology and the origin of the disease could not be ascertained [264].

The H1N1 and H3N2 virus antibodies were detected in swine sera collected from an abattoir in North-West United States. Interestingly, a higher IAV seroprevalence was observed during the Fall and early winter months. Virus isolation and sequencing identified that the H1N1 viruses were closely related to the classical H1 swine influenza virus [267]. Classical swine-like H1N1 and triple-reassortant H3N2 viruses were identified in swine samples collected across 23 states in the USA during 1998–1999 [268].

The Minnesota Veterinary Diagnostic Laboratory (MVDL) detected large number of H1N1, H1N2 and H3N2 subtypes of IAV in swine samples during 1998–2001 and again during 2007–2009. Interestingly, some of the samples were co-infected with H1N1 and H3N2 viruses [269–271]. A second-generation reassortant H1N2 virus having genes from a reassortant H3N2 and classical H1 swine influenza viruses was obtained from the lung tissue samples of a dead sow at an Indiana swine farm in November 1999 [272].

A novel subtype of H3N1 virus termed as "*A/Swine/Minnesota/00395/2004 (H3N1)*" was identified during a severe respiratory disease outbreak on a swine farm in Minnesota in October 2004. Sequencing observed that the HA gene of this strain was closely related to swine influenza H3N2 virus while the NA gene was related to classical H1N1 virus which suggested that the novel H3N1 virus emerged due to reassortment between H1N1 and H3N2 viruses in the Midwest United States [273]. Further an H2N3 subtype of IAV which may have emerged as a result of a reassortment between avian and swine influenza viruses was identified on a commercial swine farm in Minnesota in April 2006 and again in September 2006 [274].

The first evidence of A(H1N1)pdm09 virus infection in US swine appeared when four A(H1N1)pdm09 and one triple-reassortant H1N2 viruses were identified and characterized in the exhibition swine in the states of Minnesota and South Dakota in 2009 [275]. During last ten years, a large number of H1N1, H1N2, H3N2, A(H1N1)pdm09 along with reassortant IAV subtypes have been reported in the US swine populations [243,276–289].

The United States has a large feral swine population which is considered a reservoir of H1N1 and H3N2 viruses [290]. The swine-like H1N1, avian-like H1N1, swine-like H1N2, swine-like H3N2, human-like H3N2, A(H1N1)pdm09 along with avian-like H6N2 and H7N2 viruses were identified in feral swine samples collected across 35 states in the USA between October 2009–September 2013 [291].

Histological examination of the lung tissues obtained from two backyard piglets suffering from pneumonia and weight loss in Colorado in November 2010 suggested that the piglets were infected with swine influenza virus which were later confirmed to be infected with IAV subtype A(H1N1)pdm09 virus. Since the piglets were raised at the house of a pharmacist hence a possible human to swine transmission was speculated given the possibility of an occupational exposure of the pharmacist to the A(H1N1)pdm09 virus at the pharmacy [292].

The first report of IBV infection in swine appeared when swine in the Midwest United States were found infected with IBV lineages of Yagamata/B and Victoria/B [37]. This was a new finding because initially IBV was thought to have a host range limited to human, pheasants, horses and seal [1–4].

A novel strain of swine influenza virus was detected in Oklahoma swine exhibiting influenza-like symptoms in April 2011. The nasal swab samples taken from the swine were negative for the IAV infection. Hence the virus isolation was attempted in swine testicle cells; the cells in culture showed influenza-like cytopathic effects by third day. Electron microscopic observations revealed particles

typical of a virus of Orthomyxoviridae family, but the RT-PCR was negative for the IBV and ICV. After ultracentrifugation was used for virus isolation, the genome of the virus was sequenced using Ion Torrent sequencing. The genome sequence analysis along with genetic and biochemical investigations revealed that the isolated virus was a novel Orthomyxovirus having 50% overall identity at amino acid level with human influenza C virus [43]. Since this novel virus was genetically and antigenically distinct from ICV therefore, later was proposed to be categorized as a new genus of Orthomyxoviridae family which was later accepted as influenza D virus (IDV) [5].

Later, two feral swine which were shot dead in a cotton field in Texas in June 2011 were found infected with A(H1N1)pdm09 virus. The significant identity of A(H1N1)pdm09 virus isolated from these two feral swine with human A(H1N1)pdm09 virus suggested a possible transmission between human and the feral swine [290]. Another study reported seroprevalence of H3N2 virus in one feral swine from Mississippi and in five feral swine from the state of California in 2005 but a negative seroprevalence was reported in the feral swine samples obtained from the states of Florida, Oklahoma and Missouri. Additionally, the seroprevalence of IAV was reported in feral swine from Texas where a total of 68 out of 472 feral swine sera were found positive for H3N2 and H1N1 viruses [293].

Another investigation detected H3N2 virus RNA in only one feral swine from a pool of samples collected across 31 states in the USA during 2011–2012 which indicated a negligible active influenza infection in US feral swine population. On the contrary, ELISA identified IAV antibodies in 182 feral swine samples while the serological subtyping identified H3N2 virus antibodies in 76 feral swine samples collected from 19 states which indicated a significant past exposure of US feral swine to the H3N2 virus [294]. Further, seroprevalence of IDV was reported in 49 feral swine samples collected from Oklahoma, Texas, Hawaii and North Carolina during October 2012–September 2013 which provided the first evidence of past IDV infections in US feral swine [295].

A study investigating virus shedding in nursery piglets found that all 81 piglets under investigation were shedding H3N2 virus starting seventh day of arrival into the barns until 29th day. Shedding was still observed in some piglets until 39th day [296]. Interestingly, 48 of these nursery piglets were also identified shedding H1N1 virus starting at the third day of arrival into the barns until 41st day over a 53-day observation period [296]. This was the new information which identified that young nursery piglets could get infected with IAV.

The oral fluid samples collected from 25 neonatal piglets at four Oklahoma based swine farms during May–August 2014 [297] were found infected with different IAV subtypes including H1, N1, H3, and N2. This study supported the use of swine oral fluid samples in IAV diagnostics [285]. The swine oral fluid samples were also collected in North and South Carolina during June to August 2014 using the cotton rope hanging method [298]. In this method of sampling, swine are encouraged to chew the rope, as a result, saliva accumulates on the rope which is later squeezed to collect the sample aseptically. One of the benefits of this method of sampling is that each sample does not represent an individual swine but rather represents multiple swine that chewed the rope while hanging inside the pen [298]. Another benefit of this sampling method is that swine oral samples may contain contaminants like feed and feces but this method minimizes the chances of such contaminations in the sample [299].

Another investigation carried out metagenomic sequencing of swine nasal and rectal swabs obtained from apparently healthy swine which identified 11 IAV positive swine at three abattoirs and a buying station in USA in August 2015 [300].

In a striking observation, an avian-lineage H4N6 virus was isolated and sequenced from 7–8-month-old gilts on a Missouri based swine farm in December 2015 [301]. The investigators collected more samples at different time points for next few months at the same farm to assess the transmission of H4N6 virus among swine. No other samples were found positive for the H4N6 virus which suggested that the H4N6 virus did not transmit from swine-to-swine and therefore disappeared from the index farm. Interestingly, this extended study identified three H1N1 viruses infecting swine [301].

One large-scale study identified that 23 percent (2 947/12,814) of the swine samples were positive for the IAV in Mid-West United States between July 2011–March 2017, however, sequencing could identify only 173 H1 and H3 subtypes among positive samples [302]. A human to swine transmission of IAV was suggested when two human-like H3N2 virus isolates were identified from an Oklahoma based swine farm in 2017 which had high similarity with the human-like H3N2 viruses reported earlier from Baltimore [303].

3.5.3. Mexico

Maya people represent ethnolinguistic groups in South and Central America. The practice of household swine keeping put the Maya people at high risk of contracting the swine influenza viruses. Thirty-one sera samples collected from the Maya people in Mexico were identified having antibodies against H1N1 and H3N2 viruses while 93 other sera had antibodies against the H3 subtype of IAV, representing a past exposure to these viruses [304]. However, this study did not include swine samples for investigation but since swine were household animals in their backyard hence the IAV seroprevalence of the Maya people could be because of a past transmission of these viruses from the backyard swine [304].

A retrospective study identified antibodies against swine-like H1N1, A(H1N1)pdm09, H3N2, and human-like H1N1 viruses in backyard swine in Mexico between 2000 to 2009. This investigation retrospectively determined that the classical-swine H1N1 virus was most widely present in Mexican swine before the 2009 influenza pandemic [305]. Further, a significant number of swine experiencing respiratory illness had H1N1 or H3N2 virus antibodies in commercial piggeries in Sonora Province of Mexico during October 2008–March 2009. The molecular diagnostics and subtyping determined four H1 and two H3 viruses while 19 other IAV positive samples could not be subtyped given the low viral load [306].

During the influenza virus pandemic in Mexico in 2009, A(H1N1)pdm09 virus was first identified in a single swine nasal swab. Additionally, H3N2, A(H1N1)pdm09 and IBV viruses were detected in four symptomatic humans [307]. The A(H1N1)pdm09 virus isolate retrieved from the swine was believed to be the first from the sister lineage of the pandemic influenza virus isolates reported in Mexico [307].

Further 59 IAV isolates were retrieved from Mexican swine having respiratory illness during 2010–2014. Intriguingly, this study identified 13 reassorted genotypes of IAV in Mexican swine [308]. This investigation also reported that IAV introduction into Mexican swine may have occurred through three different routes; human to swine transmission; reassortment between human-like H3N2 and A(H1N1)pdm09 virus; and through the long-distance movement of the swine from USA and Europe. A periodic introduction of IAV in Mexican swine occurred with the import of American and European swine to Mexico over two decades in 1980s and 1990s before the 2009 influenza pandemic [33].

Fifty-eight IAV whole genome sequences were retrieved from Mexican swine during 2010–2014. Genome sequence analysis identified classical H1N1, H3N2, and A(H1N1)pdm09 viruses. Interestingly, the data obtained in this study suggested independent evolution of IAV in the Mexican swine population in different regions of the country. Phylogeny determined that Mexico City was the source of the 2009 influenza pandemic which erupted during March–May 2009 [33]. Later a reassortant H1N2 virus which had the genes from human and swine influenza viruses was isolated and sequenced from a swine in November 2014 [309].

3.5.4. Guatemala

The molecular diagnostics identified a total of 104 IAV positive commercial and backyard swine in Guatemala during 2010–2011 which resulted into three A(H1N1)pdm09 and one H3N2 virus isolates [310].

3.5.5. Cuba

The first report of A(H1N1)pdm09 virus in commercial piggeries in Cuba appeared in November 2010 when 24 swine were found positive for A(H1N1)pdm09 virus across five swine farms [311]. Further, five more IAV positive swine were detected in Pinar del Rio province of Western Cuba having respiratory illness and interstitial pneumonia. However only one IAV positive sample could be successfully subtyped as A(H1N1)pdm09 virus having reassorted internal genes, all except the NA gene [312].

3.5.6. Trinidad and Tobago

In a more recent investigation, a high seroprevalence of IAV (114/309) was detected in swine in Trinidad and Tobago which later identified H3N2 and A(H1N1)pdm09 viruses in swine [313].

In summary, the H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses were reported in North American swine population. Interestingly, the avian influenza virus strain H4N6 was detected in US based swine while H3N3 and H4N6 were identified in the Canadian swine and H5N2 was reported in the Mexican swine in 2018 (Figure 4E). Mexico City was identified to be the origin of 2009 influenza pandemic. It was also ascertained that A(H1N1)pdm09 virus was present in Mexican swine well before 2009 pandemic erupted.

3.6. South America

3.6.1. Argentina

After influenza virus outbreak hit a swine farm in Buenos Aires in November 2008, one of the five dead swine were diagnosed with viral pneumonia through immunohistochemistry. A full genome of H3N2 virus sharing 96–98% nucleotide sequence identity with H3N2 viruses reported in North America during 2000–2003 was recovered from the swine [314].

An H1N1 virus was reported from a swine after a swine farm manager along with his spouse experienced influenza-like symptoms few days before the outbreak erupted in the swine at a Buenos Aires based farm in June 2009. The influenza disease symptoms lasted for about a week in nursery piglets. Immunohistochemistry identified necrotizing bronchiolitis in four of the swine post-mortem samples while one sample had severe inflammation in the bronchiolar epithelia. The serological investigation detected IAV antibodies in most of the sera samples collected after 15 days of onset of clinical symptoms however the active infection was reduced to only six swine [315].

The third investigation carried out histopathology which identified lung lesions compatible to the influenza virus infection in nine swine necropsy samples at a Buenos Aires based swine farm in October 2009 and later in eight swine necropsy samples originated from a Santa Fe based farm in May 2010. The swine at Buenos Aires farm were found infected with H1N1 virus while the swine at the Santa Fe farm retrieved one H1N2 and three human-like reassortant A(H1N1)pdm09 virus isolates which had triple reassortant internal genes. This was the first report of human-like reassortant A(H1N1)pdm09 virus in swine in Argentina [316]. Later two more investigations using histopathology, immunohistochemistry, serology, and molecular analyses reported H1N2, H3N2, and reassortant H3N2 viruses with A(H1N1)pdm09 internal genes in swine in Argentina during 2011–2012 [317,318].

3.6.2. Brazil

Several H1N1, H1N2, H3N2, human-like H1N1, and A(H1N1)pdm09 viruses have been identified in Brazilian swine from the Minas Gerais, Parana, Rio Grande do Sul and Sao Paulo provinces in Brazil during and after 2009 [319–325]. A technician who visited a Minas Gerais swine farm experiencing influenza outbreak developed similar respiratory disease symptoms. The nasal swab sample was obtained from the technician, as a result, one A(H1N1)pdm09 virus was isolated which was closely related to the A(H1N1)pdm09 viruses reported from the swine herd in the Minas Gerais which was

recently visited by the technician. Hence it was concluded that a zoonotic transmission from swine to the technician occurred at the Minas Gerais swine farm [326].

An immunohistochemical investigation demonstrated microscopic lesions suggesting broncho-interstitial pneumonia in the lung tissues of four severely sick piglets at a swine farm located in Parana province in February 2011. The A(H1N1)pdm09 viruses were isolated from two piglets. Additionally, a novel reassortant H1N2 virus was also recovered [327]. One more investigation identified that A(H1N1)pdm09 virus was the most prevalent IAV subtype in sows. The co-infections of sows with A(H1N1)pdm09, H1N2, or H3N2 subtypes were also documented in Rio Grande do Sul province. These findings were noteworthy because the coinfections may trigger reassortments and thus may facilitate emergence of novel strains of IAV [328]. Later two more H1N2 viruses were isolated and characterized from swine in Rio Grande do Sul province during 2013. The sequences of both the isolates had high nucleotide similarity to each other in different genome segments in the range of 98.9% to 100% which suggested a common source of origin of both isolates [329].

3.6.3. Colombia

The A(H1N1)pdm09 virus was identified in seven swine farms in Colombia during 2008–2009 [330].

3.6.4. Peru

The A(H1N1)pdm09 virus antibodies were detected in 110 backyard swine in Peru during March 2009–October 2011. Total four A(H1N1)pdm09 virus isolates were retrieved and sequenced which determined that there were at least two separate events of A(H1N1)pdm09 virus transmission from human to backyard swine in Peru [331].

3.6.5. Chile

The backyard productive systems (BPS) for raising swine, cattle, and poultry are popular in Chile. A molecular investigation reported a negative active IAV infection across 113 BPS units within ten counties in Chile during 2012–2014 but the serological investigation detected IAV antibodies in swine at two BPS units which suggested a past exposure of swine to the IAV [332]. Interestingly, the HA gene sequence of an H12 virus was obtained from a domestic Muscovy duck at one of the BPS which appeared to have originated from a wild bird. This suggested a spillover of the IAV from wild reservoir to the domestic poultry [332].

Another study reported the prevalence of H1N2 virus in swine reared at 40 different BPS having poultry and swine in El Yali wetland during 2013–2014 [333]. One more study identified four swine sera samples (4/64; 6.3%) that were found positive for IAV antibodies collected from different BPS in Central Chile. One pool of swine nasal swab samples (1/39; 2.6%) was also detected IAV positive with real-time RT-PCR. Interestingly, 7.9% chicken, 4.3% ducks and 11.1% geese samples collected from 329 BPS in Central Chile also had active IAV infections. The breeding practice of poultry and swine in the BPS was determined to be a major risk factor for IAV transmission [334].

Briefly, the IAV strains of H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses have been reported from the swine in Argentina and Brazil while A(H1N1)pdm09 virus was reported in swine in Colombia and Peru. Swine in Chile were found infected with H1N2 virus (Figure 4F).

In summary, total 281 research articles were identified which reported several influenza viruses in swine populations globally. The highest number of studies were reported from Asia (n = 107), followed by North America (n = 76), Europe (n = 55), South America (n = 21), Africa (n = 18) and Australia (n = 4). The highest number of reports per country were documented in United States (n = 40) followed by China (n = 39) and Canada (n = 24). Until February 2020, influenza viruses have been reported from 53 countries worldwide. Four subtypes of IAV including H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses were most frequently detected in swine populations (Table 1).

Most of the large-scale studies used serological investigations including ELISA, hemagglutinin inhibition (HI), neuraminidase inhibition (NI), virus neutralization (VN), or microneutralization

(MN) assays for the determination of the seroprevalence and subtyping of the influenza viruses in swine. Several investigations used virus isolation for the confirmation and subtyping of IAV. Most of the virological investigations used one-step real-time RT-PCR and/or reverse-transcription PCR for influenza virus detection and subtyping. Sanger sequencing or next-generation sequencing using MiSeq or Ion Torrent sequencing successfully generated the influenza virus sequences from the swine samples for epidemiological interpretations. Histological examinations including immunohistochemistry or immunofluorescence were used to examine the swine lung or other internal organ tissue samples for the influenza virus diagnostics (Table 2).

4. Discussion

As of February 2020, influenza viruses have been identified and reported in swine from 53 countries worldwide (Table 1; Figure 3). The influenza viruses have been detected in different sample types including swine sera, nasal, tracheal, oropharyngeal, nasopharyngeal swabs as well as oral fluids collected from the live swine. Nasal and snout wipes, lung homogenates and fecal slurry samples were also used. Additionally, the lung as well as other internal organ tissues (Table 2) obtained from either dead or sacrificed swine have also been used for the detection of IAV symptoms i.e., lesions in lungs, pneumonia, bronchitis, bronchiolitis etc.

Various methods have been used for the detection of influenza viruses in swine samples depending on the sample type, sample numbers and objective of the study. Virus isolation methods, using either MDCK, Caco-2, HRT18, or swine testicle cells or the pathogen free embryonated chicken eggs, although considered the gold standard [335–337] have largely been taken over lately by the sequencing approaches which tend to provide a considerably faster identification of the IAV subtypes. The additional benefit of sequencing over virus isolation is that the sequences would be useful for analyzing the influenza virus outbreak clusters [338], virus evolution or reassortment [339] using phylogenetic analyses in different gene segments. A recent study reported that next-generation sequencing can be useful in the influenza virus diagnosis and for the identification of the novel virulence markers and drug resistance [340].

Most of the studies have used real-time RT-PCR with matrix-gene specific oligonucleotide primers and TaqMan probe for IAV detection [308,341,342]. The conserved sequences of the matrix-gene specific primers can detect any IAV subtype in the swine samples [343]. Most of the studies used subtype-specific real-time RT-PCR for the IAV subtyping, however, few studies opted for the conventional approach of reverse-transcription PCR followed by Sanger sequencing for amplification of the HA and/or NA genes for retrieving the sequences for phylogenetic analyses to identify the subtypes. Although the real-time RT-PCR is a powerful and rapid tool for the subtyping of IAV strains, it is more expensive than reverse-transcription PCR. A few studies have reported reverse-transcription PCR based amplification of all the eight gene segments of IAV to generate the whole genome sequences [33,91,128,231] but in most cases, the whole genome sequences were generated using MiSeq next-generation sequencing approach [150,214,325,329]. A great advantage of this sequencing approach is that it can identify novel influenza viruses in the swine samples [80,90,301].

Most of the serological investigations used one or more methods for influenza virus detection and subtyping in the swine samples e.g., ELISA, HI, NI, MN, or VN assays. The serological methods are useful in large-scale surveillances for screening large number of samples in a limited time. However, the molecular detection assays are more reliable than the serological methods given the higher sensitivity, but the serological assays are rapid and affordable hence are preferred for large-scale surveillance studies. The molecular and serological investigations report either active infections (viral RNA) or past exposures (antibodies) in swine samples, respectively. The molecular detection approaches followed by sequencing are largely used for the research focusing on the influenza virus epidemiology [73,78,82,87,94,128,146,150].

Table 2. An overview of swine sample types and methods used for detection of influenza viruses in swine populations worldwide.

S. No.	Swine Sample Types	Methods Used for Influenza Virus Detection	Virus Types/Subtypes Detected	References
1.	Nasal swab	RNA extraction, real-time RT-PCR, reverse transcription PCR, multiplex RT-PCR, ligation, partial/whole genome Sanger sequencing, Next-generation sequencing, phylogenetic analysis, virus isolation (MDCK cells/Caco-2 cells/HRT18 cells/SPF chicken eggs), transmission electron microscopy, HI assay, NI assay, ultracentrifugation	H1N1, H1N2, H3N2, H4N6, A(H1N1)pdm09, Reassortant H1N1, Reassortant A(H1N1)pdm09, Triple-reassortant H3N2, H1N7, H5N2, IBV, IDV	[5,37,43,45,46,61,74,88,137,157, 171,176,188,191,199,207,215, 219–221,241,243,248,250,263, 289,290,301,306,307,326,334]
2.	Tracheal swab	RNA extraction, real-time RT-PCR, reverse transcription PCR, Sanger sequencing, virus isolation (MDCK cells, SPF chicken eggs)	H5N1, IAV, ICV, H1N1, A(H1N1)pdm09,	[38,59,103,321,331]
3.	Nasal wipe	Real-time RT-PCR	H3N2	[289]
4.	Snout wipe	RNA extraction, real-time RT-PCR, virus isolation	IAV	[286]
5.	Oropharyngeal swab	RNA extraction, real-time RT-PCR, Sanger sequencing	IAV, A(H1N1)pdm09	[211,276]
6.	Nasopharyngeal swab	RNA extraction, real-time RT-PCR, virus isolation (SPF chicken eggs), Sanger sequencing	H3N2, A(H1N1)pdm09, IBV, ICV	[38,141,211,277]
7.	Oral fluid	RNA extraction, real-time RT-PCR, virus isolation, Sanger sequencing, Next-generation sequencing (MiSeq)	IAV, IDV, H1N1, H1N2, H3N2, A(H1N1)pdm09	[45,192,227,282,285,287,302,322]
8.	Blood/ serum	IDEXX Ab test, ELISA, HI assay, NI assay, VN assay, MN assay, western blot, virus isolation (MDCK cells)	H1N1, H1N2, H3N2, A(H1N1)pdm09, H5N1, H5N2, H5N3, H9N2, H7N9, H3N1, H4N8, H6N6, H6N2, H7N2, IBV, ICV, IDV	[37,38,46,52,59,70,79,96,97,135, 138,153,186,195,200,215,291, 306]
9.	Lung/liver/internal organ tissues	RNA extraction, real-time RT-PCR, reverse transcription-PCR, ligation, HI assay, virus isolation (MDCK cells/SPF chicken eggs), Sanger and Next-generation sequencing, hematoxylin-eosin staining, immunohistochemistry, Immunofluorescence	H1N1, H1N2, Reassortant H1N1, H3N2, H2N3, A(H1N1)pdm09, H7N2, IDV	[45,121,127,128,130,201,203,243, 245,248,274,314,320,325,327]
10.	Lung homogenate	RNA extraction, real-time RT-PCR, multiplex RT-PCR, single step RT-PCR, virus isolation (MDCK cells/Caco-2 cells/SPF chicken eggs), Sanger sequencing, membrane enzyme immunoassay, HI assay	H1N1, H1N2, H3N2, Reassortant H1N2, A(H1N1)pdm09	[130,213,218,292]
11.	Fecal slurry	RNA extraction, qRT-PCR	IAV	[88]
12.	Rectal swab	Nucleic acid extraction, reverse transcription, metagenomic sequencing	IAV	[300]

Several studies used histological examinations e.g., immunohistochemistry or immunofluorescence to identify the IAV symptoms in lung or other internal organs of either dead or severely sick swine sacrificed for the investigations [130,189,315,318]. Immunohistochemistry provides a rapid and affordable diagnosis of the influenza virus disease using swine tissue samples [344]. One major benefit of immunohistochemistry is that it can be used in the retrospective analysis of the archived tissue samples [245].

A large number of investigations have reported sub-clinical influenza virus infections in asymptomatic (apparently healthy) swine [78,103,150,161,171,278,300], indicating that influenza infections can go undetected while the swine may be shedding virus and hence may infect other swine and farm workers in contact [183]. Intriguingly, most of the swine samples processed in Australia, Europe, and North America were obtained from the symptomatic swine while most of the Chinese swine samples were collected from asymptomatic swine (Figure 5).

Symptomatic swine may exhibit mild or severe influenza like symptoms [59,202,252], including fever, coughing, sneezing, pneumonia, bronchitis, reduced appetite, diarrhea, nasal and/or ocular discharge, conjunctivitis, weakness, anorexia, prostration, weight loss, abortion in sows, and mortality in some cases [89,224,231,239,248,282,318]. Most studies where the swine were severely infected reported reduced appetite and weight loss [129,246,247,292]. Due to IAV infection, the swine takes longer to weigh 100 kg body mass [226], hence the IAV disease burden affects the swine farmers economically.

Varying rates of mortality of swine due to IAV infections were reported from around the world ranging from 0.5% to 30% [138,206,213,218,221,273,296,327]. This wide difference in mortality rate could be due to novel virus strains emerged through reassortments within the swine [273,315,327] or inter-species transmission, e.g., avian to swine transmission, resulting into severe disease outbreaks [27,80,119,133]. One example of the emergence of a novel influenza virus strain is the emergence of A(H1N1)pdm09 strain due to reassortment between avian and swine IAV strains in swine which resulted into 2009 influenza pandemic [33]. Additionally, the emergence of IAV subtype H1N2 is another classic example of influenza virus reassortment which resulted into severe disease outbreaks in Japanese and Korean swine populations during 1990s and 2000s [27,121,140].

Strains of IAV can infect the swine of any age group; piglets as young as one week may become infected with IAV naturally. Interestingly, a study in Denmark observed a piglet as young as just three days was infected with IAV despite having maternally derived IAV antibodies [183], suggesting that the infection might have occurred from the infected sow which was shedding the virus [183]. However, the symptoms of the influenza-like illness in swine may last only for one week but the virus shedding may still persist until 41 days after appearance of the influenza-like symptoms [183,296]. This phenomenon may have serious implications in influenza virus spill over to the non-infected swine as well as to the exposed farm workers due to prolonged virus shedding. Three other studies observed virus shedding in swine and reported that the virus shedding may persist until the 11 day [256], 20 day [255], or 29 day [296] after onset of the clinical symptoms in swine. This variation in the duration of the virus shedding might be strain dependent, which needs to be further investigated.

A higher rate of virus shedding and IAV prevalence was reported during the fall and early winter months than summer season because the high relative humidity present in the environment during summer decreases the transmission of influenza virus [267]. The high relative humidity in summer facilitates the generation of larger droplets which are less likely to be aerosol transmitted to a longer distance as they tend to fall on the ground quickly after their formation [240,258].

Several cases of inter-species transmission were identified which documented transmission of IAV between human and swine or between birds and swine. The occurrence of the avian influenza virus strains in swine in China (H5N1, H9N2, H4N1, H4N6, H5N3, H10N5, H4N8, H6N6, H7N9), United States (H4N6, H6N2, H7N2), Canada (H4N6, H3N3), South Korea (H7N2, H5N2), Nigeria (H5N1), and Egypt (H5N1, H5N2, H9N2) serve as the evidence of interspecies transmission of avian influenza viruses to swine. The first evidence of avian influenza virus active infection in

swine appeared in 1999 in Canada when H4N6 virus was isolated from a swine. Later several other avian-origin IAV strains were detected and sequenced in swine in China, Canada, and South Korea (Figure 6).

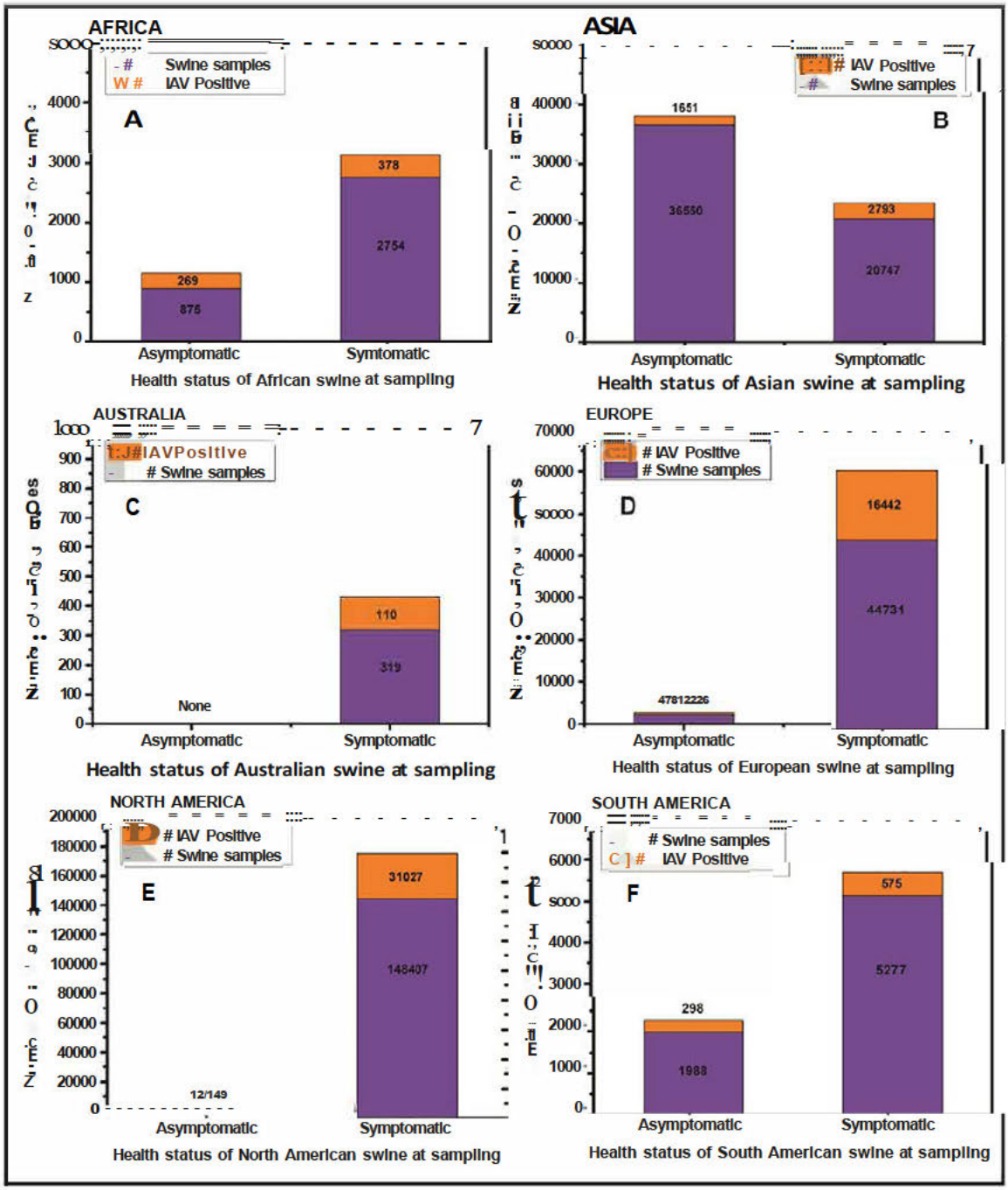


Figure 5. Health status of the swine at the time of sampling in (A) Africa, (B) Asia, (C) Australia, (D) Europe, (E) North America, and (F) South America. The asymptomatic swine were apparently healthy with no clinical symptoms while the symptomatic swine had mild to severe influenza-like disease symptoms. Most of the swine in North America, Australia, Europe, South America, and Africa represented influenza-like illness at sampling while most of the Asian swine were apparently healthy at the time of sampling. It should be noted that asymptomatic cases may not be detected if that was not an aim of the surveillance study. These graphs do not represent the severity of disease as the swine populations and the objectives of surveillances may vary among countries.

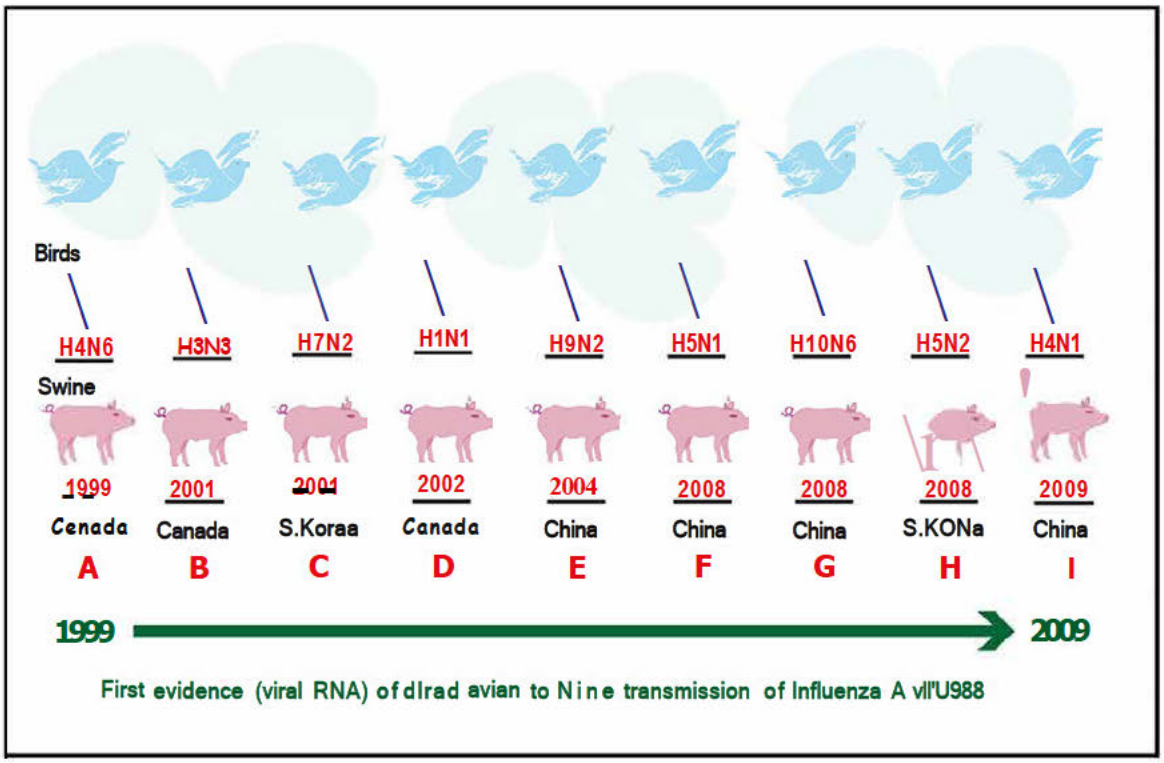


Figure 6. The first evidence of direct avian to swine transmission of avian-origin IAV strains. Viral RNA was detected through real-time RT-PCR and the partial or whole genomes were sequenced for the characterization of avian-origin IAV strains retrieved from the swine for the first time in Canada, South Korea and China.

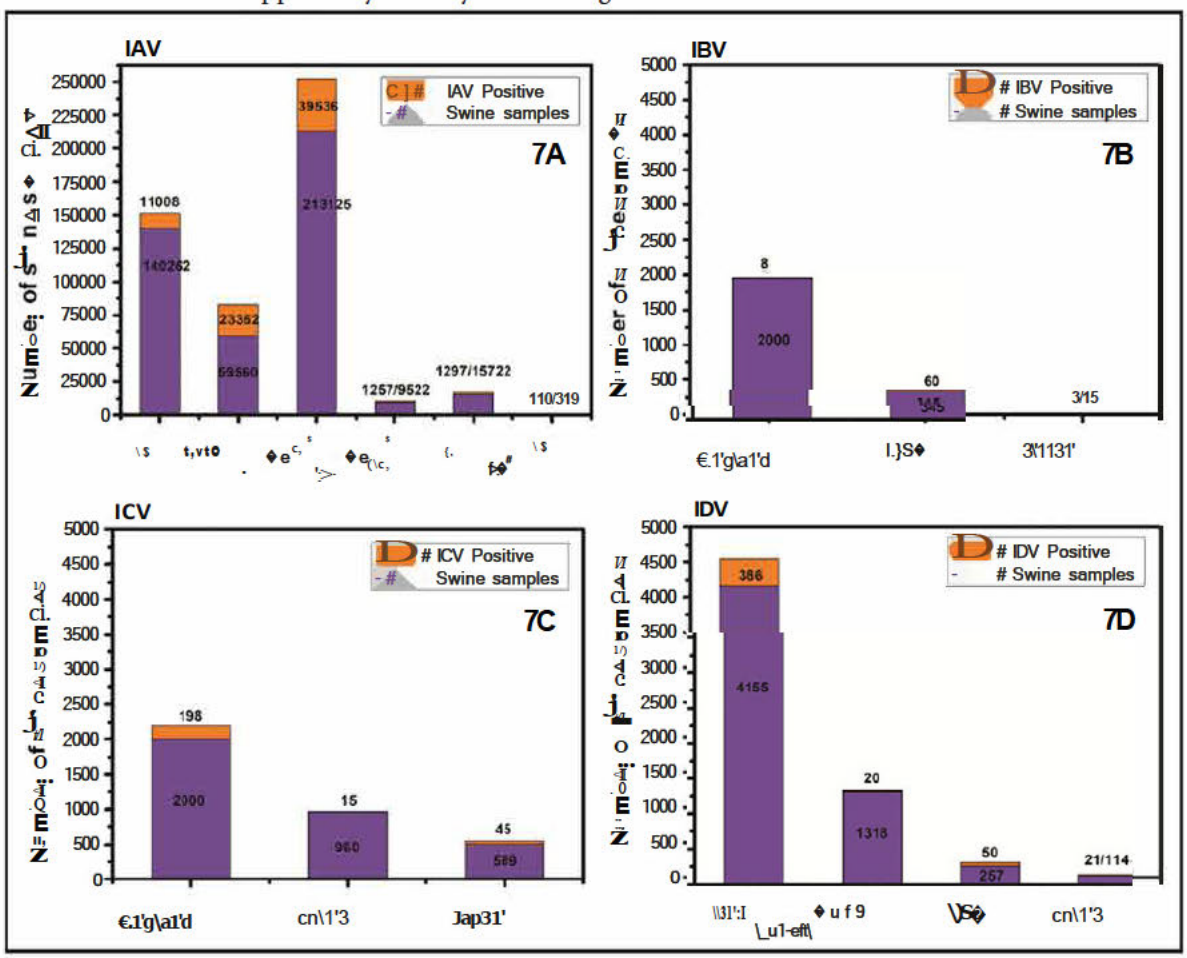
Various studies have spotted wild birds visiting the swine farms or in the vicinity which suggested that wild birds may have served as the carriers for the introduction of the different avian-origin IAV subtypes to the swine populations [59,80,100,246,332]. The highest number of avian-origin IAV strains were reported in Chinese swine which shows frequent avian-swine interaction in China, a country that has historically been an epicenter for influenza virus disease [69].

Egypt is recognized as a "hot spot" for the influenza virus reassortment due to its geographical location [345). The role of migratory wild birds in the introduction of avian influenza in Egypt has been already established [346,347], and the highly pathogenic strains of the IAV have previously been detected in migratory birds in Egypt [348). Since migratory wild birds were reported to harbor in the vicinity of Cairo [60) therefore, the probability of the migratory bird-swine interaction in the regions remain high which very well explains the occurrence of highly and low pathogenic strains of avian-origin IAV in swine in Cairo, Egypt. Given the "mixing vessel" nature of the swine, the occurrence of avian-origin IAV strains in swine is alarming in terms of IAV reassortment and evolution which may trigger the emergence of novel IAV strains of pandemic potential in the future.

Further, the multiple reports of double or triple reassortant IAV strains in swine are evidence that IAV co-infections may facilitate the antigenic diversity of the influenza viruses; and as a result, new HA and NA subtypes oflAV maybe continually added to the existing 18 HA and 11 NA subtypes in the future. Intriguingly, the frequency of the occurrence of double or triple-reassortant IAV strains in swine has dramatically increased in the recent decades [76,81,87,109,161,250,268,278]. One unique example of the reassortment and evolution of the pandemic strain of IAV in swine was the emergence of A(H1Nl)pdm09 virus in swine in Mexico which evolved due to the reassortment between avian and swine IAV strains [33).

While an overwhelming majority of investigations reported IAV in the swine across the world (Figure 7A), there were only a few reports which documented either active infections or past exposures

of the swine to the influenza virus types IBV [37,171,186,277] (Figure 7B), ICV [38,117,186] (Figure 7C) or IDV [5,43-46,217] (Figure 7D). A low prevalence of IBV was observed in swine given that only one study reported the IBV antibodies in swine samples in England during 1991-1992 [186] with no evidence of further spill over to other European countries. The active infection of IBV was later reported in US swine when two IBV isolates were obtained in 2009. A recent study from Taiwan reported three strains of the Victoria/B lineage of IBV in naturally infected swine in 2014 [171], again there was no further report of dissemination to nearby Asian countries. The IBV infected swine were apparently healthy with no signs of influenza disease.



Fi$_{gu}$ re 7. Prevalence of all the fom types of influenza vimses; (A) IAV, (B) IBV, **(C)** ICV and (D) IDV in swine populations worldwide. The highest IAV prevalence was reported in North American swine population followed by Europe and Asia. A significantly lower prevalence of IBV, ICV, and IDV was detected in certain Asian, Emopean and North American countries.

The first report of ICV appeared in Chinese swine after the virus was isolated from apparently healthy swine in 1981 in a routine diagnostic procedure at an abattoir in Beijing [38]. Later ICV seroprevalence was reported in English and Japanese swine during 1980s-1990s [117,186] with no further evidence of circulation anymore thereafter.

The IDV was first detected and characterized in 2011 in Oklahoma based swine in the United States which appeared to have made a species jump from cattle to swine [5,43]. Interestingly, a complete IDV genome was retrieved from a symptomatic sow in Italy in 2015 which was found closely related to the IDV genome reported in 2011 from Oklahoma, USA [217]. This might have happened due to the trade of the cattle or swine between Italy and the United States. A recent study from China identified IDV sequences which shared a high similarity (99%-100%) with the IDV sequences reported earlier from the cattle in China [102] which was another evidence of bovine to swine transmission of IDV.

The IDV has been in circulation in swine in the current decade with reports emerging from swine in Italy, Luxembourg, China and the United States.

In summary, IAV was first isolated from a swine in USA in 1930 [34,349] and later antibodies for the human influenza viruses were reported in swine at the State Prison of New Jersey in 1937 [259]. More IAV outbreaks and cases in swine in North America were reported during 1981–2000; the frequency has now dramatically fallen in the last two decades (Figure 8). This might be due to improved swine influenza surveillance and vaccination in North America in recent decades. The H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses were reported from the commercial, backyard, exhibition, feral swine and wild boars in the United States.

As of February 2020, the highest number of reports of influenza virus infections in swine in a country were documented in the United States (n = 40) followed by China (n = 39) and Canada (n = 24). The highest number of IAV positive swine samples were reported in the United States (36128/200384) followed by China (5031/90760). One of the factors behind the higher number of IAV cases in swine in the United States compared to China would be related to the disease symptoms. A majority of the North American swine samples that were screened for the IAV infections had either mild or severe symptoms of influenza-like illness which would have made it visually easier to identify IAV infected swine in the United States. On the contrary, a smaller number of Chinese swine exhibited influenza-like disease symptoms while a large proportion of the Chinese swine population appeared to have sub-clinical infections with no symptoms. This would have made it difficult for identifying the influenza virus infected swine during surveillances in China.

The first report of IDV in 2011 in Oklahoma swine reflected the antigenic diversity and evolution of influenza viruses in the US swine. However, the recent influenza virus disease prevalence in North American swine appeared to have declined, nevertheless, given the large swine population of the continent, the surveillance should continue to track the influenza virus diversity and evolution.

The first serological evidence of IAV in European swine was documented from the Czechoslovakia during 1969–1972, but the first H1N1 virus in European swine was isolated in Belgium in 1979 which was apparently transmitted from wild ducks in Germany to the swine in Belgium. Since then several H1N1, H1N2, H3N2, and A(H1N1)pdm09 viruses have been detected in commercial and backyard swine as well as in wild boars within Europe. The incidence of IAV in European swine has increased several folds in the past two decades with a relatively high number of IAV positive swine samples (19644/49814). Most of the IAV positive European swine were reported having influenza-like symptoms at the time of sampling. Germany reported the highest number of IAV positive swine in Europe where the pork industry is considered the third largest globally after China and the United States.

Importantly, the IDV was more recently identified in the European swine, first in Italy in 2015, and later a retrospective study identified IDV infection in swine samples collected in Luxembourg during 2014–2015 which indicated that the circulation of IDV in European swine took place only after 2014. The evidence has suggested the bovine to swine transmission of IDV. This observation is interesting because until recently more emphasis has been given to the avian-swine interaction and the bovine–swine interactions have been neglected from the influenza virus spill over perspective.

The first occurrence of IAV in Asian swine can be traced back to 1969, but the IAV prevalence has increased multi-fold in the recent two decades. The IAV subtypes H1N1, H1N2, H3N2, and A(H1N1)pdm09 have become endemic in swine in several Asian countries. The highly pathogenic avian-origin IAV strains of H5N1 and H7N9 have been reported from Chinese swine while H5N1 has been documented in swine in Viet Nam and Indonesia. The highly pathogenic strains of H5N2 have been reported in South Korean swine. Several LPAIV strains including H4N1, H4N6, H4N8, H6N6, H9N2, and H10N5 have also been documented in Chinese swine. The studies suggested a frequent interaction between wild birds and swine in China which appeared to have transmitted avian-origin IAV strains in the Chinese swine. Occurrence of equine influenza virus H3N8 in Chinese swine further expanded the genetic diversity of swine influenza viruses.

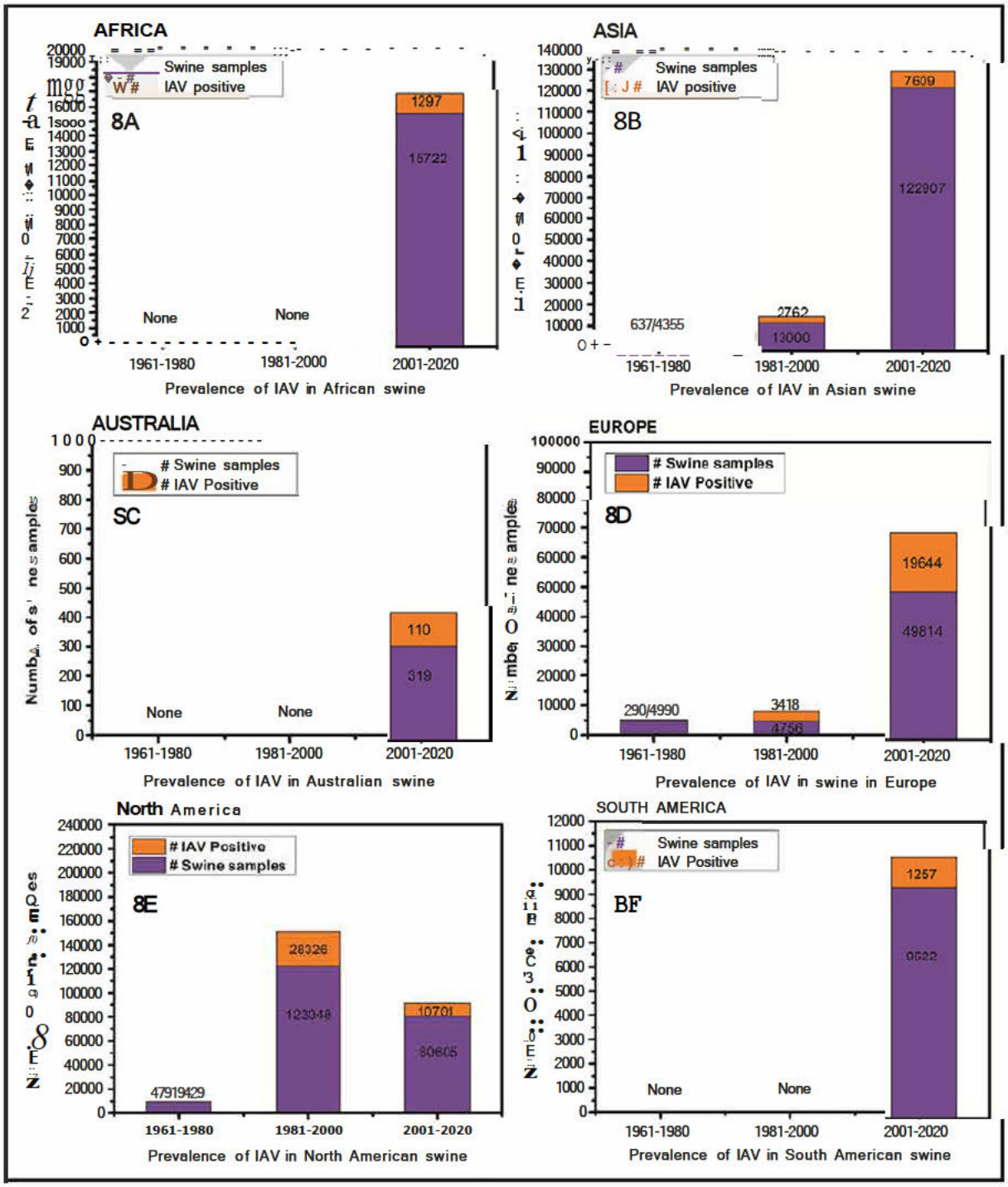


Figure 8. Historical progression (1961-2020) of IAV prevalence in swine populations in (A) Africa, (B) Asia, (C) Australia, (D) Europe, (E) North America, and (F) South America. An increasing IAV prevalence was observed in all the continents except North America where a smaller number of cases were observed during last two decades than 1981-2000.

Despite an avian to human transmission of certain avian influenza virus strains including H5N1 virus, only a limited human to human transmission of avian influenza viruses was established in the past [350,351]. With the passage of these avian-origin IAV strains in a mammalian host like swine, a high probability remains of these avian influenza virus strains to adapt and gain the ability of the human to human transmission, if happens, the consequences would be devastating for the public health.

Australian swine were free from influenza virus until the year 2009 when a New South Wales swine farm reported an influenza-like outbreak in the swine. The zoonotic transmission of the

A(H1N1)pdm09 virus was reported to the farm workers and the farm owner. Until now, there have only been four IAV reports in Australian swine which reflects a low prevalence of IAV in Australian swine. New Zealand is yet to officially report the influenza virus prevalence in swine and remains free from the disease.

A retrospective study identified that the A(H1N1)pdm09 virus was present in Mexican swine as early as 2000, well before the influenza pandemic occurred during March–May 2009. A high genetic diversity of IAV in Mexican swine due to live swine imports from North America and Europe during 1980s laid the foundation of the emergence of zoonotic strain of A(H1N1)pdm09 virus in Mexican swine [33]. The highest number of IAV positive swine in Central America were reported from Mexico followed by Guatemala. Interestingly, a report of the highly pathogenic H5N2 virus in Mexican swine in 2018 further triggers the alarm in the context of a potential novel IAV reassortment. Outbreaks of IAV in the South American swine populations occurred during the last two decades, with the highest prevalence reported in the Brazilian swine. A considerable proportion of cases showed sub-clinical infections with no symptoms which might have made it more difficult to detect the infected swine.

The reports of IAV active infections or the seroprevalence appeared in the African swine only during last two decades. Until February 2020, IAV have been detected in swine in Cameroon, Nigeria, Egypt, Kenya, Reunion Island, Uganda, Togo, and Ghana. However, South Africa has a considerable swine population [352], but currently there is no published report on the prevalence of active IAV or other influenza virus infections in the South African swine. This might be because of the lack of an active surveillance for the detection of the influenza virus disease in the swine in South Africa.

5. Conclusions

The reports of reassortant, double-reassortant and triple-reassortant influenza viruses in Asian, North American and European swine strengthens the concept of swine being the "mixing vessel" in terms of influenza virus reassortment and evolution. The multiple reports of avian-origin IAV strains including highly pathogenic H5N1, H5N2 and H7N9 in swine are alarming given the fact that the avian-origin strains may adapt in swine to facilitate the emergence of a reassortant pandemic strain. The highest number of influenza virus studies in swine population have been reported from the United States (n = 40) followed by China (n = 39). Also, the United States reported the highest numbers of IAV cases in swine. Due to widespread active surveillance, the United States has significantly brought down the influenza virus disease in swine in the last two decades. Conversely, the IAV disease burden has increased multi-fold in Chinese swine in the last two decades. Additionally, the occurrence of several high- and low-pathogenic avian-origin IAV strains in the Chinese swine population may put the country at greater risk of an influenza pandemic for the future. Given the "mixing vessel" nature of swine physiology, the occurrence of several avian-origin IAV strains and multiple reports of double-reassortant and triple-reassortant IAV subtypes in Chinese swine are alarming because reassortments in swine may facilitate the emergence of a new IAV strain of pandemic potential.

In the background of the current Corona virus pandemic (COVID-19) which originated in China, the presence of avian-origin IAV strains in Chinese swine must be considered a serious threat for the future and hence must be dealt accordingly. An active nationwide swine surveillance similar to that of North America which as a result, has brought down the current prevalence of influenza virus in the North American swine, should be in place in the rest of the world to safeguard the public health and the economics of the swine farming. A better and active worldwide swine influenza surveillance would be useful for upgrading the current diagnostic protocols and vaccines to prevent future influenza virus outbreaks.

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CHAPTER 4

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REVIEW

Open Access

A systematic review of influenza A virus prevalence and transmission dynamics in backyard swine populations globally

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Abstract

Background: Backyard swine farming is critical to generating subsistence and food security in rural and peri-urban households in several developing countries. The objective of this systematic review was to analyze the molecular and serological prevalence of influenza A virus (IAV) in backyard swine populations globally.

Results: We identified 34 full-text research articles in NCBI-PubMed and Google Scholar databases that have reported IAV sero- and/or virological prevalence in backyard swine up to 11 July 2021. The highest number of studies were reported from Asia (n = 11) followed by North America (n = 10), South America (n = 6), Africa (n = 6), and Europe (n = 1). While the maximum number of studies (44.12%) reported human-to-swine transmission of IAV, swine-to-human (5.88%), poultry-to-swine (5.88%), and wild birds-to-swine (2.94%) transmissions were also reported. An overall higher IAV seroprevalence (18.28%) in backyard swine was detected compared to the virological prevalence (1.32%). The human-origin pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 was the more frequently detected IAV subtype in virological studies (27.27%) than serological studies (18.92%). In addition, the avian-origin highly pathogenic H5N1 and H5N8 viruses were also detected, which further substantiated the evidence of avian-swine interactions in the backyards.

Conclusion: Human-swine and avian-swine interactions in backyards may transmit IAV between species. Monitoring the circulation and evolution of IAV in backyard swine would help stakeholders make informed decisions to ensure sustainable backyard swine farming and public safety.

Keywords: Avian influenza, Backyard swine farming, Biosecurity, Influenza A virus, IAV outbreak, IAV pandemic, Interspecies IAV transmission

Background

Swine farming is the largest meat-producing industry, with gross production of more than one-third of all the meats consumed globally (1, 2]. While pork is produced mainly by large-scale commercial farms to meet the demand, many small-scale backyard farms co-exist, primarily for subsistence and food security, within the rural or peri-urban households in developing countries [2]. A backyard farm is a household unit in rural, agrarian, or peri-urban communities that rears one or more animal species, including swine *(Sus scrofa domesticus)*, chicken, ducks, turkey, and cattle, raised for either household consumption or supplying within the local community for subsistence. One prominent challenge backyard swine farming faces is the lack of suitable biosecurity, which may facilitate the dissemination of zoonotic pathogens, including influenza A virus (IAV), endangering backyard farming and public health. Negligence of biosecurity at the backyard farms may provide a suitable environment

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for disseminating IAV within the backyard animals, resulting in IAV disease outbreaks causing economic losses to the swine growers [3–10]. Several reports in the recent past have documented highly and low pathogenic avian influenza viruses in migratory and other birds [11–15] that can disseminate the IAV strains between the countries and continents [16]. Interactions of wild birds with domestic poultry and swine may transmit the IAV in the backyards. Additionally, the probability of zoonotic and reverse zoonotic transmission of IAV between swine and occupationally exposed household members threatens public health. A schematic representation of IAV transmission in backyards is illustrated in Fig. 1.

For the first time, we reported the serological and molecular prevalence and the transmission dynamics of IAV exclusively in backyard swine populations globally.

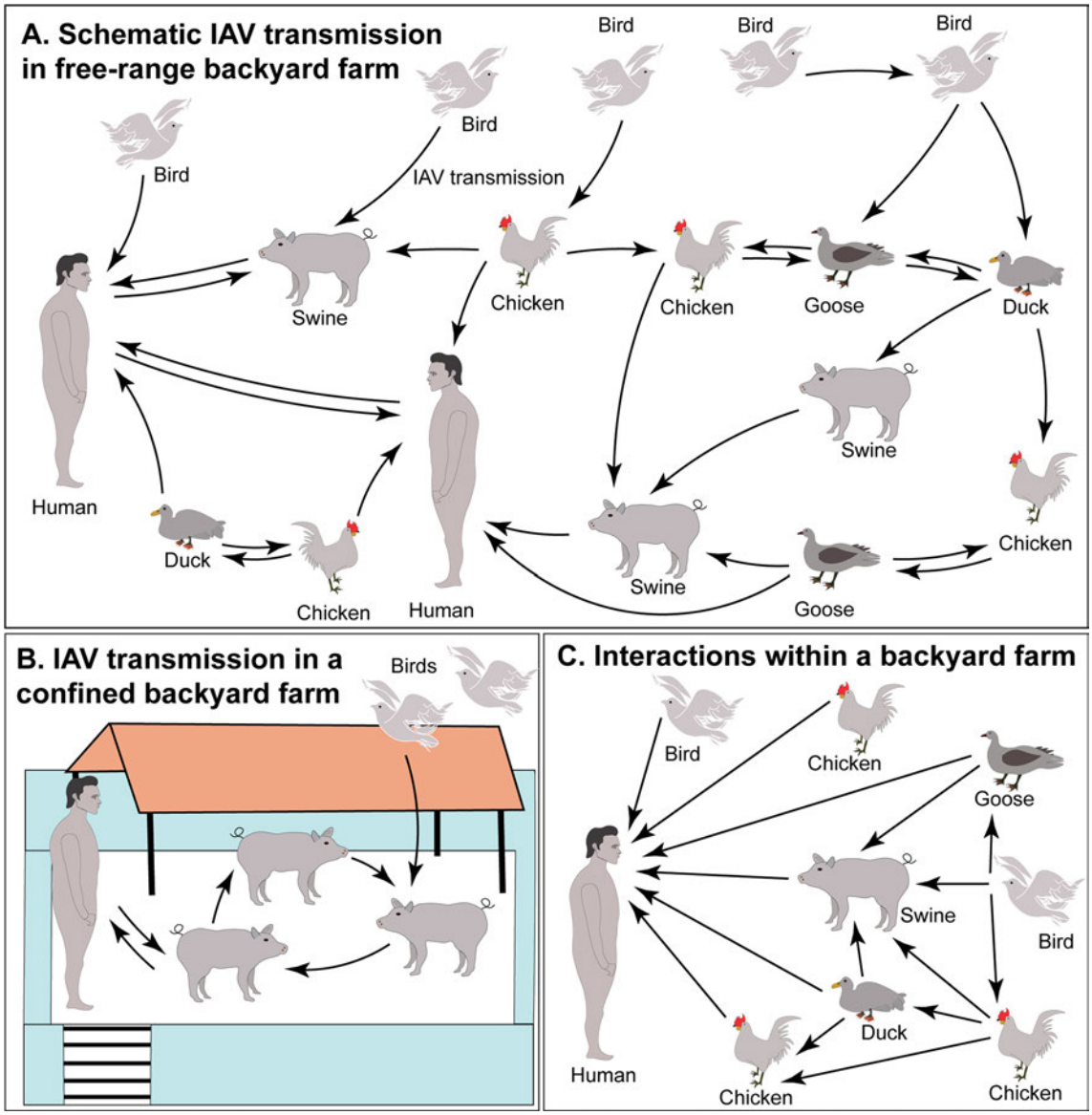

Fig. 1 A schematic representation of IAV transmission within the backyard farms. **a** Birds may disseminate IAV strain(s) to the poultry and/or swine. A high probability remains for the zoonotic and reverse-zoonotic transmission of IAV between swine and household members, which may also trigger human-to-human transmission. **b** A schematic representation of IAV transmission in backyard swine kept within the pens. Birds may disseminate IAV to the swine confined in the pens, resulting in swine-to-swine transmission. Occupational exposure may facilitate zoonotic or reverse zoonotic IAV transmission between swine and household members. The IAV transmission may occur in both production types through contaminated feed, water, or bird faeces. **c** Humans, swine, chickens, ducks, geese, and birds may frequently interact within the backyard which increases the risk of IAV transmission among them

This study describes the existing IAV circulation dynamics in backyard swine farms, which is essential to assess the current threat regarding the IAV disease outbreak. This study would also assist stakeholders in making an informed decision regarding minimizing the risk of IAV disease dissemination in backyard farms to ensure sustainable backyard swine farming.

Methods

Search criteria

Our objective was to analyze the transmission of IAV to the swine populations raised in the backyards, which may occur through swine–swine, poultry–swine, human–swine, and wild birds–swine interactions. A comprehensive search of NCBI-PubMed and Google Scholar databases was conducted up to 11 July 2021 for identifying the available research articles reporting IAV disease in backyard swine populations globally. The search terms including "Influenza A virus in backyard swine," "Influenza A virus in backyard pigs," "Influenza A virus in backyard production systems," "Influenza A virus in rural pigs," "Influenza A virus in household pigs," and "Swine backyard production systems" were entered in Google Scholar and NCBI-PubMed databases one by one for identifying relevant full-text research articles. The research articles from the database search investigating IAV serological and/or molecular prevalence in backyard animals, including swine, poultry, and cattle, were segregated into groups for analysis based on the interaction patterns. An overview of Preferred Reporting Items for systematic reviews and meta-analysis (PRISMA) flowchart [17] used to screen the relevant articles is depicted in Fig. 2.

Inclusion and exclusion criteria

The full-text original research articles which investigated molecular (virological) and/or serological prevalence of IAV exclusively in the backyard or household swine populations were included in this systematic review. The analysis did not include research articles that reported IAV sero- and virological prevalence in commercial, feral, exhibition swine, and wild boars. Conference abstracts, review articles, editorials, letters, commentaries, and opinions were not included in the analysis. We excluded the conference abstracts (n = 2) from the analysis because they did not provide sufficient information on the methodology as well as the sample size in one of the studies. Only English-language articles were included in the analysis. The relevant records were thoroughly screened through the titles, abstracts, and/or methodology, emphasizing the swine holding types for determining their relevance for inclusion in the study. The references of the identified relevant research articles were also screened to find other eligible research articles to be included in the analysis. All the relevant full-text research articles were downloaded for a detailed analysis.

Results

The first study that documented H1N1 virus antibodies in backyard swine sera was reported from a family backyard farm in Wisconsin, USA, in 1977 [18]. After that, 33 other studies have been conducted for detecting IAV sero- and/or virological prevalence in backyard swine populations in 26 countries. Interestingly, most of these studies (n = 32) were conducted in the last two decades (Fig. 3), suggesting that the IAV surveillance in backyard swine populations attracted significant attention only during the recent decades.

The studies included active and passive IAV surveillance in backyard swine from various countries, including clinically healthy and symptomatic swine. The clinical signs of IAV disease symptoms in backyard swine populations included nasal and ocular discharge, coughing, sneezing, anorexia, lethargy, incoordination, paralysis of the hindquarters, rapid weight loss, pneumonia, and mortality [18–24]. The backyard swine without clinical signs of illness had a significantly higher IAV seroprevalence (2897/15693; 18.46%) than virological prevalence (69/9389; 0.73%). On the other hand, backyard swine with clinical signs of illness had a comparable serological (89/635; 14.01%) and virological (66/797; 8.28%) IAV prevalence. An overview of IAV sero- and virological prevalence in clinically healthy and sick backyard swine is provided in Fig. 4.

The highest number of studies that investigated IAV sero- and/or virological prevalence in backyard swine populations were reported from Asia (n = 11) followed by North America (n = 10), South America (n = 6), Africa (n = 6), and Europe (n = 1). We analyzed the sero- and virological prevalence of IAV in backyard swine in a continent-wise manner as presented below.

Africa

Benin and Cote d' Ivoire

Following a previous outbreak of highly pathogenic avian H5N1 virus in West Africa, a study was conducted to determine the prevalence of other IAV subtypes in backyard swine populations of Benin and Cote d' Ivoire during 2009–2010. In this surveillance, backyard farms were preferred for monitoring because the previous H5N1 outbreak, during 2006–2008, predominantly occurred in backyard flocks. Sixty-two and 1548 nasal swab samples were collected from backyard pigs in Benin and Cote d' Ivoire, respectively, and tested using real-time RT-PCR; none of them were IAV positive, resulting in a negative molecular prevalence. In addition, 457 blood samples

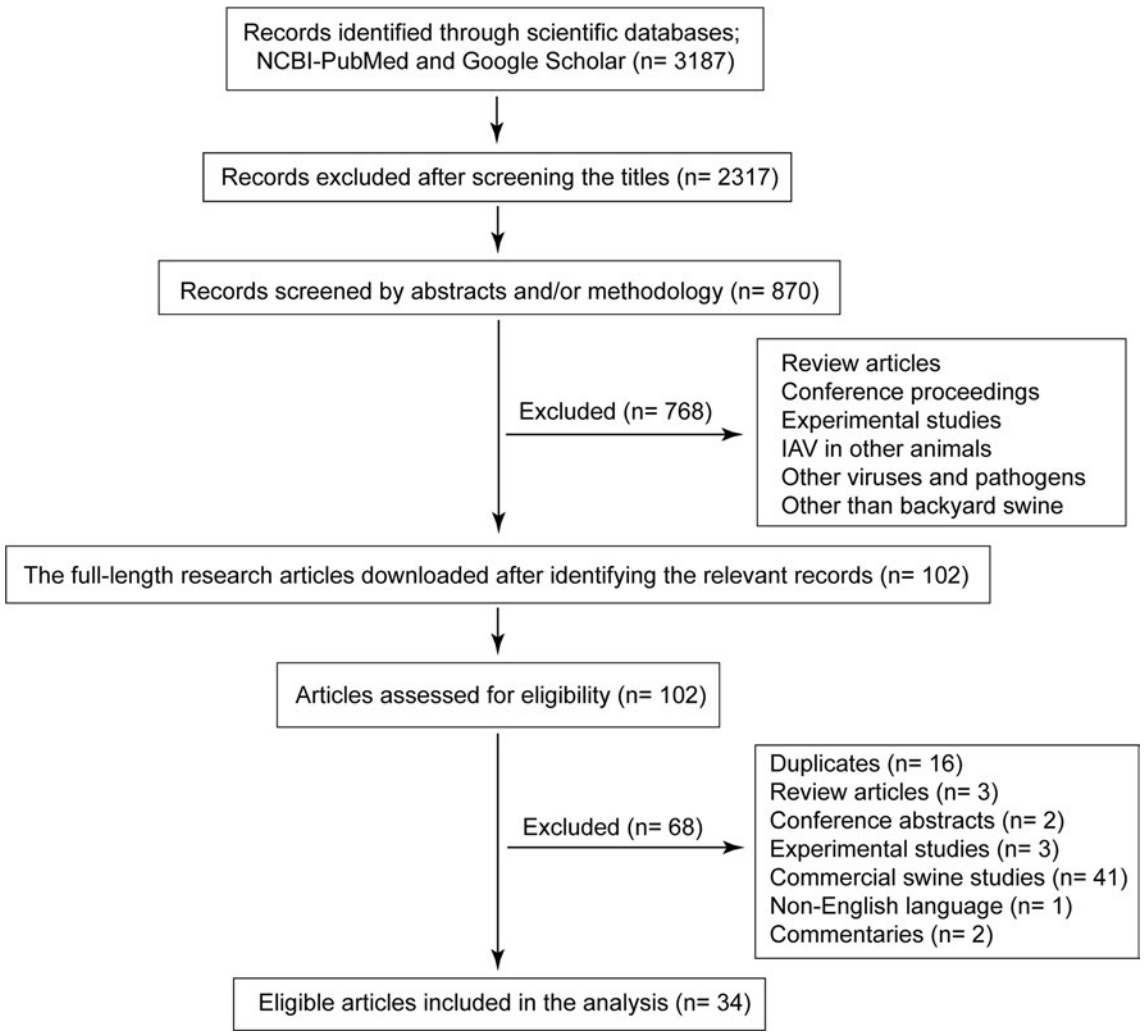

Fig. 2 PRISMA chart illustrating the search strategy for identifying the relevant full-text research articles available in NCBI-PubMed and Google Scholar databases up to 11 July 2021 for inclusion in the study. Full-text original research articles which investigated molecular and/or serological prevalence of IAV exclusively in backyard swine populations were included in systematic review

obtained from backyard pigs in Cote d' Ivoire were tested with ELISA and hemagglutinin inhibition (HI) assays, which resulted in a negative IAV seroprevalence [25].

Cameroon

The second study in Africa, which investigated IAV in backyard swine, was conducted in rural Cameroon during December 2009 and August 2012. Here, nasal swabs and sera samples were collected from 325 backyard swine. Only two swine were found infected with pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 through real-time RT-PCR, while the HI assay detected pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 and H3N2 virus antibodies in one swine serum [26], suggesting a low IAV sero- and virological prevalence.

Kenya

A total of 1491 nasal swab samples were collected from backyard swine in Kibera, Nairobi, during 2010–2012. Real-time RT-PCR and virus isolation identified 11 (0.7%) pigs infected with pandemic A(H1N1)pdm09 virus clade 1A.3.3.2. The complete genomes of three of the pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses were sequenced in this study. Additionally, 13 (10.2%) out of 127 swine sera samples were found IAV positive using the ELISA assay, while the HI assay detected H1N1 and H3N2 virus-specific antibodies. The IAV specific antibodies were also detected in poultry raised simultaneously with swine. The HA genes of the isolated pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses were closely related to the human

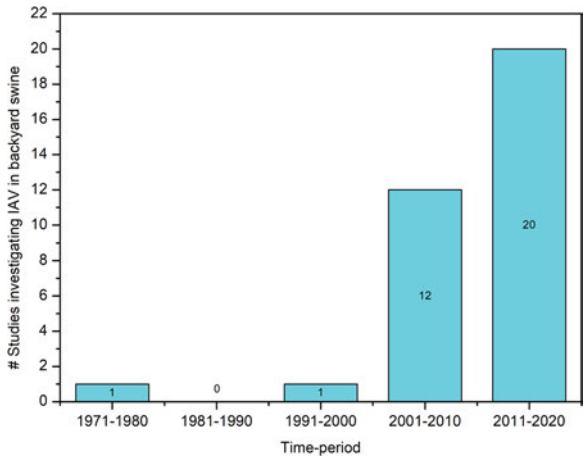

Fig. 3 The trend of IAV investigations in backyard swine populations globally

A(H1N1)pdm09 virus clade 1A.3.3.2 viruses reported from Kenya in 2009, which suggested human-to-swine transmission of the pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses in Kenya [27]. The second study in Kenya investigated IAV sero- and virological prevalence in backyard swine sera (n = 1990) and nasal swab (n = 2066) samples, respectively, during September 2013–April 2014. While the ELISA detected IAV-specific antibodies in 230 (11.56%) sera samples, suggesting past infection, none of the nasal swabs could amplify IAV specific sequences using RT-PCR, suggesting the absence of active IAV infection in these Kenyan backyard swine populations at the time of the surveillance [28].

Uganda

There was only one study that investigated the serological prevalence of IAV in Ugandan backyard swine. This study reported that the IAV specific antibodies were detected in 26 (4.98%) of 522 clinically healthy swine sera samples which were collected from household swine in the Lira and Masaka districts in 2015 [29].

Nigeria

Surveillance for IAV was conducted in Nigerian backyard swine during 2015–2016. Blood samples from 500 backyard pigs were collected for serological investigation, and 129 tracheal swabs and lung tissues were collected for virological investigation. The ELISA detected IAV-specific antibodies in 222 sera by HI assay, which confirmed pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 (n = 14) and highly pathogenic H5N1 virus (n = 6) antibodies in sera samples. Real-time RT-PCR detected 43 IAV positive samples suggesting active IAV infections. Sanger sequencing confirmed the presence of highly pathogenic H5N1 viruses (n = 5) in Nigerian backyard swine [12]. Overall, while the IAV seroprevalence was detected in backyard swine in Cameroon, Kenya, Nigeria, and Uganda, active IAV infections (virological prevalence) were identified in Cameroon, Kenya, and Nigeria (Fig. 5).

Asia

A significantly low rate of past IAV infections (seroprevalence) and active infections (virological prevalence) were determined by various studies that were conducted in Asian countries, with a few exceptions, including China, India, Bangladesh, and Bhutan, where a relatively higher seroprevalence (25.49%, 19.83%,

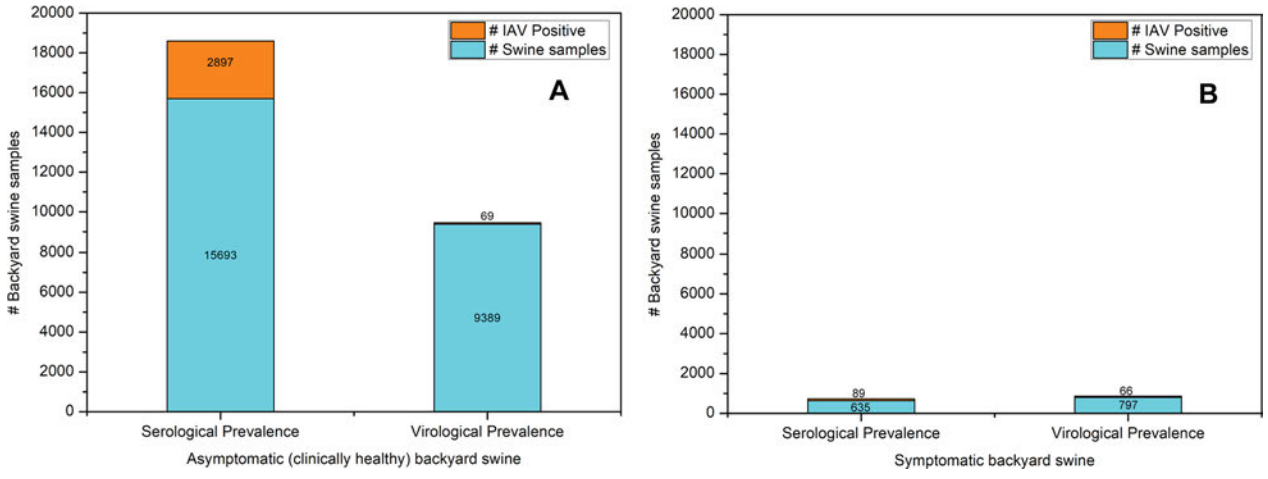

Fig. 4 Serological and virological prevalence of IAV in **a** asymptomatic (clinically healthy) and **b** symptomatic (sick) backyard swine populations globally

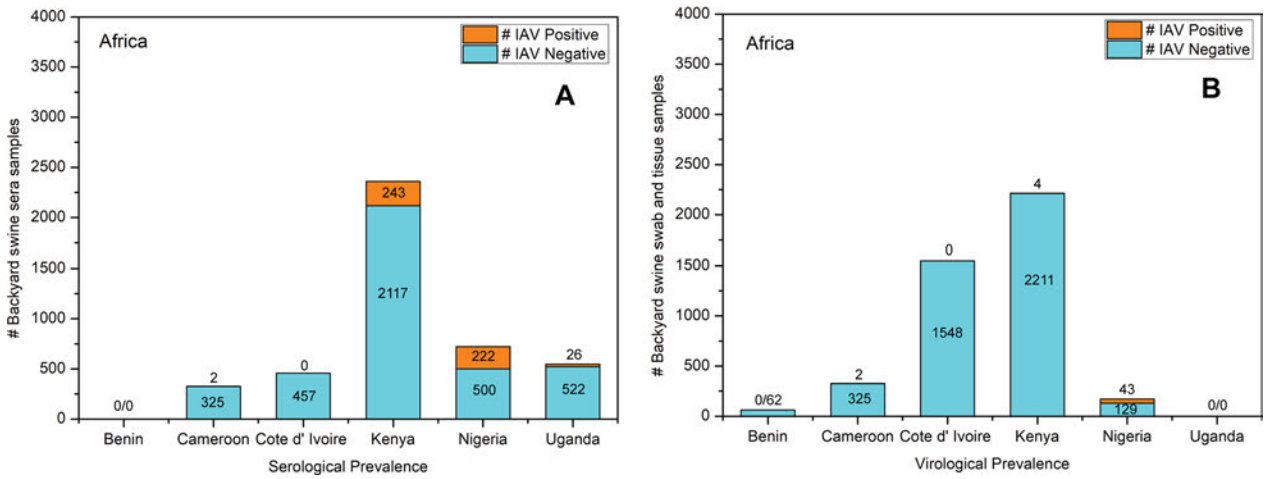

Fig. 5 **a** Serological and **b** Virological prevalence of IAV in African backyard swine populations

12.22%, and 7.74%, respectively) was reported in the backyard swine. An overview of IAV sero- and virological prevalence in Asian backyard swine is represented in Fig. 6.

Bangladesh

There was only one study that reported IAV serosurveillance in backyard swine in Bangladesh. This study collected 180 sera samples from the backyard swine from July to December 2013. The ELISA assay detected IAV-specific antibodies in 22 (12.22%) swine sera [30] which suggested the circulation of IAV in backyard swine in Bangladesh in the past.

Bhutan

A cross-sectional study during October 2011–February 2012 was conducted to identify the seroprevalence of IAV in rural backyard swine in Bhutan. Sera samples were collected from 465 backyard pigs in 69 villages and subjected to an ELISA test which detected IAV antibodies in 55 sera samples. All the ELISA positive sera were confirmed with the HI assay, which identified that 36 sera had pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 antibodies while 35 sera had European swine H1N1 virus clade 1C.1 antibodies [31].

Cambodia

Active IAV surveillance in rural Cambodia included 198 nasal swabs collected from backyard swine in 159

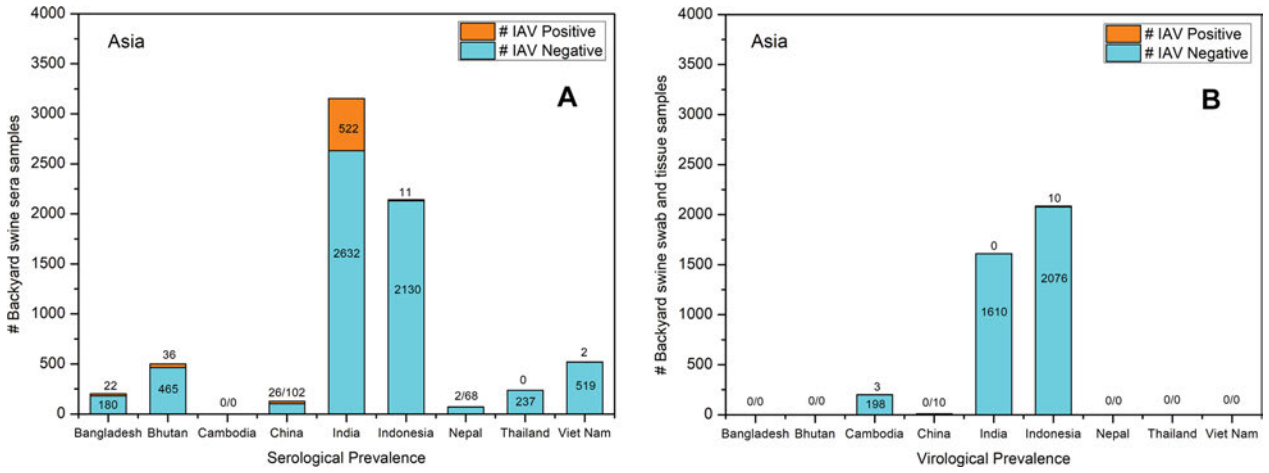

Fig. 6 **a** Serological and **b** Virological prevalence of IAV in Asian backyard swine populations

households located across 30 villages during May 2011–March 2013. Nasal swabs collected from a pig in 2011 and from two other pigs in 2012 were found IAV positive with real-time RT-PCR. The full-length genome sequencing of IAV positive swine identified H3N2 viruses in these three backyard swine [32].

China

A sero-surveillance study detected H1N1 and H3N2 virus antibodies in 12 backyard swine sera samples collected during November 1993 in backyard farms in Nanchang, China. Households that reared swine and poultry in the backyards were selected for the study. Samples were also taken from the household members which determined the occurrence of H1N1 and H3N2 virus antibodies in human sera. Additionally, four H3N2 viruses were isolated from the human nasal swab samples. The data suggested transmission of H1N1 and H3N2 viruses from household members to the backyard swine [33]. Additionally, five H3N8, H4N4, H7N4, and H11N2 viruses were isolated from duck faeces [33], suggesting the circulation of various IAV subtypes in some of these backyard farms.

In another study, a European avian-like swine H1N1 virus clade 1C.1 was isolated from the tracheal specimen of a 3-years and 8-months old deceased boy at a family backyard farm who developed fever and dyspnea, which led to his admission to a local hospital where he later died due to further complications. Further investigation at the family backyard farm detected European avian-like swine H1N1 virus clade 1C.1 antibodies in two swine sera samples. Another family member was also found to be H1N1 positive while being asymptomatic. The swine and poultry were raised free-range in this backyard farm; however, poultry was found seronegative for IAV infections. Several of the pigs raised on this backyard farm were reported to have developed fatigue and had been slaughtered before the commencement of the study [23]. While the deceased boy was reported to not have had any known contacts with the backyard swine, which an elderly family member primarily raised, the data suggested a zoonotic transmission event from swine to the elderly family member who may have consequently transmitted the virus to the young boy in the family [23].

India

Co-circulation of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 and seasonal human H1N1 influenza viruses were reported from Indian backyard swine during 2009-2016. A total of 2632 sera and 1610 nasal swabs from backyard swine were either collected or received from ten Indian provinces. All the sera samples were analyzed for pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 seroprevalence. While no virus could be isolated from the swine nasal swabs, 522 (19.83%) sera samples had antibodies for the pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 suggesting a past exposure of these swine to the pandemic A(H1N1)pdm09 virus clade 1A.3.3.2. Additionally, 14.3% of the tested samples also had antibodies only for seasonal human H1N1 influenza virus, suggesting a past co-circulation of these viruses in Indian backyard swine populations [34].

Indonesia

Two IAV surveillance studies were conducted in Indonesian backyard swine. The first surveillance collected 344 sera and 304 nasal and throat swabs from backyard swine in Indonesian villages during 2005. The microneutralization (MN) assay could not detect IAV antibodies in the sera samples. Similarly, all the 304 nasal and throat swabs were also negative for IAV with virus isolation and reverse-transcription-PCR [35], suggesting that the backyard swine populations under study were free from IAV. The second serological and virological surveillance took place in 2006 when 1786 sera and 1772 nasal swab samples were collected from backyard swine in Indonesian villages. The HI assay detected H5 antibodies in 11 swine sera samples, while the RT-PCR and Sanger sequencing identified ten H5N1 viruses in nasal swab samples [36]. This suggested previous as well as active H5N1 infection in Indonesian backyard swine.

Nepal

A single sero-surveillance for IAV was conducted in the Nepalese backyard swine from August 2016–February 2017. Total 68 sera samples were collected from free-range backyard swine and tested for IAV antibodies using ELISA assay. Only two sera samples were found positive for IAV antibodies [37].

Thailand

Only one sero-surveillance detected no IAV antibodies in 237 backyard swine sera samples collected from 74 households in Thailand during September 2016–February 2017 [38].

Viet Nam

A serological study collected 519 sera samples from backyard swine in 158 villages located in Northern Viet Nam during April 2005–August 2006 for determining IAV seroprevalence. ELISA tests detected IAV antibodies only in two swine sera samples [39], suggesting a significantly low rate of past infection of these backyard swine.

Europe

France

One highly pathogenic avian influenza H5N8 virus was detected in a backyard swine in France during an H5N8 outbreak among French poultry during 2016–2017. The HI assay confirmed that one backyard swine had antibodies against H5 clade 2.3.4.4b and identified that avian origin H5N8 virus would have been transmitted from domestic ducks raised on the backyard farm to the swine [40]. No clinical signs of disease were reported from the infected backyard swine. This was the only report of IAV in European backyard swine.

North America

Costa Rica

In Costa Rica, nasal swabs were collected from 509 backyard swine from 25 observation units with clinical signs of influenza-like illness during October–November 2010. An observation unit was defined as a space that confined the pigs either within an entire backyard or the pigs physically confined within an individual barn. Real-time RT-PCR detected 11 (2.16%) pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 positive nasal swab samples, which indicated a low rate of active IAV infection in these backyard swine [24].

Dominican Republic

Fifty-four sera samples collected from backyard swine in 36 premises in the Dominican Republic during August 2010 were tested for IAV seroprevalence using the HI assay, which detected H1N1 and H3N2 virus antibodies in 12 (22.2%) and 17 (31.5%) samples, respectively. These results suggested a past exposure of these backyard swine to the IAV. In this study, the human-to-swine transmission of IAV was suspected [22].

Guatemala

A study included collecting nasal swabs and sera samples from 426 backyard swine in 2010 and 2011 in Guatemala. Sera samples were tested with ELISA and HI assays, which identified 13 sera with A(H1N1)pdm09 virus clade 1A.3.3.2 and/or swine H1 and H3 virus antibodies. Interestingly, 52 nasal swabs were determined to be IAV positive, out of which four viruses were successfully isolated. While three isolates were confirmed to be pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses, one isolate was a human-like H3N2 virus. This investigation suggested a reverse-zoonotic transmission of human A(H1N1)pdm09 virus clade 1A.3.3.2 and H3N2 viruses to the backyard swine in Guatemala [19].

Haiti

Sera samples were collected from 109 backyard swine from 10 regions in Haiti during April 2010. The HI assay identified H1N1 virus antibodies in 24 (22.01%) and H3N2 virus antibodies in 13 (11.92%) sera samples [41] which suggested past infections of H1N1 and H3N2 viruses in backyard swine populations under investigation.

Mexico

Three studies have been conducted to detect IAV in Mexican backyard swine populations. The first study retrospectively analyzed 2094 backyard swine sera samples for the IAV antibodies from 2000 to 2009. The HI assay identified the highest seroprevalence of swine H1N1 virus (74%), which was followed by swine H3N2 virus (24.2%), pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus (17.8%), and human H1N1 virus (1.3%) [42]. Intriguingly, the findings revealed the seroprevalence and, therefore, the past exposure of these Mexican backyard swine to the pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses. This was an interesting observation because it suggested that the pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses were circulating in the Mexican swine well before the emergence of the 2009 swine flu pandemic, which originated in Mexico. In the second study, nasal and rectal swabs from 23 backyard swine were collected from four backyard swine farms located in rural areas in Mexico during February–July 2016. Next-generation sequencing was conducted to study the virome of the backyard swine, which identified several RNA and DNA viruses in the samples under investigation; however, IAV was not detected in these samples [43]. The third study included nasal swabs (n = 175) of backyard swine, which were tested for IAV prevalence using a real-time RT-PCR assay. These samples were collected from a wetland which was located at a wild duck-backyard livestock interface in Mexico. The study's objective was to determine IAV circulation at this interface and identify and characterize the IAV subtypes. While none of the swine nasal swabs tested IAV positive, which ruled out active IAV infection in the backyard swine, three IAV subtypes, including H1N1, H3N2, and H5N2, were detected in the Mexican duck (*Anas diazi*), which emphasized the significance of active IAV surveillance in the region to monitor the possible future spillover [44].

Trinidad & Tobago (West Indies)

In a sero-surveillance conducted during October 2013–February 2015, 139 swine sera were collected from small backyard farms located on the island of Trinidad. In addition, 45 swine sera were collected from the small

backyard farms located on the island of Tobago. While the ELISA assay detected IAV antibodies in 14 (10.07%) sera obtained from Trinidad, the sera from Tobago were negative for IAV seroprevalence. Among the ELISA positive samples from Trinidad, H3N2 and pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus antibodies were detected by the HI assay [45]. These data suggested a past circulation of IAV, which appeared to be confined to the island of Trinidad, while no past IAV exposure was detected in the backyard swine on the island of Tobago in this investigation.

United States

Following a respiratory disease outbreak in the swine on a family farm in Wisconsin in October 1975, characterized by sneezing, coughing, and anorexia in the swine, an 8-year-old boy who had close contact with the swine in the household became ill with fever, headache, chills, abdominal pain, and sore throat. The swine H1N1 virus antibodies were detected in the serum sample of the boy 3 weeks after the onset of the illness. As a result, an investigation was initiated to ascertain the source of infection to the boy. Interestingly, the antibodies for the swine H1N1 virus were also detected in eight of the ten swine sera samples collected from the boy's family farm [18]. These data suggested swine-to-human zoonotic transmission of the swine H1N1 virus on the Wisconsin family farm.

In a more recent study, after observing the symptoms of pneumonia and rapid weight loss in one-month-old piglets at a small backyard piggery during November 2010 in Colorado, intestine, and lung tissue samples were submitted to the laboratory for diagnosis. Molecular diagnostics followed by virus isolation and genome sequencing for the hemagglutinin (HA) gene identified that the two piglets were infected with pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus. Since the piggery was owned by a pharmacist who may have had occupational exposure to the A(H1N1)pdm09 virus clade 1A.3.3.2 virus, reverse-zoonotic transmission from the owner to the piglets was suspected [21]. An overview of IAV sero- and virological prevalence in North American backyard swine populations is represented in Fig. 7.

South America

Brazil

An IAV serological surveillance during 2012 and 2014 consisted of 1667 backyard swine sera from 479 subsistence swine holdings in the Rio Grande do Sul province of Brazil. All the swine sera were initially screened for IAV antibodies using the ELISA assay. The ELISA positive sera (n = 111, 6.65%) were subtyped using the HI assay, which detected pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus antibodies in 92 (5.51%) swine sera, while six sera had antibodies for H1N2 and five sera had antibodies for H3N2 viruses [46]. This was the only investigation from Brazil reporting IAV seroprevalence in backyard swine.

Chile

Chile reported the highest number of studies (n = 4) that conducted IAV surveillance in backyard swine populations. The first study investigating both IAV virological and serological prevalence was conducted in Central Chile during 2012–2014. A total of 67 nasal swabs and

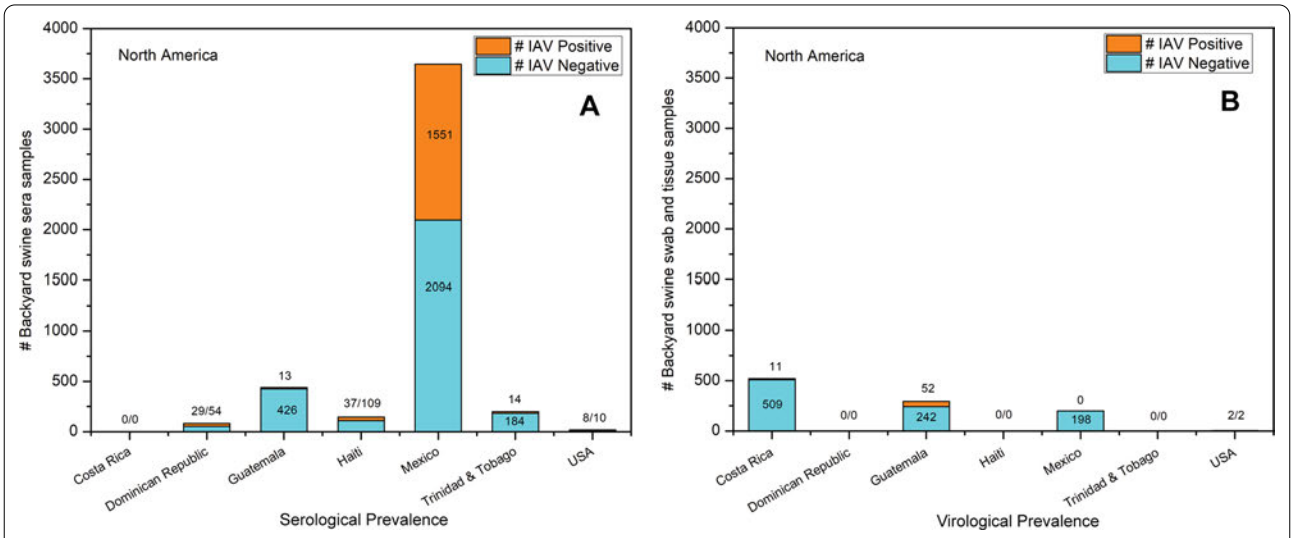

Fig. 7 Serological (**a**) and virological (**b**) prevalence of IAV in North American backyard swine populations. The highest seroprevalence was reported in the Mexican backyard swine. The highest virological prevalence was identified in the backyard swine in Guatemala

127 sera were collected from backyard swine which were subjected to real time-PCR and ELISA tests, respectively. While all the nasal swabs were negative for IAV, only two sera had IAV antibodies suggesting a low circulation of IAV in backyard swine under investigation [47]. The second study reported active IAV infection in backyard swine during Spring 2013 and Fall 2014 and identified an H1N2 virus from a swine nasal swab sample [20]. Interestingly, poultry and geese were also identified as IAV positive at the same backyard farm, suggesting the risk of interspecies transmission of IAV. The third study identified an H1N2 virus from a nasal swab sample of a backyard swine in Central Chile [48]. This study included three nasal swabs and 266 sera of backyard swine collected during 2013–2015 in Central Chile. While the ELISA detected IAV antibodies in 86 (32.33%) swine sera samples, the HI assay detected IAV antibodies in only 22 (8.27%) sera. Subtypes identified were human H1N1, swine H1N1, swine H1N2, and pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses. Interestingly, 15 of the swine sera samples were positive for multiple IAV subtypes, as determined by the HI assay, suggesting the co-circulation of multiple IAV subtypes in backyard swine in Chile. In addition, active infection was detected in one of the nasal swabs which was identified as an H1N2 virus after sequencing [48]. The fourth surveillance study was conducted in backyard swine during September 2013 and July 2015. In this study, 64 sera and 39 nasal swab samples were collected. While four swine sera were IAV seropositive with ELISA, only one nasal swab was IAV positive using a real time-PCR assay [49]. None of the IAV positive samples in this study could be subtyped.

Peru

A total of 1303 sera and 923 tracheal swab and lung tissue samples were collected at the time of slaughter from apparently healthy backyard swine in Tumbes, Peru, at four different times during March 2009–October 2011. While the HI assay detected pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus antibodies in 110 (8.44%) sera, virus isolation and reverse transcription-PCR detected pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses in one lung tissue sample and four tracheal swabs only. Despite a significantly low molecular prevalence, the phylogenetic analysis determined more than one human-to-swine transmission events for these pandemic A(H1N1) pdm09 virus clade 1A.3.3.2 viruses in Tumbes [50]. An overview of IAV sero- and virological prevalence of South American backyard swine is represented in Fig. 8.

In summary, an overall significantly higher seroprevalence (18.28%; 2986/16328) (Fig. 9a) was detected in backyard swine populations compared to the virological prevalence (1.32%; 135/10186) (Fig. 9b). A relatively higher IAV seroprevalence was reported in backyard swine populations in Brazil, India, Kenya, Mexico, Nigeria, and Peru (Fig. 9c). More precisely, a higher seroprevalence of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses were reported than other IAV subtypes in backyard swine in various countries. On the other hand, IAV active infections (virological prevalence) were reported from backyard swine in Cameroon, Kenya, Nigeria, Cambodia, Indonesia, Guatemala, Chile, Peru, and the USA (Fig. 9d). Notably, the IAV active infections included the subtypes of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2, H1N2, H3N2, and H5N1 viruses in backyard swine populations.

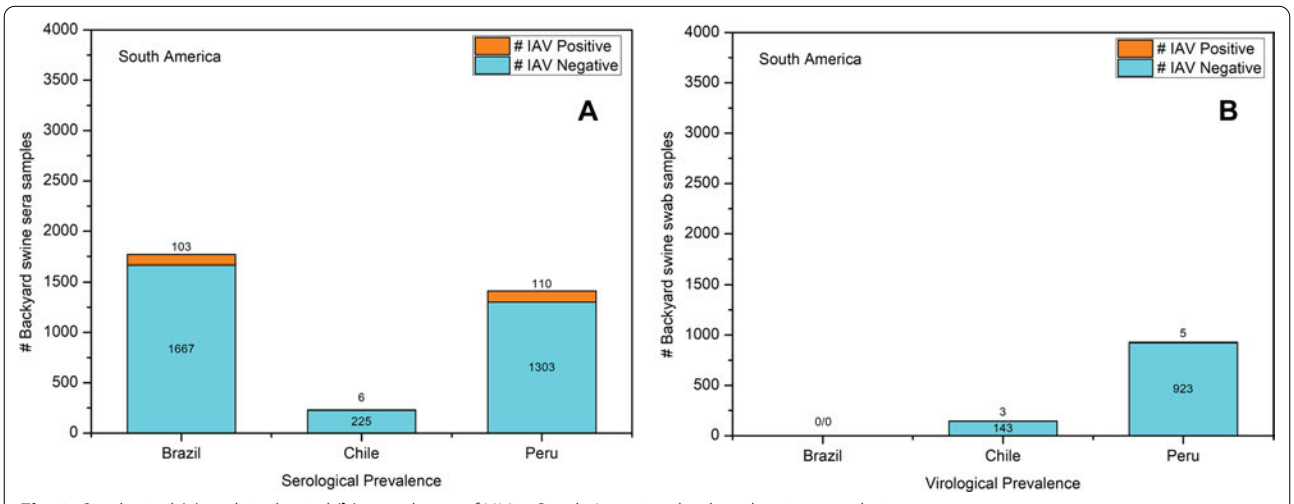

Fig. 8 Serological (**a**) and virological (**b**) prevalence of IAV in South American backyard swine populations

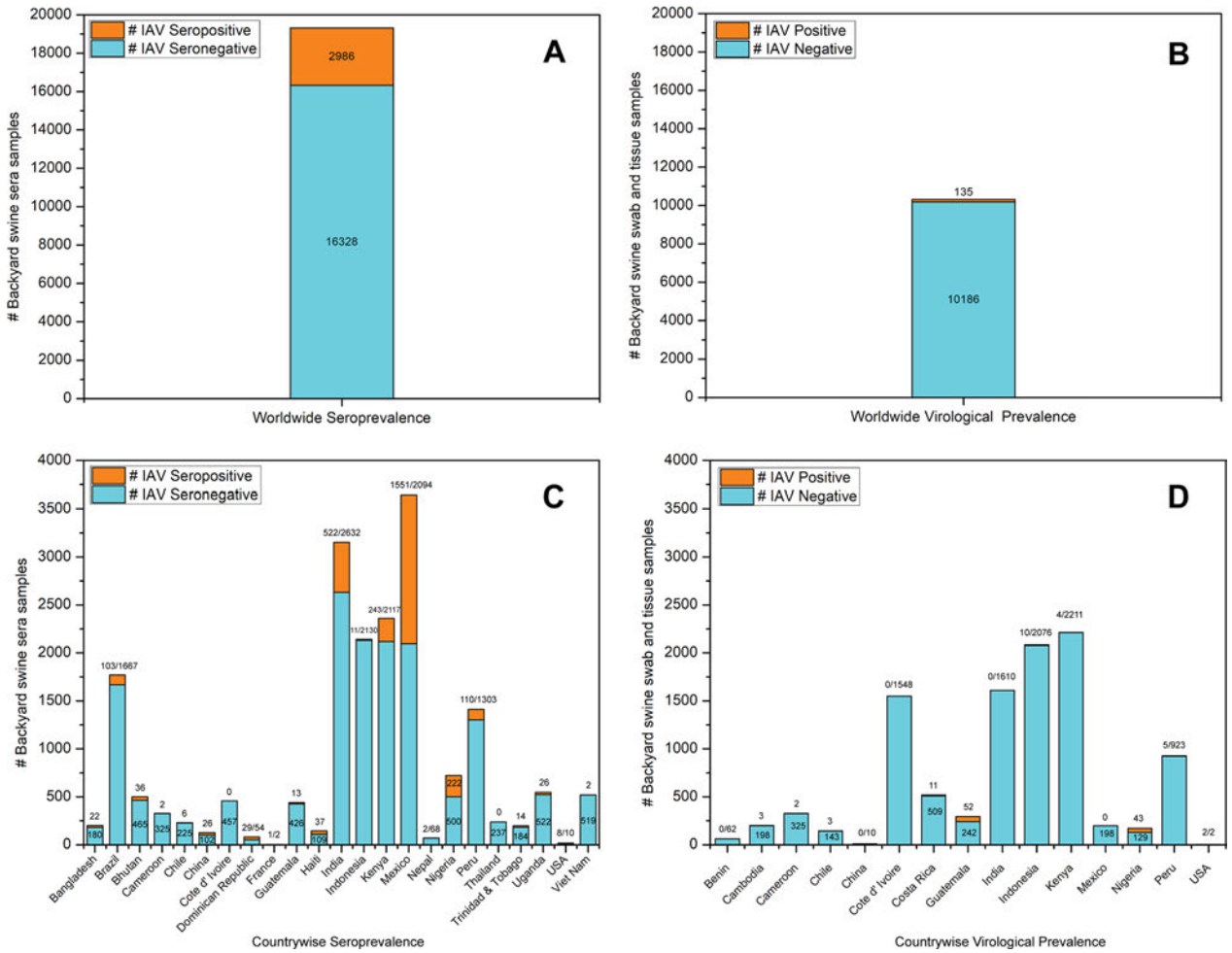

Fig. 9 **a** Serological and **b** Virological prevalence of IAV in backyard swine populations globally **c** Serological and **d** Virological prevalence of IAV in backyard swine populations in various countries. It should be noted that the objectives of IAV surveillance may vary among countries; hence the data presented here may only be used as an indicator of IAV sero- and virological prevalence in the backyard swine populations

IAV transmission dynamics in backyard swine

While a few studies (n = 13; 38.24%) reported that swine were the only household animals in the backyards [19, 21, 22, 41, 43, 45], most of the studies reported either the presence of swine and poultry (n = 19; 55.88%) [20, 24, 44, 47–49] or swine, poultry, and cattle (n = 2; 5.88%) [42, 46] (Fig. 10a). Interestingly, 13 (38.24%) studies reported that swine and poultry regularly interacted in the backyards [12, 20, 23, 27, 28, 33, 35, 37, 39, 40, 47–49], while seven (20.58%) studies reported interactions among the wild birds, swine, and poultry [24–26, 30, 32, 36, 44]. Two (5.88%) studies reported interactions among the swine, poultry, and cattle [42, 46], while the other remaining studies (n = 12; 35.29%) did not mention any interactions between backyard swine and other animal species [18, 21, 22, 29, 31, 34, 38, 41, 45] (Fig. 10b). As far as the reports of interspecies IAV transmission were concerned, numerous studies suggested human-to-swine (reverse zoonotic) transmission of IAV (n = 15; 44.12%) [19, 21, 22, 24, 26, 27, 29, 31, 33, 34, 41, 42, 45, 46, 50] while swine-to-human (n = 2; 5.88%) [18, 23], poultry-to-swine (n = 2; 5.88%) [36, 40], and wild birds-to-swine (n = 1; 2.94%) [47] IAV transmissions within the backyards were also suggested. On the other hand, several studies (n = 14; 41.18%), including those that conducted sero-surveillance only [29–31, 37–41], could not determine the epidemiology of IAV transmission in the backyards (Fig. 10c). While only a few studies were aimed towards investigating IAV in swine having clinical signs of disease (n = 8; 23.53%) [18–24, 41], most of the studies included clinically healthy swine (n = 26; 76.47%) [12, 25–40, 42–50] for the detection of IAV (Fig. 10d).

The molecular investigations that utilized real-time RT-PCR and Sanger sequencing for detection

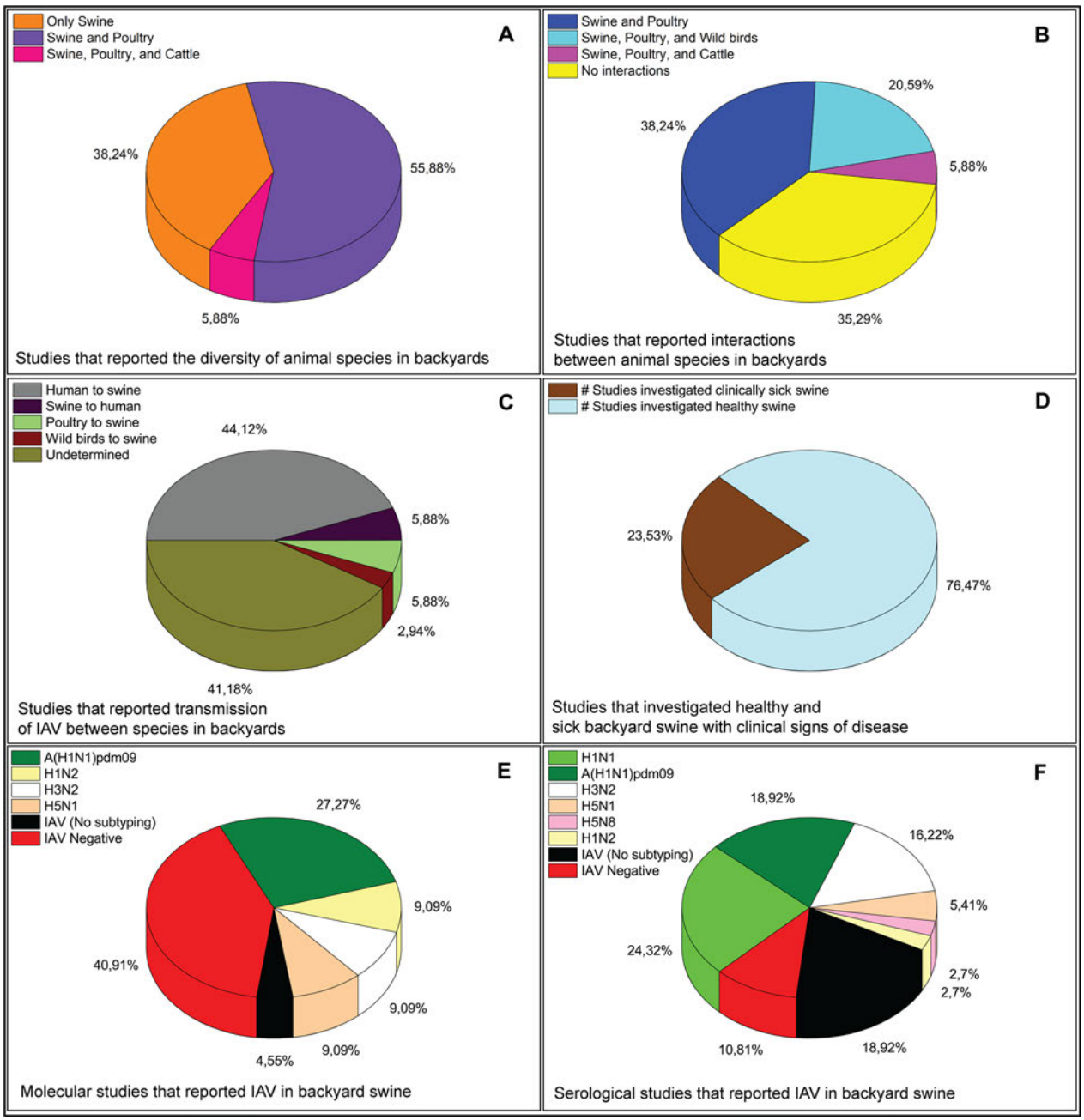

Fig. 10 The global status of backyard swine farming along with transmission dynamics of IAV. **a** Several studies reported the presence of various animal species in swine backyard farms. **b** The number of studies that reported the interactions between backyard swine and other animal species in the backyards. **c** The number of studies that reported IAV interspecies transmission in the backyards. **d** The number of studies that investigated symptomatic and clinically healthy backyard swine. **e** Many molecular studies identified and characterized various IAV subtypes in backyard swine swabs and tissue samples. **f** The number of serological studies that identified and characterized different IAV subtypes in backyard swine sera samples.

and characterization of IAV reported that pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 was the more frequently present IAV subtype (n = 6; 27.27%) [19, 21, 24, 26, 27, 50] in backyard swine populations compared to H1N2 (n = 2; 9.09%) [20, 48], H3N2 (n = 2; 9.09%) [19, 32], and H5N1 (n = 2; 9.09%) [12, 36] subtypes. Several molecular investigations (n = 9; 40.91%) could not detect IAV in backyard swine swab or tissue samples under investigation [23, 25, 28, 34, 35, 43, 44, 47],

which resulted in a negative IAV prevalence, suggesting the absence of IAV active infection at the time of the investigation (Fig. 10e). A few serological investigations (n = 7; 18.92%) that detected IAV antibodies in backyard swine sera but did not report the subtype was because they only used ELISA assay [28–30, 37, 39, 47, 49] (Fig. 10f).

Serological studies that used HI assay reported the antibodies for H1N1 (n = 9; 24.32%), pandemic A(H1N1) pdm09 virus clade 1A.3.3.2 (n = 7; 18.92%), H3N2 (n = 6; 16.22%), H5N1 (n = 2; 5.41%), H5N8 (n = 1; 2.7%), and H1N2 (n = 1, 2.7%) viruses (Table 1).

Discussion

Though numerous reports were available on IAV prevalence in organized (commercial) swine farms globally during the twentieth century [51], the IAV surveillance in rural backyard swine populations remained neglected until the emergence of the swine flu pandemic in 2009 in Mexican swine [52]. The swine flu pandemic of 2009 appears to have acted as a catalyst for IAV surveillance in backyard swine populations because most of the studies (85.29%; 29/34) were commenced after the swine flu pandemic hit in March 2009. Within a relatively short period during 2009–2021, total 34 studies have been reported providing valuable insights on IAV transmission dynamics in backyard swine populations in various countries [19, 38, 49]. While the human-origin pandemic A(H1N1) pdm09 virus clade 1A.3.3.2 viruses were more commonly reported in backyard swine, the human H1N1 and H3N2 viruses were also detected in backyard swine in various countries suggesting reverse-zoonotic transmission events from human-to-swine. Intriguingly, the presence of avian-origin highly pathogenic viruses including H5N1 [12] and H5N8 [40] suggest the possibilities of avian-to-swine transmission. In particular, the presence of highly pathogenic avian H5N1 viruses in Nigerian backyard swine is of interest because Nigeria falls within the East Africa–West Asia flyway of the migratory birds [53] used by long-distance migratory wild birds for intercontinental migration for overwintering [54]. The occurrence of significantly large numbers of highly pathogenic H5N1 viruses in poultry populations in Nigeria [12, 15], might be attributed to the migratory wild birds in the country. Since these birds can potentially introduce exotic IAV subtypes into the domestic bird populations, the interactions of swine, poultry, and wild birds in backyard farms pose a high risk of IAV disease transmission.

Similarly, South Africa, due to its unique geographic location, is a favourable destination for wild birds for over-wintering, and falls within the East Africa–West Asia flyway and East Atlantic flyway of wild migratory birds [53]. In recent years, numerous avian influenza virus subtypes have been reported in wild and domestic avian species in South Africa which poses a significant threat in terms of avian to swine spillover of these viruses [11, 55–60]. We recently reported avian (chicken, duck, pigeon, mallard, and other wild birds) to swine spillover and adaptation of eleven IAV subtypes in swine populations globally [61] which suggested that several of these viruses have been already adapted in swine. The adaptation of IAV in swine poses a threat regarding further spillover and disease outbreaks and threatens public health. A recent study from Chile suggested that the interaction of backyard swine with domestic poultry or wild birds may facilitate the transmission of IAV within the backyards [49].

While IAV active infection was detected only in a few backyard swine in Asia (Cambodia and Indonesia) [32, 36], seroprevalence was reported from Bangladesh [30], Bhutan [31], China [23, 33], India [34], Indonesia [36], Nepal [37], and Viet Nam [39]. We believe this was due to the surveillance objectives, which varied between countries. For example, six studies attempted only to detect seroprevalence of IAV in backyard swine in Asia [30, 31, 33, 37–39], while four investigated both sero- and virological prevalence [23, 34–36] and remaining one study investigated only virological prevalence in the Asian backyard swine [32]. Of note was the occurrence of only two surveillance studies in Chinese backyard swine [23, 33] despite China being the largest swine producer globally [62, 63]. Most importantly, it has been considered an epicentre of IAV disease [64, 65].

In contrast, numerous studies have reported several IAV subtypes in Chinese commercial swine populations [51]. The limited investigation in Chinese backyard swine suggests that emphasis in China has been placed on large-scale commercial swine farms, which are the major suppliers of pork meat to the Chinese consumers. The neglect of IAV disease surveillance on backyard swine farms in China could have far-reaching consequences given the region's widespread circulation of avian and human origin IAV subtypes [51]. For example, a recent study from China documented a novel subtype (G4 EA H1N1), which is a reassortant avian-like H1N1 swine IAV subtype of clade 1C.2.3 with genes from pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 at multiple Chinese commercial swine farms with evidence of zoonotic transmission to the swine farm workers [66]. While many studies have reported virological and serological prevalence of IAV on commercial large-scale swine farms in China [51, 67], limited information of IAV disease in backyard swine populations in China reflects the negligence regarding IAV surveillance in backyard swine farms in China. Similarly, the limited number of sero- and/or virological surveillance studies in other Asian

Table 1 A summary of studies that reported IAV subtypes in backyard swine populations globally

Continent	Country	Serological methods	Antibodies detected	Citations	Molecular methods	IAV subtypes identified	Citations
Africa	Benin	None	None	[25]	Real time RT-PCR	None	[25]
	Cote d'Ivoire	ELISA, HI	None	[25]	Real time RT-PCR	None	[25]
	Cameroon	ELISA, HI	A(H1N1)pdm09 virus clade 1A.3.3.2	[26]	Real time RT-PCR, Sanger sequencing	A(H1N1)pdm09 virus clade 1A.3.3.2	[26]
	Kenya	ELISA, HI	IAV, H1N1, H3N2	[27, 28]	Real time RT-PCR, virus isolation	A(H1N1)pdm09 virus clade 1A.3.3.2	[27]
	Nigeria	ELISA, HI	A(H1N1)pdm09 virus clade 1A.3.3.2, H5N1	[12]	Reverse transcription-PCR, real time RT-PCR, Sanger sequencing	H5N1	[12]
	Uganda	ELISA	IAV	[29]	None	None	
Asia	Bangladesh	ELISA	IAV	[30]	None	None	
	Bhutan	ELISA, HI	A(H1N1)pdm09 virus clade 1A.3.3.2, swine H1N1 clade 1C.1	[31]	None	None	
	Cambodia	None	None		Real time RT-PCR, Sanger sequencing	H3N2	[32]
	China	HI, NI	H1N1, H3N2	[23, 33]	Real time RT-PCR, virus isolation	None	[23]
	India	ELISA, HI	H1N1, A(H1N1)pdm09 virus clade 1A.3.3.2	[34]	Virus isolation	None	[34]
	Indonesia	HI, MN	H5N1	[35, 36]	Virus isolation, reverse transcription-PCR, Sanger sequencing	H5N1	[35, 36]
	Nepal	ELISA	IAV	[37]	None	None	
	Thailand	ELISA	None	[38]	None	None	
	Viet Nam	ELISA	IAV	[39]	None	None	
Europe	France	ELISA, HI	H5N8	[40]	None	None	
North America	Costa Rica	None	None		Real time RT-PCR, virus isolation, Sanger sequencing	A(H1N1)pdm09 virus clade 1A.3.3.2	[24]
	Dominican Republic	HI	H1N1, H3N2	[22]	None	None	
	Guatemala	ELISA, HI	A(H1N1)pdm09 virus clade 1A.3.3.2, H3N2	[19]	Real time RT-PCR, virus isolation, Sanger sequencing	A(H1N1)pdm09 virus clade 1A.3.3.2, H3N2	[19]
	Haiti	ELISA	H1N1, H3N2	[41]	None	None	
	Mexico	HI	Human H1N1, Swine H1N1, Swine H3N2, A(H1N1)pdm09 virus clade 1A.3.3.2	[42]	Real time RT-PCR, MiSeq	None	[43]
	Trinidad & Tobago	ELISA, HI	A(H1N1)pdm09 virus clade 1A.3.3.2, H3N2	[45]	None	None	
	USA	HI	Swine H1N1	[18]	Real time RT-PCR, virus isolation, Sanger sequencing	A(H1N1)pdm09 virus clade 1A.3.3.2	[21]
South America	Brazil	ELISA, HI	H1N2, H3N2, A(H1N1) pdm09 virus clade 1A.3.3.2	[46]	None	None	
	Chile	ELISA	IAV	[47, 49]	Real time RT-PCR, virus isolation, Sanger sequencing	H1N2	[20, 48, 49]
	Peru	HI	A(H1N1)pdm09 virus clade 1A.3.3.2	[50]	Reverse transcription-PCR, Sanger sequencing	A(H1N1)pdm09 virus clade 1A.3.3.2	[50]

countries also reflects the neglect of IAV disease surveillance in rural Asian settings having backyard swine compared to commercial swine populations [51]. The true disease status and evolution of IAV in backyard animals, including swine, cannot be determined in the absence of active IAV surveillance.

Most intriguingly, the seroprevalence of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus in backyard swine populations in Mexico during 2000–2009 suggested the occurrence and circulation of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses in Mexican backyard swine well before the emergence of the 2009 swine flu pandemic in Mexico [42]. This suggested that the continued circulation of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 virus in Mexican swine resulted in the acquisition of mutations for efficient mammalian transmission, and thus triggered the pandemic. The sero- and virological prevalence of human-origin H1N1, H3N2, and pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses in North American [21, 45] and H1N2, H3N2, and pandemic A(H1N1)pdm09 virus clade 1A.3.3.2 viruses in South American [48, 50] backyard swine populations indicated multiple human-to-swine transmission events. These data reiterated that human–swine interactions within the households or backyards may trigger zoonotic and reverse-zoonotic transmission of IAV endangering public health.

It has been established that the IAV infected swine may start virus shedding one day post-infection (dpi) while the virus can be shed up to 5 dpi [68] and hence can be detected successfully in swine nasal swab samples up to 5 dpi [69]. Additionally, swine's onset of disease symptoms occurs 1–3 dpi, while recovery starts about a week post-infection [70]. These data suggested that a delay in obtaining the nasal swabs from a clinically sick swine might result in a negative molecular detection. Interestingly, it should also be noted that other virus pathogens, including Porcine astrovirus type-4 (PAstV-4) can also cause acute respiratory illness in swine mimicking the symptoms related to influenza-like illness [71]. However, most of the studies investigated IAV in clinically healthy backyard swine which had no clinical signs of influenza disease, it appears to be one of the reasons why a low rate of active IAV infection was detected in clinically healthy backyard swine under investigation (69/9389; 0.73%). On the other hand, the HI assay can effectively detect IAV antibodies in swine sera at seven dpi while the peak may reach up to two-to-three weeks post-infection [72, 73] therefore the HI assay would be able to detect the IAV antibodies in clinically symptomatic swine, which appears to be the case in backyard swine sero-surveillances where a comparable percentage of serological IAV prevalence was detected in clinically healthy (2897/15693; 18.46%) and clinically symptomatic backyard swine (89/635; 14.01%).

It is evident that widespread prevalence and circulation of various avian influenza viruses in wild birds and domestic poultry poses a constant threat to backyard swine farming, given the inadequate biosecurity measures, and therefore needs continuous monitoring of IAV disease. Therefore, adequate biosecurity on the backyard swine farms is recommended, with minimized direct human–swine interactions to reduce the possibility of zoonotic and reverse-zoonotic IAV transmission, thereby safeguarding public health. These findings reiterate the need for ongoing surveillance to track IAV circulation and evolution in backyard swine populations.

Conclusions

Backyard swine farms rearing both swine and poultry remain at a high risk of IAV interspecies transmission from domestic poultry to swine. In addition, migratory wild birds pose a significant threat and may introduce exotic IAV subtypes to the backyard swine. The possibility of zoonotic and reverse-zoonotic transmission between swine and humans also persists within the backyard farms. The occurrence of pandemic A(H1N1)pdm09 virus clade 1A.3.3.2, highly pathogenic avian H5N1, and several other IAV subtypes in backyard swine populations should be of concern as this may cause disease outbreaks in swine as well as in the exposed human populations. The occurrence of both human and avian IAV subtypes in backyard swine may facilitate their evolution, representing public health risk. A policy of active IAV surveillance in backyard swine populations should be implemented to track their molecular evolution.

Abbreviations

Dpi: Days post-infection; ELISA: Enzyme-linked immunosorbent assay; IAV: Influenza A virus; HI: Hemagglutinin inhibition; MN: Microneutralization; PRISMA: Preferred reporting items for systematic reviews and meta-analysis; RT-PCR: Reverse-transcription-polymerase chain reaction.

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Authors' contributions

Conceptualization: RPC and MLG, Data acquisition and analysis: RPC, Preparation of figures and table: RPC, Writing-original draft preparation: RPC, Writing-reviewing and editing: RPC and MLG, Supervision: MLG. Both authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

This study is part of a research project which has been approved by Animal Research Ethics Committee of the University of KwaZulu-Natal; Reference: AREC/041/019O. Section 20 permit In terms of Animal Diseases Act, 1984 (Act No. 35 of 1984) was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa; Reference: 12/11/1/5/4 (1425).

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing Interests.

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CHAPTER 5

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Review of genome sequencing technologies in molecular characterization of influenza A viruses in swine

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Abstract. The rapidly evolving antigenic diversity of influenza A virus (IAV) genomes in swine makes it imperative to detect emerging novel strains and track their circulation. We analyzed in our review the sequencing technologies used for subtyping and characterizing swine IAV genomes. Google Scholar, PubMed, and International Nucleotide Sequence Database Collaboration (INSDC) database searches identified 216 studies that have utilized Sanger, second-, and third-generation sequencing techniques to subtype and characterize swine IAV genomes up to 31 March 2021. Sanger dideoxy sequencing was by far the most widely used sequencing technique for generating either full-length (43.0%) or partial (31.0%) IAV genomes in swine globally; however, in the last decade, other sequencing platforms such as lllumina have emerged as serious competitors for the generation of whole-genome sequences of swine IAVs. Although partial HA and NA gene sequences were sufficient to determine swine IAV subtypes, whole-genome sequences were critical for determining reassortments and identifying unusual or less frequently occurring IAV subtypes. The combination of Sanger and second-generation sequencing technologies also greatly improved swine IAV characterization. In addition, the rapidly evolving third-generation sequencing platform, MinION, appears promising for on-site, real-time sequencing of complete swine IAV genomes. With a higher raw read accuracy, the use of the MinlON could enhance the scalability of swine IAV testing in the field and strengthen the swine IAV disease outbreak response.

Keywords: IAV subtyping; IAV surveillance; influenza A virus; MiniON, next-generation sequencing; Sanger sequencing; swine IAV sequencing, whole-genome sequencing.

Influenza A virus (IAV; ***O 'thomyxoviridae, Alphainfluenza-virus)*** is the most prevalent of the 4 influenza virus types that have been reported in swine populations globally.[18] The tracheal receptors that efficiently bind human- and avian-origin IAVs make swine a favorable host or a mixing vessel for IAV inter-species transmission, reassortment, and evolution.[93] A broad range of antigenic diversity in hemagglutinin (HA) and neuraminidase (NA) genes exists in currently circulating swine IAV genomes,[10 4 10 5] and forms the basis of IAV subtyping;[3 110] however, mutations[118] and reassortments in internal gene segments are also critical for swine IAV evolution. The 2009 flu pandemic originated primarily from the interspecies transmission and reassortment of avian and human IAV strains within swine in Mexico, which triggered the emergence of a new IAV subtype termed the "A(HINI) pdm09" virus.[65] In addition, by utilizing various sequencing technologies, numerous other novel and reassortant IAV subtypes have been reported in swine in recent years.[9 7 100 103 106]

Sanger dideoxy sequencing was the first sequencing platform that was introduced in 1977,[90] and, given the long (~800 bp) and high-quality reads produced, is considered the gold standard for DNA sequencing.[11] Sanger sequencing utilizes a chain-termination strategy that relies on a modified DNA polymerase enzyme, which incorporates dideoxynucleotides (ddNTPs) at a specific position in the DNA template. The initial studies for sequencing swine IAVs used ^{32}P-end- labeled oligonucleotide primers[95] and had to be manually read from the sequencing gel, making this method hazardous as well as labor- and time-intensive. The introduction of automated DNA sequencers, such as the ABI 3730 DNA sequencer9 and the ABI 3130/3130x/ genetic analyzer,[66 69 70] as well as the replacement of radioactive-labeled primers with rhodamine-based fluorescent dyes, overcame these challenges and, as a result, this technology was used extensively to obtain up to 900 bases of IAV gene sequences per sequencing reaction.

The automation of Sanger sequencing along with the advent of next-generation sequencing (NGS) platforms, including the Roche 454 GS Junior in 2005, lllumina in

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2007, and the Ion Torrent PGM in 2010, also facilitated the whole-genome sequencing (WGS) of IAV in swine.[2,14,21] Although all next-generation technologies are capable of high-throughput sequencing, they use different sequencing strategies. Briefly, the Roche 454 GS Junior Titanium platform uses pyrosequencing by which it detects, in real-time, luminescence that results from the release of pyrophosphate at the incorporation of each nucleotide into the growing complementary DNA strand.[35] The Illumina platform utilizes a reversible chain termination strategy that uses fluorescently labeled reversible terminators (specially designed ddNTPs) that terminate primer extension during the sequencing reaction. In contrast, the Ion Torrent PGM works on the principle of proton detection by measuring the pH change that results from the release of a hydrogen ion upon incorporating a dNTP to the growing DNA template, using an ion semiconductor chip that offers a higher sequencing speed.[54]

One of the significant limitations of Roche 454 pyrosequencing was the generation of false signals for homopolymers of adenine (A) in the sequencing reaction.[86] Similarly, one of the limitations of the Ion Torrent PGM lies in its low accuracy in recognizing homopolymers of >6 nucleotides in the DNA template. Illumina sequencing offers a highly specific "base-by-base" sequencing technology for eliminating homopolymer errors. Various Illumina sequencers, namely, Genome Analyzer IIx (GAIIx), NextSeq, MiSeq, and HiSeq, have been used to identify and characterize IAV genomes in swine populations. Notably, the MiSeq platform of Illumina, because of its user-friendly interface, is a popular second-generation sequencer for generating full-length IAV genomes in swine.

The advent of the Nanopore MinION, a third-generation sequencing platform, has further enhanced the existing capabilities of generating complete swine IAV genomes.[84] Given that high accuracy and low sequencing cost are critical for large-scale swine IAV testing, the continuous upgrading of the sequencing platforms, over a relatively short period of time, has made swine IAV WGS more affordable. The advancement and automation of sequencing technologies has therefore made it possible to conduct genomic surveillance of emerging subtypes, and monitoring of IAV evolution, in real-time. We present here a comprehensive overview of the use of various genome sequencing technologies and their applications, reliability, and cost-effectiveness in swine IAV testing, and their capacity for the characterization of novel and reassortant IAV subtypes in swine.

Review protocol and search criteria

We adopted a 2-tier approach for identifying the relevant records for IAV genome sequencing in swine. First, a comprehensive search of scientific databases, including NCBI-PubMed and Google Scholar, was conducted to identify full-text research articles that reported genome sequencing for identifying and characterizing IAV subtypes in swine populations globally. Search terms, including "influenza A virus outbreak in pigs", "influenza A virus outbreak in swine", "influenza A virus in swine", "sequences of influenza A virus in swine", and "influenza virus disease in swine", were entered into the NCBI-PubMed and Google Scholar databases one by one. The title, abstract, methodology, and/or supplementary information associated with the research articles that emanated from the online database searches up to 31 March 2021 were screened for relevance for inclusion in our study. The supplementary data of publications were also used to assess the significance of articles for inclusion in our study. In a few cases, if the full-text research articles were not accessible online, those were requested from the authors through ResearchGate. The availability of full-length and partial IAV genomes and the information on sequencing methods were thoroughly verified through the NCBI-GenBank database using the accessions provided in each research article included in the analysis.

A further search of the International Nucleotide Sequence Database Collaboration (INSDC) database using the terms "influenza A virus in swine" and "influenza A virus genome sequencing" identified an additional 14 BioProjects reported from 9 countries that have attempted IAV genome sequencing in swine populations during 2017–2020 that were not available in the NCBI-PubMed and Google Scholar databases. The INSDC database is maintained by a collaboration of NCBI-GenBank, the European Bioinformatics Institute (EMBL-EBI), and the DNA Data Bank of Japan (DDBJ). INSDC publishes the technologies used for library preparation and sequencing. We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA)[53] 2009 flowchart to screen the literature and identify the relevant research articles because the PRISMA flowchart systematically explains each step for screening the literature and therefore helps to identify the relevant records (Fig. 1).

Inclusion and exclusion criteria

We included full-text original research articles and INSDC BioProjects that utilized genome sequencing to generate partial or complete IAV genomes in swine populations. Reviews, experimental studies, and serologic studies that did not attempt IAV genome sequencing in swine were not included in our analysis. Research articles in a language other than English were excluded from our analysis.

Records selected

As of 31 March 2021, we identified 216 records that have reported various methods for either partial or complete genome sequencing of IAV subtypes in swine populations globally (Fig. 1). The primary use of IAV genome sequencing was the subtyping and molecular characterization of IAV genomes to unravel the growing antigenic diversity of IAV in

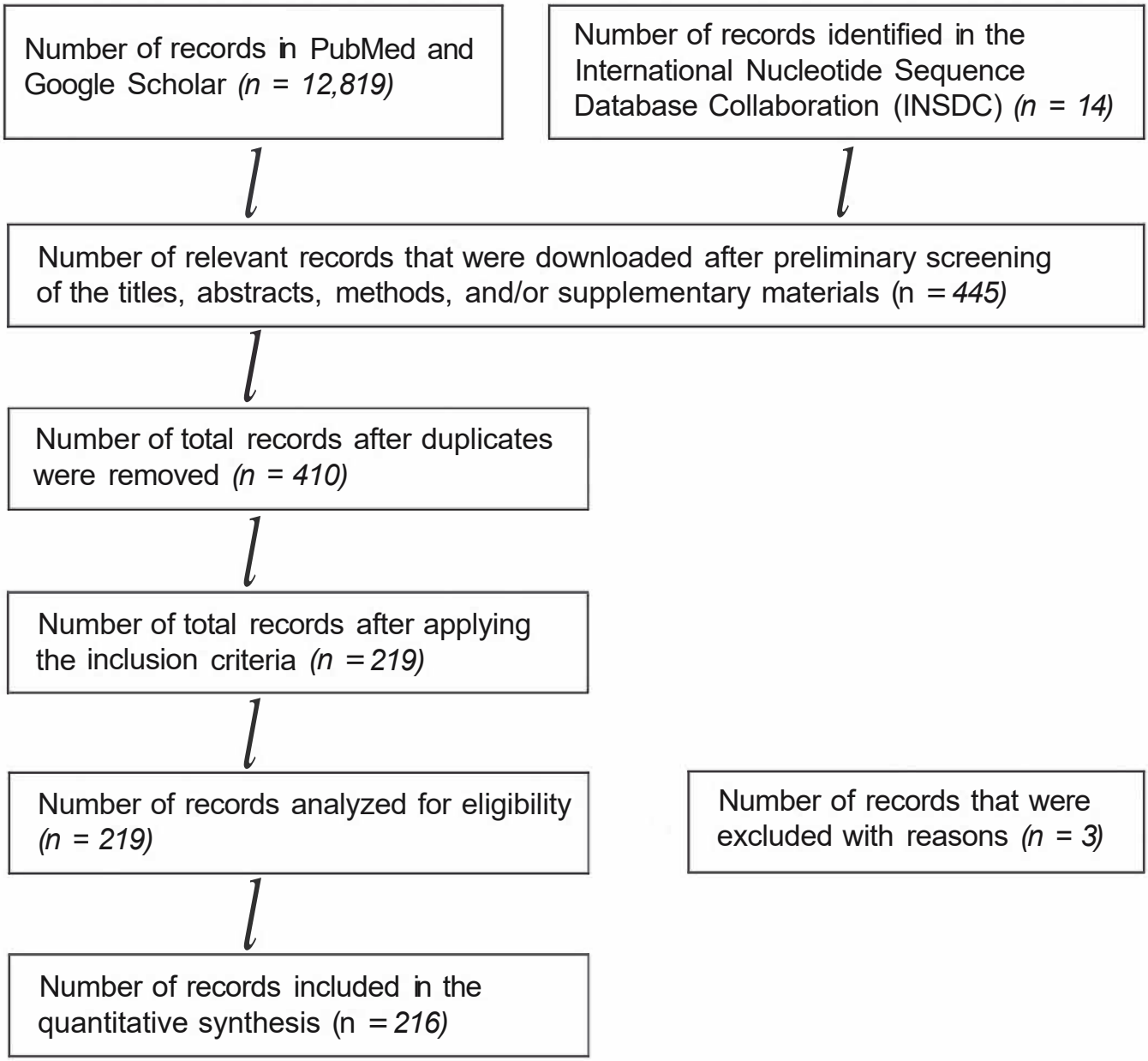

Figure I. The **PRISMA** chart illustrates the search strategy for relevant records that have utilized sequencing methods for subtyping and characterizing influenza A virus genomes from swine globally. We included 216 relevant records in our study, including 202 full-text research articles available in NCBI-PubMed and Google Scholar databases, and 14 BioProjects available in the INSDC database.

swine. Sanger dideoxy sequencing was the first method utilized for partial IAV genome sequencing in swine during the 1970s and 1980s in passive surveillance programs.[11][6] The full-length swine IAV genomes started appearing in the 1990s, still using Sanger technology.[78] The lllumina GAIIx was the first NGS platform to generate the complete genome of the A(H1Nl)pdm09 virus from ill swine during the "swine-flu" pandemic in Mexico in 2009.[26] Although the Roche 454 GS Junior Titanium was among the earliest NGS platforms launched in 2005, it was not until 2010 that the Roche 454 GS Junior platform was utilized to sequence HINI and H3N2 viruses in swine in Spain.[62] The Ion Torrent PGM, another second-generation sequencing platform, generated a complete genome of a HIN2 subtype termed *"Al* swine/Denmark/10302-2/2012(HIN2)" during passive surveillance in pigs in Denmark in 2012.[14] Further technologic advancements in recent years enabled the launch of third-generation sequencing platforms. For example, Oxford Nanopore Technologies launched a real-time sequencing platform termed "MinION" for rapid WGS. To date, only one investigation has reported full-length swine I*A*V genomes using Nanopore MinION sequencing,[84] in which 13

full-length IAV genomes were sequenced and characterized from symptomatic swine on-site overnight at a swine exhibition in Iowa, USA.[84]

Sanger sequencing

Sanger dideoxy sequencing was the most widely used sequencing method overall, with 93 (43.0%) studies using this technology to generate all 8 gene segments of IAV genomes; 67 (31.0%) studies amplified and sequenced only partial IAV genomes, including HA and NA genes for IAV subtyping. It was noteworthy that most of the novel and reassortant IAV subtypes were detected and characterized by Sanger dideoxy WGS (Table 1), which was also the most widely used sequencing method for whole genomes. Of the numerous studies that generated only partial IAV genomes in swine, there were also a few that identified novel or reassortant IAV subtypes.

Second-generation sequencing

The introduction of various NGS platforms facilitated the detection and characterization of swine IAV WGS in active and passive surveillance undertaken in several countries. A total of 23 (10.6%) studies used only Illumina sequencing, which was the most used NGS platform for generating complete swine IAV genomes. In addition, 3 (1.8%) studies generated partial IAV genomes using Illumina sequencing alone. Ion Torrent PGM was used in 7 (3.2%) studies to generate complete IAV genomes; 1 (0.5%) study utilized a combination of Illumina and Ion Torrent PGM for generating full-length swine IAV genomes. Only one (0.5%) study utilized the Roche 454 GS Junior Titanium platform for IAV WGS.

Even though second-generation sequencers offered much promise for generating full-length IAV genomes, there were certain limitations, one of which was the generation of short reads.[5,31] The alignment of short reads into a contiguous sequence (contig) is challenging, especially in cases in which there are repetitions in the genome sequences, often resulting in gaps in the genome assembly (e.g., where genome repeats are longer than the individual read lengths or where there is insufficient coverage).[81] Although mapping the assembled genome with a reference genome may resolve these gaps, in the case of de novo assembly, filling these gaps becomes challenging and usually requires a bioinformatics pipeline with a sufficiently trained workforce.

Sanger sequencing and NGS in combination

A total of 13 (6.0%) studies used a combination of Illumina and Sanger dideoxy sequencing to generate complete swine IAV genomes. Most of these studies were large-scale and spanned a long period of time, with the incorporation of the Illumina platform once it became available.[114-116] In addition, 3 (1.4%) studies combined Ion Torrent PGM and Sanger dideoxy sequencing for generating full-length IAV genomes, whereas the Roche 454 GS Junior Titanium and Sanger dideoxy sequencing were used for full-length sequencing in 2 (0.9%) studies.

Third-generation sequencing

Oxford Nanopore Technologies developed a portable sequencing device called the "MinION" to overcome the challenges with NGS technologies, and to offer single-molecule real-time sequencing.[39] To date, one (0.5%) study utilized only the MinION sequencing platform for generating complete IAV genomes in swine; one study (0.5%) utilized the combination of Illumina and MinION sequencing to generate swine IAV whole genomes (Fig. 2). One of the significant advantages of the MinION is the generation of long reads (~5,000 bases). The portable Nanopore MinION device is also available at an affordable price of ~USD 1,000. The Nanopore MinION device detects the sequences using an applied electric current as the DNA template passes through the biological nanopore.[39] Other salient features of the MinION include its portability, miniature size, rapid results, and laptop-based sequence analysis. These features make the MinION an attractive option as a scalable sequencing technique for large-scale real-time IAV surveillance in swine. Interestingly, the MinION can generate swine IAV sequences much faster than the other NGS platforms. For example, the pipeline steps from RNA extraction until sequencing using the MinION may take 14 h[43] or 14.5 h[84] compared to Illumina (39 h),[84] Roche 454 GS Junior (24 h),[54] Ion Torrent PGM (33 h),[43] and Sanger sequencing (15 h). The advent of third-generation sequencing technology appears promising for real-time and large-scale IAV surveillance in swine populations.

Sequencing approaches used by NGS studies

Overall, 5 different sequencing platforms have been used to generate IAV genomes in swine populations based on various reaction chemistries (Fig. 3; Table 2). Although amplicon sequencing was the more popular sequencing approach in NGS studies (70.4%), a few studies (27.3%) used random primers for cDNA synthesis for library preparation; the one remaining (2.3%) NGS study did not mention the sequencing approach utilized.

Advantages of WGS over partial IAV genome sequencing

Although partial genome sequences (specifically HA and NA genes) are sufficient to determine IAV subtypes and their origin, in recent decades, more studies have focused on generating complete swine IAV genomes because it provides crucial information about swine IAV evolution (Fig. 4). An example of this is a recent whole genome study on swine in China in

Table 1. Novel influenza A virus (IAV) subtypes in swine characterized by various sequencing approaches.

Technique/Novel IAV subtypes reported	Virus strains sequenced	Year	PMID	Citation
Sanger dideoxy WGS				
Reassortant H9N2	A/Swine/Hong Kong/9/98(H9N2)	1998	11559800	Ref. [78]
H4N6	A/Swine/Ontario/01911-1/99	1999	10982381	Ref. [40]
Avian H3N3	A/Swine/Ontario/42729A/01	2001	15365042	Ref. [41]
	A/Swine/Ontario/K01477/01	2001		
Reassortant H7N2	A/swine/KU/16/2001	2001	21741185	Ref. [45]
Avian H1N1	A/Swine/Saskatchewan/18789/02	2002	15365042	Ref. [41]
Reassortant H3N1	A/Swine/Minnesota/00395/2004	2004	16641303	Ref. [59]
Equine H3N8	A/swine/Chibi/01/2005(H3N8)	2005	19396578	Ref. [102]
	A/swine/Anhui/01/2006(H3N8)	2006		
H5N1	A/swine/Banten/UT2071/2005(H5N1)	2005	20875275	Ref. [76]
	A/swine/Banten/UT3063/2005(H5N1)	2005		
	A/swine/Banten/UT6001/2006(H5N1)	2006		
Reassortant H2N3	A/swine/Missouri/4296424/2006(H2N3)	2006	18093945	Ref. [60]
	A/swine/Missouri/2124514/2006(H2N3)	2006		
Reassortant avian-origin H9N2	A/Swine/Guangxi/7/07(H9N2)	2007	18403137	Ref. [112]
Reassortant H5N2	A/Swine/Korea/C12/08	2008	19359528	Ref. [49]
	A/Swine/Korea/C13/08	2008		
Avian H10N5	A/swine/Hubei/10/2008/H10N5	2008	23166264	Ref. [106]
H5N1	A/swine/Jiangsu/1/2008	2008	23836394	Ref. [36]
	A/swine/Jiangsu/2/2009	2009		
Novel H4N1	A/Swine/HuBei/06/2009(H4N1)	2009	23166273	Ref. [38]
Novel reassortant H3N2	A/Swine/Guangxi/NS2783/10(H3N2)	2010	25008935	Ref. [52]
Eurasian avian-like H1N1 genotype 1	A/swine/Henan/201/2011	2011	32601207	Ref. [99]
Reassortant H3N1	A/swine/Chachoengsao/NIAH105583-062-46/2012	2012	26115167	Ref. [1]
Eurasian avian-like H1N1 genotype 1	A/swine/Hebei/156/2012	2012	32601207	Ref. [99]
	A/swine/Jilin/625/2013	2013		
Eurasian avian-like triple reassortant H1N1 genotype 5	A/swine/Shandong/S113/2014	2014	32601207	Ref. [99]
Eurasian avian-like triple reassortant H1N1 genotype 6	A/swine/Anhui/1227/2015	2015	32601207	Ref. [99]
Eurasian avian-like triple reassortant H1N1 genotype 4	A/swine/Shandong/16/2016	2016	32601207	Ref. [99]
	A/swine/Hebei/0113/2017	2017		
	A/swine/Henan/SN10/2018	2018		
Novel reassortant H1N1	A/swine/China/Qingdao/2018(H1N1)	2018	31535780	Ref. [113]
Sanger dideoxy (partial) genome sequencing				
Reassortant H3N2	A/swine/Potsdam/35/1982(H3N2)	1982	32868846	Ref. [116]
Reassortant H1N7	A/Swine/England/191973/92	1992	9191869	Ref. [15]
H9N2	A/swine/Henan/2/2004(H9N2)	2004	18401696	Ref. [21]
	A/swine/Henan/3/2004(H9N2)	2004		
H5N1	A/swine/Egypt/165/2015(H5N1)	2015	29075888	Ref. [29]
H9N2	A/swine/Egypt/151/2015(H9N2)	2015		
Avian-origin H5N1	A/swine/Nigeria/49/2016(H5N1)	2016	29651056	Ref. [66]
Roche 454 GS Junior WGS				
H1N1, H1N2	A/swine/Ontario/13-1/2012(H1N1)	2012	26030614	Ref. [32]
	A/swine/Ontario/68/2012(H1N2)	2012		
Illumina WGS				
Novel reassortant H3N2	A/swine/Rietberg/19732/2014(H3N2)	2014	32868846	Ref. [116]
Avian-origin H4N6	A/swine/Missouri/A01727926/2015(H4N6)	2015	28841443	Ref. [2]
Unusual reassortant H1N1	A/swine/Siberia/1sw/2016(H1N1)	2016	28883131	Ref. [98]
H1N2 variants	A/swine/Denmark/18-6662-26_PB2/2018 (H1N2)	2018	32927910	Ref. [12]
Ion Torrent PGM WGS				
H5N2	A/swine/Estado de Mexico/ EdoMexDMZC03/2015(H5N2)	2015	30126057	Ref. [88]
Avian H5N2	Feral swine/Campeche/DMZC-DEFSAL-UIFMVZ19-12 (H5N2)	2019	32403268	Ref. [63]
Oxford Nanopore MinION WGS				
H1N1	A/swine/Iowa/18Tosu0505/2018(H1N1)	2018	32024713	Ref. [84]
H1N2	A/swine/Iowa/18TOSU0374/2018(H1N2)	2018		
H3N2	A/swine/Iowa/18Tosu0394/2018(H3N2)	2018		

WGS = whole-genome sequencing.

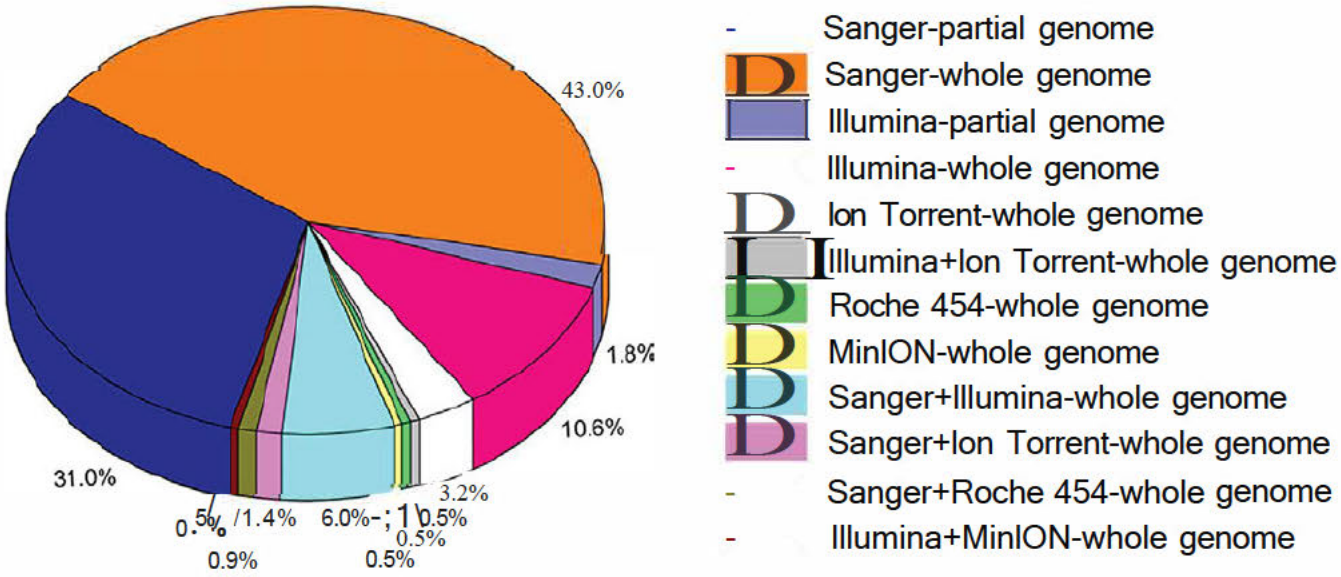

Figure 2. Illustration of sequencing approaches for partial and full-length genome sequencing of influenza A virus (IAV) subtypes from swine globally. Various sequencing techniques were used in 216 studies to generate IAV genomes in swine up to 31 March 2021. Sanger dideoxy sequencing[76,97] was the sequencing technique used most widely, followed by second-generation Illumina sequencing.2 Since 2014, a third-generation sequencing method, MinION, has also been used for generating IAV genomes from swine.84

which they found reassortant HA and NA genes ofEurasian-avian origin, the PA gene of avian-origin, NS gene of the triple-reassortant lineage, and other internal genes of the A(H1Nl)pdm09 virus lineage. These sequences, therefore, provided information on how the swine viruses had evolved to facilitate human infection.[99] Another example is a 2017 study in the United States, in which whole-genome phylogenetic analyses of swine IAVs identified numerous swine genotypes with triple-reassortant internal genes (TRIGs).[83] In addition, whole-genome sequences have been used to identify potential mammalian adaptation markers in IAVs isolated from swine.[61,82] These markers indicate that circulating human[82] as well as avian IAVs[17,61] are adapting to swine. Other advantages of sequencing whole IAV genomes include the use of this information for reverse genetics. An example illustrating this use is a study in which reverse genetics experiments showed that the 2009 pandemic emerged not only as a result of IAV gene reassortments, but also mutations in the genome.[118]

Applications of swine IAV WGS

WGS has also indirectly assisted with IAV vaccine development.[57,105] For example, genetic information from a swine-like triple-reassortant H3N2 virus allowed scientists to develop a live attenuated vaccine against the A(HINI) pdm09 virus[79] through modifications of the PBI and PB2 polymerase genes of the swine virus. Similarly, genetic information from IAV genomes enabled the expression of a truncated NSI protein from swine H3N2 virus for a modified-live virus vaccine.[85] Sequences from circulating strains have also provided information about amino acid substitutions that affect vaccine efficacy.[105] For this type of application, Sanger sequencing and NGS technologies have an advantage because they can efficiently measure the occurrence of variants of concern (resistant or capable of immune escape) in a complex IAV population.[57] In addition, swine IAV, like several other RNA viruses, may form quasi-species given their error-prone polymerase enzyme.[23] NGS deep-sequencing platforms, such as lllumina, have efficiently detected minority variants in a diverse IAV population,[10,56] and have shown their strength in analyzing antigenic drift[50] and identifying existing antigenic diversity in swine IAVs, which are helpful with vaccine-related decisions.[13,51,104]

Most NGS studies using amplicon sequencing for generating all 8 swine IAV genome segments[28] have facilitated the identification of complex IAV populations. For example, identification of avian-origin H4N6 virus in swine in Canada used amplicon sequencing to generate the entire H4N6 virus genome.[2] Furthermore, generating whole-genome swine IAV sequences using NGS has enabled the analysis of antigenic shift (reassortments)[74] in the genomes, giving insights into swine IAV evolution.[8]

Advantages of NGS over RT-PCR

Although commonly occurring IAV subtypes, such as HI and H3, as well as NI and N2, can be determined using reverse-transcription PCR (RT-PCR) assays targeting the HA and NA genes, the existing broad genetic diversity of IAVs in swine could make subtyping challenging for some of these variants as well as other less-frequently occurring IAV subtypes,

	First-generation sequencing	Second-generation sequencing			Third-generation sequencing
	Sanger Dideoxy	Roche 454 GS Junior	Illumina	Ion Torrent PGM	Oxford Nanopore MinION
Year of inception	1977	2005	2007	2010	2014
Reaction chemistry	Dideoxy chain termination	Pyrosequencing	Reversible chain termination	Proton detection	Real-time single molecule sequencing
Read-length/run (bp)	800	500	2x150/ 2x300	200	>5,000
# Reads/run	1	100,000	300 million (MiSeq) 400 million (NextSeq) 640 million (GAIIx) 5 billion (HiSeq)	5 million	60,000
Sequencing cost per million bases (USD)	2,400	9	0.1	1	<1
Platform cost (USD)	95,000	100,000	125,000	80,000	1,000
Sequencing cost per sample (USO)	968	1,100	475-1,177	658	475
Pipeline steps	R Sample	R Sample	R Sample	R Sample	R Sample
RNA extraction	QIAamp viral RNA minikit	MagMAX 96 viral RNA isolation kit	QIAamp viral RNA minikit	QIAamp viral RNA minikit	TruTip
Whole-genome amplification	SSIII Platinum Taq DNA polymerase	MuLVRT-PCR	SSIII Platinum TaqDNA polymerase	SSIII Platinum Taq Hi Fidelity DNA polymerase	SSIVTaq DNA polymerase/Q5
Multiplexing and library preparation	Agarose gel-purification of DNA	454 rapid library multiplex identifier system	Nextera XT	Ion Xpress plus fragment library kit, Emulsion PCR	Q5/LSK-108
Genome sequencing	ABI Prism 3130x or ABI 3730 DNA analyser	Roche 454 GS Junior Titanium	MiSeq/ HiSeq/ Nextseq/ GAIIx	Ion Torrent PGM	MinION
Sequence analysis	Server	Server	Server	Server	Laptop
Total time	15 h	24 h	39 h	33 h	14.5 h

Figure 3. Comparison of Sanger, second-, and third-generation sequencing technologies that have been used for influenza A virus (IAV) sequencing in swine populations. The sequencing cost varied among different sequencing platforms, especially between Sanger and second-generation sequencing platforms. The Oxford Nanopore MinION sequencing generated IAV genomes from swine samples within 14.5 h, the shortest reaction time among all next-generation sequencing platforms.

especially in events of spillover from other host species to swine. Genetic information gained from sequencing, therefore, has an advantage. For example, one nasal swab sample obtained from a pig with clinical signs of influenza-like illness was found to be RT-PCR positive during the IAV screening assay. The virus was isolated on Madin-Darby canine kidney (MDCK) cells, but it could not be subtyped using H1 and H3 as well as N1 and N2 subtyping assays; however, IAV screening RT-PCR remained positive. The virus isolate was sequenced on the Illumina MiSeq, which successfully generated the complete genome of an avian-origin H4N6 virus.2 In addition, nonspecific amplification during PCR might result in a false IAV positive. For example, 4 swine oropharyngeal swabs that had been identified incorrectly as IAV positive by RT-PCR were subsequently identified as non-target contigs and not actual viral RNA contigs, using the MiSeq platform.[55] NGS has also shown potential in IAV testing with full-length IAV genomes generated from RT-PCR-positive swine

Table 2. An overview of sequencing approaches to generate influenza A virus (IAV) genome sequences in swine.

Sequencing technology	IAV subtypes reported	Strategy for IAV sequencing	Citation
First-generation sequencing			
Sanger dideoxy WGS	H1N1, H1N2, H3N2, A(H1N1)pdm09, H2N3, H3N1, H3N3, H3N8, H4N1, H4N6, H5N1, H5N2, H7N2, H9N2, H10N5	All 8 segments were fully amplified using IAV gene-specific/universal primers. The amplicons were sequenced.	Refs. [1,4,9,24,36–38,40,41,45,49,52,59,60,75,76,78,94,99,102,106,108,117]
Sanger dideoxy sequencing (partial genome)	H3N2, H9N2	All 8 segments were partially amplified. The amplicons were sequenced.	Refs. [21,95]
	H1N1, H1N2, H3N2, A(H1N1)pdm09, H5N1	The HA and NA genes were amplified using gene-specific primers. The amplicons were sequenced.	Refs. [34,66,80,96,101]
	Avian H5, H9	The HA gene was amplified using gene-specific primers. The amplicons were sequenced.	Ref. [29]
Second-generation sequencing			
Illumina MiSeq/HiSeq 2000/GAIIx WGS	H1N1, H1N2, A(H1N1) pdm09, reassortant H1N1, reassortant H3N2, reassortant A(H1N1)pdm09	Reverse transcription used random hexamers. Double-stranded cDNA was used for library preparation.	Refs. [20,47,98,114–116]
Illumina MiSeq	H3N2, A(H1N1)pdm09	Reverse transcription used IAV universal primers. Double stranded cDNA was used for library preparation.	Ref. [67]
Illumina MiSeq (partial genome)	H1, H3	Reverse transcription used random hexamers. Only HA gene was amplified using gene-specific primers.	Ref. [46]
	H1	Only HA gene was amplified using gene-specific primers. The amplicons were used for library preparation.	Ref. [100]
Illumina MiSeq/HiSeq/ NextSeq 500/GAIIx WGS	H1N1, H1N2, reassortant H1N2, H3N2, H4N6, A(H1N1)pdm09	All 8 segments were fully amplified using IAV gene-specific primers. The amplicons were used for library preparation.	Refs. [2,12,16,19,22,25,64,65,68,71,73,77,87,91,92,109]
Ion Torrent PGM	H1N1, H1N2, H3N2, A(H1N1)pdm09, H5N2	All 8 segments were fully amplified using IAV gene-specific primers. The amplicons were used for library preparation.	Refs. [7,14,44,58,63,72,88,89]
Roche 454 GS Junior	H1N1, H1N2, H3N2	Reverse transcription used random primers. Double-stranded cDNA was used for library preparation.	Refs. [32,33,62]
Third-generation sequencing			
Oxford Nanopore MinION	H1N1, H1N2, H3N2	All 8 segments were fully amplified using IAV gene-specific primers. The amplicons were used for library preparation.	Ref. [84]

WGS = whole-genome sequencing.

samples with Ct values <30.[27,107] However, real-time RT-PCR–positive samples with Ct values >35 failed to generate IAV sequences because of the very low abundance of virus sequences in those samples with high Ct values.[27]

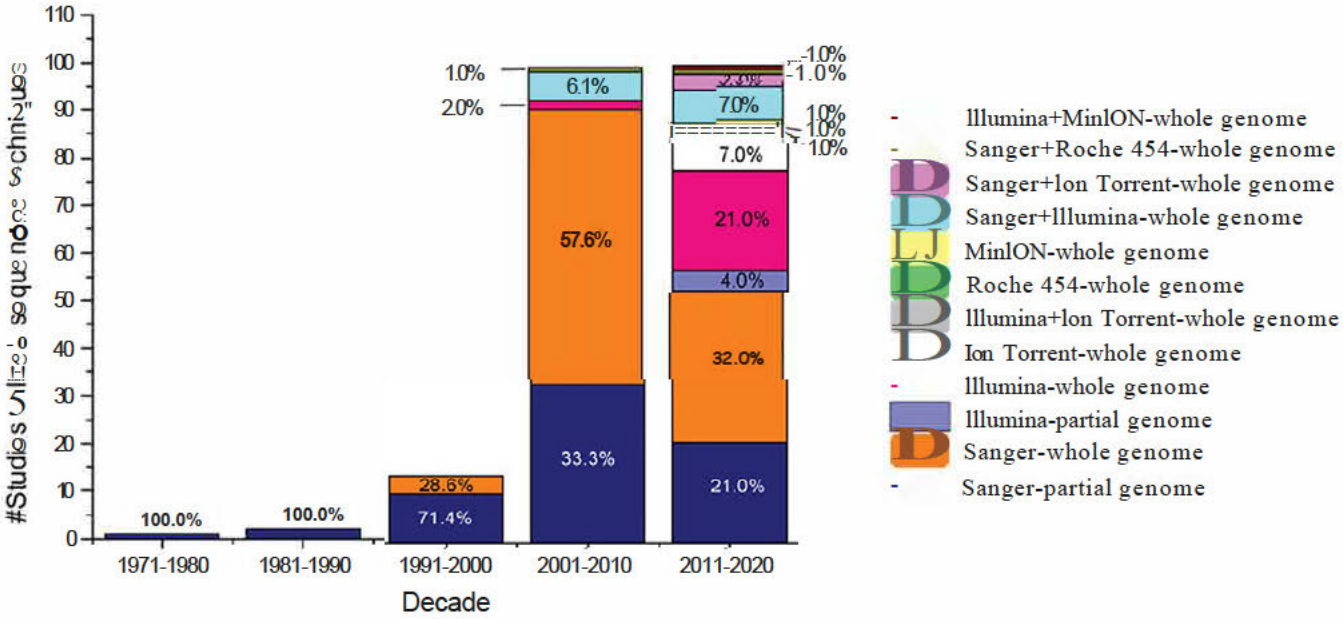

Figure 4. A graphical illustration of the trend of reporting sequencing approaches for either partial or complete genome sequencing of influenza A virus (IAV) from swine globally. Partial swine IAV gene sequencing started during the 1970s. Although Sanger dideoxy sequencing continues to be the sequencing method used most widely for generating IAV genomes from swine, the emergence of next-generation sequencing platforms has challenged the dominance of Sanger dideoxy sequencing since ~2010. Various second- and third-generation sequencing methods have generated full-length IAV genomes from swine samples in recent decades. A few long-term, large-scale studies utilized a combination of Sanger and second-generation sequencing techniques to generate IAV genomes in swine.

Cost-effectiveness of various sequencing platforms

Although Sanger sequencing is still cost-effective for partial swine IAV sequencing for subtype identification, NGS platforms have reduced the sequencing cost per million bases compared to Sanger dideoxy sequencing. For example, Sanger sequencing cost ~USD 2,400per megabase (Mb), compared to the Roche 454 GS Junior platform cost ofUSD 9. The Ion Torrent PGM further reduced this cost to USD I; Illumina sequencing costs only USD 0.1 per Mb,[54,111] and the Nanopore MinION costs <USD I per Mb. A 2021 study analyzed the cost-effectiveness of Sanger and NGS platforms for influenza virus WGS in the reference laboratories in Europe and determined that Sanger WGS at the Friedrich-Loeffler-Institut (FLI; Germany) costs€ 836 (USD 968) per sample.[6] In comparison, the Ion Torrent PGM costs € 568 (USD 658) per sample at the FLI.[6] The Illumina MiSeq WGS at the Animal and Plant Health Agency (APHA, UK) costs€ 1,017 (USD 1,177).[6] It was reported that the NGS cost varies between the laboratories because of several factors, including batch size for sample preparation as well as supplier related costs of equipment and consumables.[6]

In our experience, the Illumina MiSeq at the Agricultural Research Council (ARC), Onderstepoort, Pretoria, South Africa costs ~USD 475per sample; Illumina MiSeq at the Michigan State University, USA starts from USD 524per sample, depending on the genome coverage (https://rtsf.natsci.msu.edu/genomics/pricing/). Although a specific cost for IAV sequencing on the Roche 454 GS Junior platform (which is no longer supported) could not be obtained, general sequencing costs are reported to be USD 1,100 per sample (http://www.personalizedgenes.com/). As a result, the competitive cost of the NGS platforms have facilitated the use of large-scale whole genome studies for IAV surveillance in swine. The competitive running cost of the Nanopore MinION at ~USD 475 per sample has the potential to establish it as a preferred sequencing application for swine IAV detection in research laboratories.

Sequencing challenges

Just as Sanger sequencing offered high accuracy of raw reads (99.9%), the second-generation sequencing platforms (e.g., Roche 454 GS Junior [99.9%] and Illumina [98%]), offered comparable accuracy when aligned to reference genomes.[54] However, one challenge that the Roche 454 GS Junior sequencer faced was its inability to correctly identify homopolymers of 6 nucleotides.[54] The Ion Torrent PGM was reported to be 1.5 times less accurate than the Illumina MiSeq for sequencing IAV genomes as a result of insertions and deletions that occurred mainly in the homopolymer regions. In contrast, the errors that occurred on the Illumina MiSeq platform were mostly nucleotide substitutions.[103]

The accuracy of MinION sequencing, when aligned to a reference genome, has been reported to be much lower than

the other NGS platforms (up to 71.5%)[48]; however, de novo assembly using the MinION offered improved assembly compared to lllumina.[30] Intriguingly, the recent advances in algorithms in MinlON are reported to improve its raw read accuracy up to 98.3% (as of May 2021; Oxford Nanopore Technologies), with further improvements ongoing to gain a higher raw read accuracy. Given that we are under constant threat of the emergence of another influenza pandemic, it is imperative for a sequencing technology such as the MinION to become deployable for a scalable outbreak response in the field.

Prospects of RNA sequencing for IAV detection

Direct RNA sequencing of IAV using the Nanopore MinION generates longer reads of the IAV genome in a shorter time by eliminating the requirements for cDNA synthesis and PCR amplification.[42] The complete coding region of an IAV genome has been sequenced directly from RNA using a reverse genetically constructed "rA/Puerto Rico/8/1934" virus, a candidate vaccine virus, and a standard laboratory strain.[42] Using a custom-designed adaptor to target the negative-sense RNA into a protein nanopore on the MinION platform, I00% nucleotide coverage was generated successfully, with 99% of reads mapped to the IAV genome[42]; the study should pave the way for performing direct RNA sequencing for other RNA viruses and may also be applied to swine IAV clinical samples.

Conclusion

The ongoing reports of novel IAV subtypes and genotypes in swine populations exemplify the need for active IAV genomic surveillance. Sanger dideoxy sequencing has contributed significantly to unravelling the existing genetic and antigenic diversity of IAV genomes in swine. More recently, the applications of second-generation sequencing, especially the lllumina MiSeq, have facilitated large-scale surveillance for investigating IAV disease burden in swine populations. Interestingly, the on-site real-time sequencing ability of the emerging third-generation Nanopore MinION technology, given the low cost, portability, and laptop-based analysis, makes it a serious competitor to the existing second-generation sequencing platforms. Optimization of the MinION for direct RNA sequencing may transform the swine IAV genome sequencing landscapes in upcoming years.

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CHAPTER 6

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ORIGINAL PAPER

Deciphering transmission dynamics and spillover of avian influenza viruses from avian species to swine populations globally

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Abstract
Genome sequences of eleven avian influenza virus (AIV) subtypes have been reported in swine populations from seven countries until August 2020. To unravel the transmission dynamics and spillover events of AIVs from avian reservoirs to swine, full-length hemagglutinin (HA) sequences of AIV subtypes (*n*= 11) reported from various avian species and swine were retrieved from the 'Influenza Research Database'. Phylogenetic analysis identified closely related avian and swine AIV sequences suggesting potential spillover events from multiple domestic and wild avian species, including chicken, duck, pigeon, goose, quail, and aquatic birds to swine. Furthermore, N-linked glycosylation analysis of these closely related AIV sequences supported the possibility of multiple spillover events of highly pathogenic H5Nl and low pathogenic H9N2 viruses from various avian species to swine. The principal coordinate analysis further validated these findings for H5Nl and H9N2 viruses; however, spillover events of the other nine AIV subtypes were limited. Interestingly, the presence of potential mammalian adaptation markers, particularly in some of the swine H5Nl, H7N9, and H9N2 viruses, suggested that these viruses may have already adapted in swine. The occurrence and circulation of these AIVsin swine, especially the H5Nl and H9N2 viruses with numerous spillover events from the avian reservoirs to swine, pose a significant threat in terms of their reassortment with endemic swine viruses or circulating human influenza viruses within the swine which may facilitate the emergence of a novel influenza virus strain with pandemic potential.

Keywords Avian influenza virus• Avian to swine spillover• Phylogenetic analysis• N-linked glycosylation •Principal coordinate analysis •IAV adaptation •Virus evolution •Influenza pandemic

Introduction

Influenza A virus (IAV) is a member of the Orthomyxoviridae family with a broad avian and mammalian host range [34, 51, 54, 55, 57-59, 63). Genomes of IAV comprise eight gene segments of a negative-sense single-stranded RNA [5]. The IAV virion particle has two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). The acquisition of subtle alterations in amino acid sequences within the HA protein triggers seasonal influenza epidemics [4, 49). In contrast, the reassortments of different IAV subtypes co-infecting a host trigger the emergence of a novel IAV strain, which may be able to start a pandemic in an immunologically naive population [3, 4, 36, 38). Due to the constant evolution of IAV, 18 HA and 11 NA subtypes have been reported infecting various avian and mammalian hosts [20, 27, 48).

The 1918 Spanish flu was the first and the deadliest of all the four influenza pandemics that occurred so far. One complete genome sequence of 1918 Spanish flu was recently obtained from a single victim buried under the permafrost in Alaska [40, 50), and four partial genome sequences were obtained from archival biopsies [40). Analyses of these sequences and other contemporary IAV sequences determined that the 1918 **HINI** pandemic virus originated from an avian reservoir that was most likely transmitted to the human population shortly before the emergence of the Spanish flu pandemic [41). More recently, the 2009 swine flu pandemic originated during March-M_{ay} 2009 in the Mexican swine [14, 34). The role of migratory birds and

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long-distance swine trades with human interaction were the primary factors responsible for the 2009 swine flu pandemic [14, 34].

While the first molecular evidence of interspecies transmission of AIV to swine was reported in Canada in 1999 when a low pathogenic H4N6 virus was detected in swine [22], followed by reports of H3N3 [23] in swine in Ontario in 2001, the majority of the reports of AIVs have been documented in Chinese swine: H9N2 in 2004 [8], H5N1, H7N9 [17] and H10N5 in 2008 [56], H4N1 in 2009 [19], H6N6 in 2010 [64], and H4N8 in 2011 [46]. Additionally, the genomes of H5N2 [30], H7N2 [29], and H9N2 viruses [12] were reported from South Korean swine in 2001, 2004, and 2008, respectively. The genomes of highly pathogenic H5N1 viruses were also reported from Indonesian swine during 2005–2007 [37]. Most recently, in 2018, the genome of an H5N2 virus was reported from Mexican swine [42].

The proteolytic cleavage of HA into HA1 and HA2 by the removal of an N-terminal signal sequence [33] facilitates the binding of IAV virion particles to the host's sialic acid (SA) receptors [26, 44, 52] and is an important step in IAV pathogenesis, as host receptor binding facilitates the internalization and membrane fusion of the IAV virion [45], triggering infection [10]. The HA protein then undergoes post-translational modification by a process termed 'N-linked glycosylation' [25] and a recent study reported the utility of using N-linked glycosylation patterns in HA protein to identify ecological spillover and adaptation of influenza viruses [24]. Addition or deletion of the glycosylation site(s) may impact IAV adaptation and infectivity in the host [9, 26]. In this context, we analysed full-length HA glycoprotein sequences of AIV subtypes ($n = 11$) reported in swine and various avian species globally to identify avian to swine spillover events and transmission dynamics.

Materials and methods

Acquisition of influenza virus sequences from the database

We downloaded all full-length HA nucleotide sequences of H3N3 (34 avian and two swine), H4N1 (13 avian and one swine), H4N6 (1394 avian and three swine), H4N8 (256 avian and one swine), H5N1 (3516 avian and 28 swine), H5N2 (1115 avian and four swine), H6N6 (419 avian and two swine), H7N2 (212 avian and one swine), H7N9 (746 avian and two swine), H9N2 (6301 avian and 22 swine), and H10N5 viruses (90 avian and one swine) available in the Influenza Research Database (https://www.fludb.org/) until 31 August 2020. The partial HA sequences were not included in the study.

Phylogenetic analysis

All full-length HA nucleotide sequences of AIV subtypes ($n = 11$) that have been reported in avian species and swine were used for phylogenetic analysis. The best substitution models for the phylogenetic analyses were identified using MEGA-X software [28]. Tamura-Nei (TN93) appeared to be the best model for the full-length nucleotide sequences under investigation. The full-length HA nucleotide sequences were then aligned using MUSCLE multiple sequence alignment in 'Geneious Prime 2020.1.2' software for constructing Neighbour-Joining phylogenetic trees. Each tree was created using the TN93 genetic distance model with 1000 bootstrap replications. We built the individual trees for each AIV subtype to determine the transmission patterns between avian species and swine. The neighbour-joining trees identified the most closely related sequences of avian and swine AIV subtypes to explore their N-linked glycosylation sites further.

Prediction of potential N-linked glycosylation sites in HA proteins

We identified the closely related AIV subtypes reported from avian species and swine (sharing the same nodes or clade) from the Neighbour-Joining trees for conducting N-linked glycosylation analysis. The N-linked glycosylation analysis was performed using NetNGlyc 1.0 web server, maintained by the Center for Biological Sequence analysis (http://www.cbs.dtu.dk/services/NetNGlyc/) at the Technical University of Denmark [15]. The NetNGlyc 1.0 web server examines the sequences in the context of Asn-Xaa-Ser/Thr sequons to predict the potential N-glycosylation sites in the protein sequence under query. The algorithm used in the NetNGlyc 1.0 web server obtains a 'jury agreement' for the nine artificial neural networks evaluated based on a threshold score of $^{>}0.5$ [15, 21]. The NetNGlyc 1.0 server was chosen for the prediction of potential glycosylation sites because it has been reported to have a high accuracy of 76% [15, 21].

Principal coordinate analysis

The distance matrix for each subtype of AIV under investigation was generated using full-length HA nucleotide sequences in 'Geneious Prime 2020.1.2' software, providing a comma-separated values (CSV) file. The CSV file of the distance matrix was converted to the excel file using Microsoft Excel. Finally, the distance matrix was used for the principal coordinate analysis (PCA) using 'PAST: Paleontological Statistics Software Package for Education and Data Analysis' version 4.02 [16]. The PAST software package is a highly cited program that integrates spread-sheet-type

distance matrix data with multivariate statistics, plotting and curve fitting, time series, and phylogenetic analysis [16].

Multiple sequence alignment

The full-length HA nucleotide sequences of swine H5N1 ($n=28$) and H9N2 ($n=22$) viruses were aligned using the MUSCLE algorithm in 'Geneious Prime 2020.1.2' software to assess their relatedness to ascertain the possibility of swine-to-swine transmission of these viruses.

Determination of mammalian adaptation markers

We identified previously reported potential mammalian adaptation markers in mature HA proteins of swine IAV subtypes ($n=11$) using the Sequence Variation (SNP) tool available at the Influenza Research Database [65]. The Sequence Variation tool utilizes the HA subtype numbering conversion based on the cross-subtype HA numbering scheme proposed by Burke and Smith [6]. Additionally, the amino acid substitutions were manually verified using multiple or pairwise sequence alignment of swine IAV subtypes using the MUSCLE algorithm in 'Geneious Prime 2021.2.2' software.

Results

The present study utilized all the available full-length HA sequences of AIV subtypes ($n=11$) in various avian species and swine populations to study avian to swine spillover events and transmission dynamics. The phylogenetics, N-linked glycosylation, and PCA analysis of AIV sequences under study identified multiple spillover events of highly pathogenic H5N1 and low pathogenic H9N2 viruses from various avian species to swine, while the spillover events of other nine AIVs were limited. We hereby presented the results of our analyses in a subtype-specific manner.

H5 subtypes (H5N1 and H5N2 viruses)

The highest number of full-length HA nucleotide sequences of AIVs in swine belonged to subtype H5N1 ($n=28$), reported from China and Indonesia which along with HA sequences reported from various avian species globally ($n=3516$) revealed the transmission dynamics and spillover events of H5N1 viruses from various avian species to swine (Fig. 1A). The phylogenetic analysis determined the closely related swine and avian H5N1 virus sequences suggesting multiple spillover events from avian species, including ducks, chicken, and wild birds to swine in Indonesia and China. The phylogenetic analysis of full-length HA nucleotide sequences of avian ($n=1115$) and swine ($n=4$) H5N2 viruses determined two independent events of avian to swine transmission of H5N2 viruses in Mexico and South Korea (Fig. 1A).

The full-length amino acid sequences of closely related avian and swine H5N1 viruses identified from phylogenetics were further analysed for the N-linked glycosylation which determined the presence of seven patterns of glycosylation (Fig. 1B). The glycosylation sites at amino acid positions 26 (NNST), 27 (NSTE), 39 (NVTV), 302 (NSSM), 499/500 (NGTY), and 558/559 (NGSL) were conserved among all swine and closely related avian H5N1 viruses. The glycosylation sites at amino acid positions 88 (NVSE), 156 (NSSF or NPSF), 170 (NNTY), 179 (NYTN), 181 (NNTN), 209 (NPTT), and 289 (NCST) varied among the swine and closely related avian H5N1 viruses. Interestingly, a few Indonesian and Chinese swine H5N1 viruses had identical glycosylation sites which might be due to a common origin of these viruses given the long-distance migration network of avian H5N1 viruses in Southeast Asia [50]. Interestingly, the presence of seven different patterns of glycosylation in swine and closely related avian H5N1 viruses strongly supported the basis of phylogenetic clustering of these viruses.

The glycosylation sites at amino acid positions 26 (NNST), 27 (NSTE), 39 (NVTV), 181 (NNTN), 302 (NSSM), 496 (NGTY), and 555 (NGSL) were conserved among all swine and closely related avian H5N2 viruses, while glycosylation sites at amino acid positions 209 (NPTT) and 252 (NDSI) varied between Mexican and Korean swine and closely related avian H5N2 viruses (Fig. 1B). These findings supported the basis of the phylogenetic clustering of H5N2 viruses and confirmed the independent introductions of H5N2 viruses from avian species to swine in Mexico and South Korea.

The principal coordinate analysis (PCA) plot also supported the observations of phylogenetics and N-linked glycosylation and further added to the fact of the multiple spillover events of H5N1 viruses while suggesting two independent introductions of H5N2 viruses in swine (Fig. 1C). Due to limited number of available swine H5N2 virus sequences, the swine-to-swine transmission of H5N2 virus could not be ascertained. To our surprise, the multiple sequence alignment of HA nucleotide sequences of swine H5N1 viruses ($n=28$) identified seven Indonesian swine with 100% nucleotide sequence identity, belonged to H5 clades 2.1.3 and 2.1.3.3 (Fig. 1D). Interestingly, these H5N1 viruses were reported from the same swine farm in Indonesia [37] which suggested a strong possibility of swine-to-swine transmission of H5N1 viruses.

H7 subtypes (H7N2 and H7N9 viruses)

The Neighbour-joining phylogenetic tree of full-length HA nucleotide sequences of avian H7N2 ($n=212$) and swine H7N2 ($n=1$) viruses determined wild bird to swine

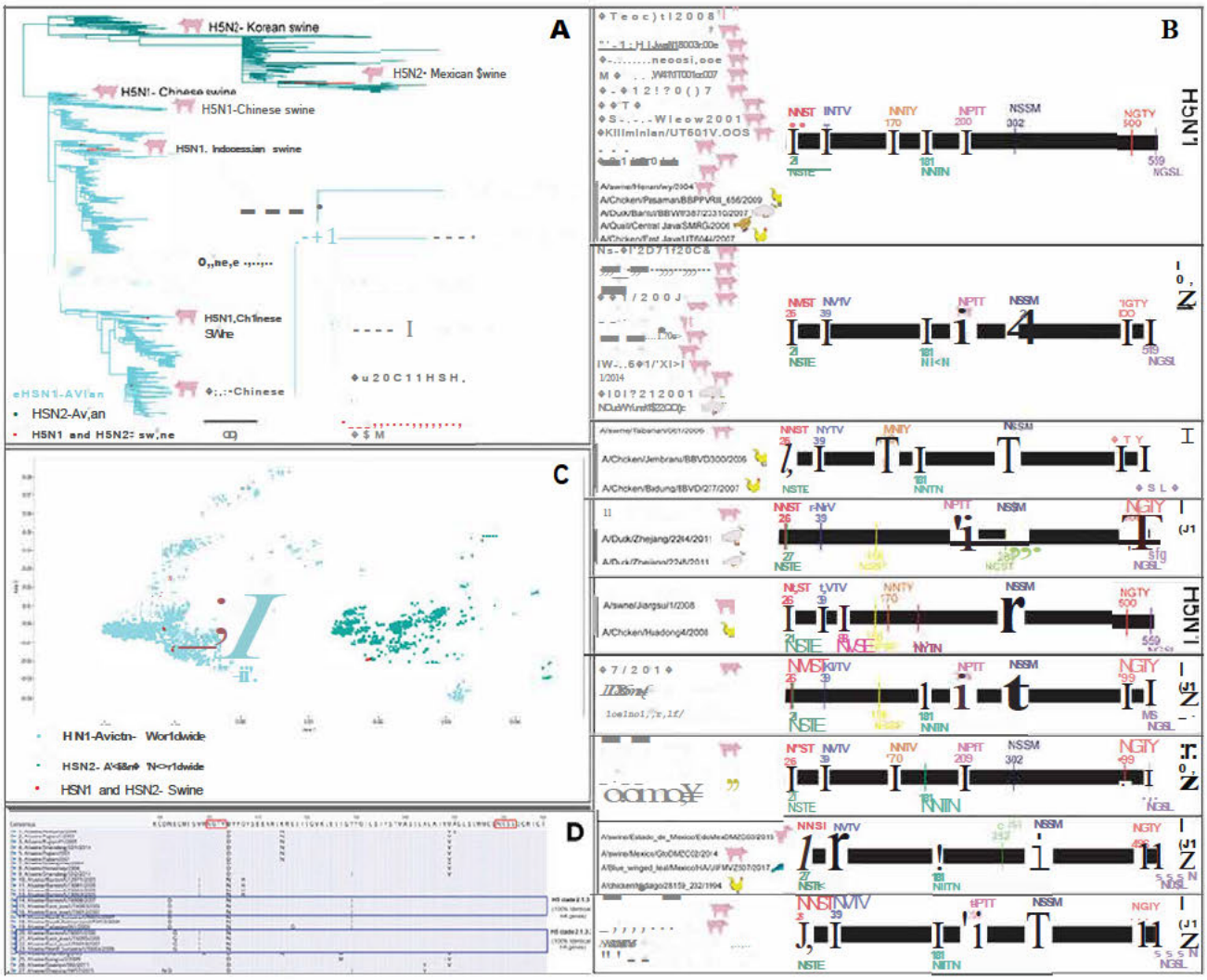

Fig. 1 **A** Neighbour-joining tree of full-length HA nucleotide sequences of avian and swine HSNl (*n*=3544) and HSN2 viruses (11 = 1119) suggested multiple spillover events from chicken, ducks and wild birds to swine. The possibility of swine-to-swine transmission of HSNl viruses in Indonesia was also observed. The cyan nodes represented HSNl virus transmission among avian species, while the dark cyan nodes represented HSN2 virus transmission among avian species. The red nodes represented HSNl and HSN2 virus transmission from avian species to swine. The cyan and red dots at the nodes of the subtree represented> 90% bootstrap support. B Seven different glycosylation patterns of swine and closely related avian HSNl viruses suggested multiple avian to swine transmissions of HSNl viruses. The identical glycosylation patterns of swine and closely related avian HSNl viruses represented the possibility of spillover within these groups. Two different patterns of N-linked glycosylation suggested independent introductions of HSN2 viruses in Mexican and Korean swine. C 1be PCA plot further confirmed multiple spillover events of HSNl and HSN2 viruses in swine populations. 1be red dots represented HSNl and HSN2 viruses in swine, while the cyan and dark cyan dots represented HSNl and HSN2 viruses, respectively, in avian species reported worldwide. D The 100% amino acid as well as nucleotide sequence identities for a few swine HSNl viruses (highlighted within two blue boxes) along with identical glycosylation sites (highlighted with red boxes) suggested the possibility of swine-to-swine transmission of these swine HSNl viruses in Indonesia

transmission of the H7N2 virus in South Korea (Fig. 2A). Two full-length HA gene sequences of highly pathogenic H7N9 viruses were reported from Chinese swine in 2017. The Neighbour-joining tree of these two Chinese swine H7N9 viruses with 746 avian H7N9 viruses reported worldwide (Fig. 2A) shows chicken to swine transmission of H7N9 viruses.

The H7N2 viruses obtained from swine and phylogenetically closely related ducks and wild birds had identical glycosylation sites (Fig. 2B) which suggested a broad circulation of H7N2 viruses in avian species in Southeast Asia. The PCA plot depicted the global pattern of avian and swine H7N2 virus transmission (Fig. 2C). The HA nucleotide sequences of H7N9 viruses reported from Chinese swine were not identical which ruled out the possibility of

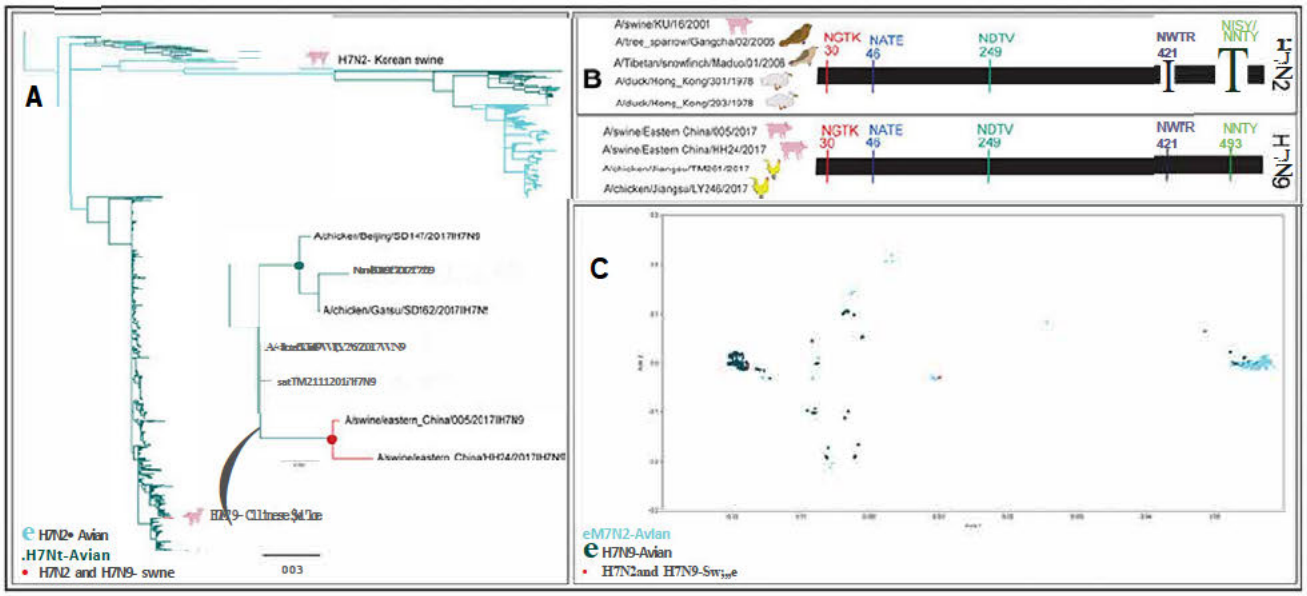

Fig. 2 **A** Neighbour-joining tree of full-length HA nucleotide sequences of 212 avian and one swine H7N2 viruses represented wild bird to swine transmission of the H7N2 virus in South Korea. Neighbour-joining tree of748 full-length HA nucleotide sequences of swine and avian H7N9 viruses determined chicken to swine spillover of H7N9 viruses in China. A subtree highlighted the circulation of H7N9 viruses between chicken and swine in China. The blue and red dots at the nodes of the subtree represented> 90% bootstrap support. B The identical glycosylation pattern in HA proteins ofH7N2 viruses obtained from multiple wild bird species and ducks suggested a broad circulation of H7N2 viruses in avian species in Southeast Asia with one spillover event to swine. The identical N-linked glycosylation patterns shared by swine and closely related avian (chicken) H7N9 viruses supported the findings of phylogenetics suggesting chicken to swine spillover of H7N9 virus in China. C PCA plot represented the global transmission of avian H7N2 viruses with one spillover event to swine and two avian to swine spillover events of H7N9 viruses. The cyan and dark cyan dots represented the circulation of H7N2 and H7N9 viruses, respectively, among avian reservoirs worldwide, while the red dots suggested the occurrence of swine H7N2 and H7N9 viruses

swine-to-swine transmission of H7N9 viruses and therefore suggested two separate spillover events from chicken to swine. The presence of identical glycosylation sites in swine and closely related chicken H7N9 viruses further supported the phylogenetic analysis suggesting chicken to swine spillover ofH7N9 viruses in China (Fig. 2B). Two overlapping red dots in the PCA plot suggested that the Chinese swine may have acquired the H7N9 virus either from the same poultry flock or a common origin (Fig. 2C).

H9N2 virus

Total 6323 full-length HA gene sequences of low pathogenic H9N2 viruses have been reported from swine (*n*= 22) and avian species (*n*= 6301) worldwide. Neighbour-joining tree suggested multiple events of avian to swine transmission of H9N2 viruses in China, Hong Kong, and South Korea (Fig. 3A).

The phylogenetic analysis was supported by the N-linked glycosylation patterns which suggested at least four evolution trajectories of H9N2 viruses in Chinese swine (Fig. 3B). Additionally, the phylogenetics and glycosylation patterns suggested only one spillover event of H9N2 viruses in each of Hong Kong and South Korean swine populations. Interestingly, identical glycosylation patterns among Chinese, Hong Kong and South Korean swine populations indicated a common origin of H9N2 viruses which might be due to either through long-distance bird migration or swine trade in Southeast Asia. The maximum number of swine H9N2 viruses were reported from China which suggested a widespread circulation ofH9N2 viruses in avian species in China and their frequent interactions with swine.

The glycosylation sites at amino acid positions 29 (NSTE), 82 (NPSC), 141 (NVSY), 298 (NTTL), 305 (NVSK), 492 (NGTY) and 551 (NGSC or NGSA) were conserved, while glycosylation sites at amino acid positions 145 (NGTS), 218 (NRTF or NKTF), and 313 (NCSK) were unstable among swine and avian H9N2 viruses. The PCA plot supported the phylogenetics and N-linked glycosylation analyses and depicted multiple spillover events of H9N2 viruses in swine (Fig. 3C). Most of the swine H9N2 viruses except two differed in nucleotide sequences among each other suggesting a low possibility of swine-to-swine transmission.

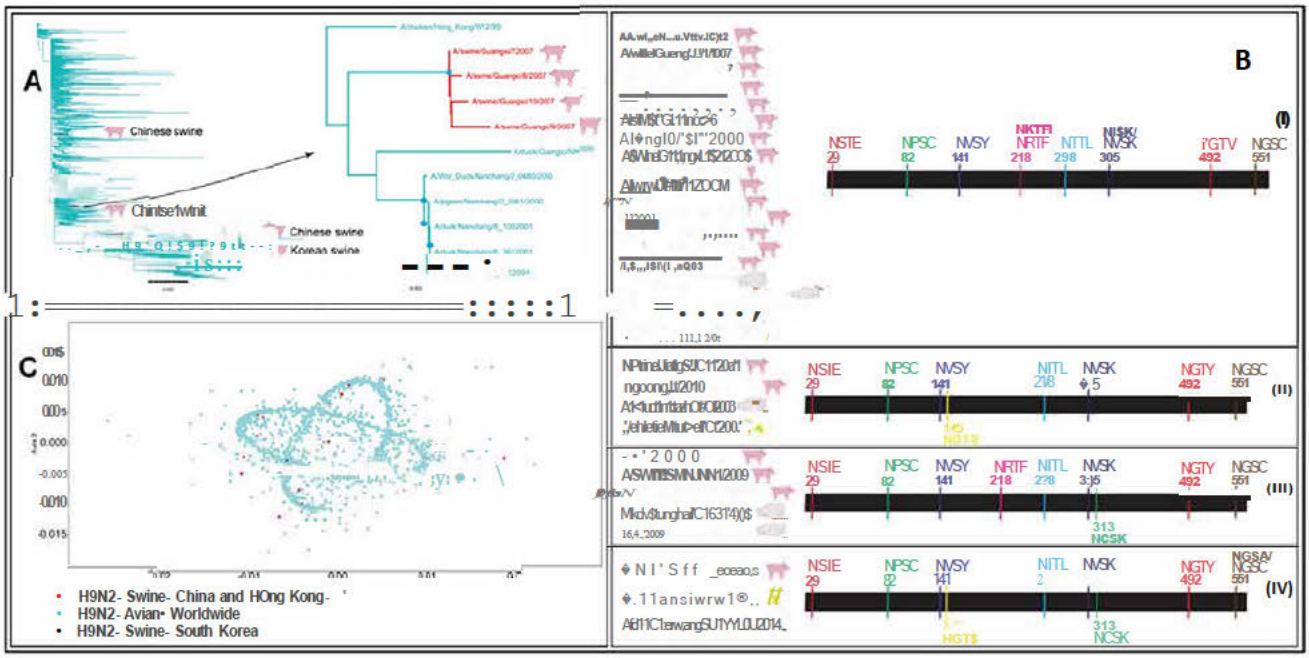

Fig. 3 A Neighbour-joining tree of 6323 full-length H A nucleotide sequences of avian and swine H9N2 viruses determined multiple spillover events from avian reservoirs to swine. The red nodes represented avian to swine transmission of H9N2 viruses in China and Hong Kong, while black nodes represented avian to swine spillover in South Korea. The cyan nodes represented the circulation of H9N2 viruses among avian species globally. The blue dots at the nodes in the subtree represented> 90% bootstrap support. B Four patterns of glycosylation sites in swine and avian H9N2 viruses suggested multiple introductions and evolutionary trajectories of H9N2 viruses in swine in Southeast Asia. C The P C A plot represented multiple spillover events of H9N2 viruses in swine

H3N3 virus

Thirty-six full-length HA gene sequences of avian and swine H3N3 viruses including two sequences from Ontario based swine were analysed. The Neighbour-joining tree suggested that the transmission ofH3N3 virus to Ontario based swine may have occurred from wild birds (Fig. 4A).

The N-linked glycosylation analysis suggested that deglycosylation at amino acid position 499 (NGTY) may have transmitted the H3N3 virus to the swine (Fig. 4B). The PCA plot determined an overlapping red dot for swine H3N3 viruses in Ontario (Fig. 4C) which suggested a common avian origin. It should be noted that the availability of limited sequence data for H3N3 virus HA genes made it challenging for us to identify the potential spillover event from avian to swine. The lack of further reports of H3N3 virus in swine suggested that the H3N3 virus might have got introduced to Ontario swine through a sporadic transmission from an avian reservoir which did not spread further and hence was never detected again.

H4 subtypes (H4N1, H4N6, and H4N8 viruses)

The phylogenetic analysis of full-length HA gene sequences ofH4N1 (13 avian and one swine), H4N6 (1397 avian and three swine), and H4N8 (256 avian and one swine) viruses determined duck to swine spillover of H4Nl and H4N8 viruses in China, and mallard to swine transmission ofH4N6 viruses in Canada and the United States (Fig. 5A).

The presence of identical glycosylation sites in HA proteins of Chinese swine and Hong Kong ducks (Fig. 5B) strengthened the outcome of phylogenetic analysis. The glycosylation at amino acid residue 14 (NSSQ) appeared crucial for duck to swine transmission of the H4Nl virus. The PCA plot supported the phylogenetic and glycosylation analyses and grouped the swine H4Nl virus along with the Asian and European avian H4Nl viruses. It was interesting to note that H4Nl viruses reported from Chinese swine and Hong Kong duck were not contemporary which suggested that the H4Nl virus may have been in circulation in avian reservoirs in Southeast Asia at a low frequency and hence remained undetected. The presence of identical glycosylation sites in closely related HA proteins of swine and avian (mallard) H4N6 viruses (Fig. 5B) suggested the circulation and long-distance dissemination of avian H4N6 viruses in North American mallards. These observations were further supported by the PCA plot which depicted independent spillover events ofH4N6 viruses in North American swine (Fig. 5C). Presence of

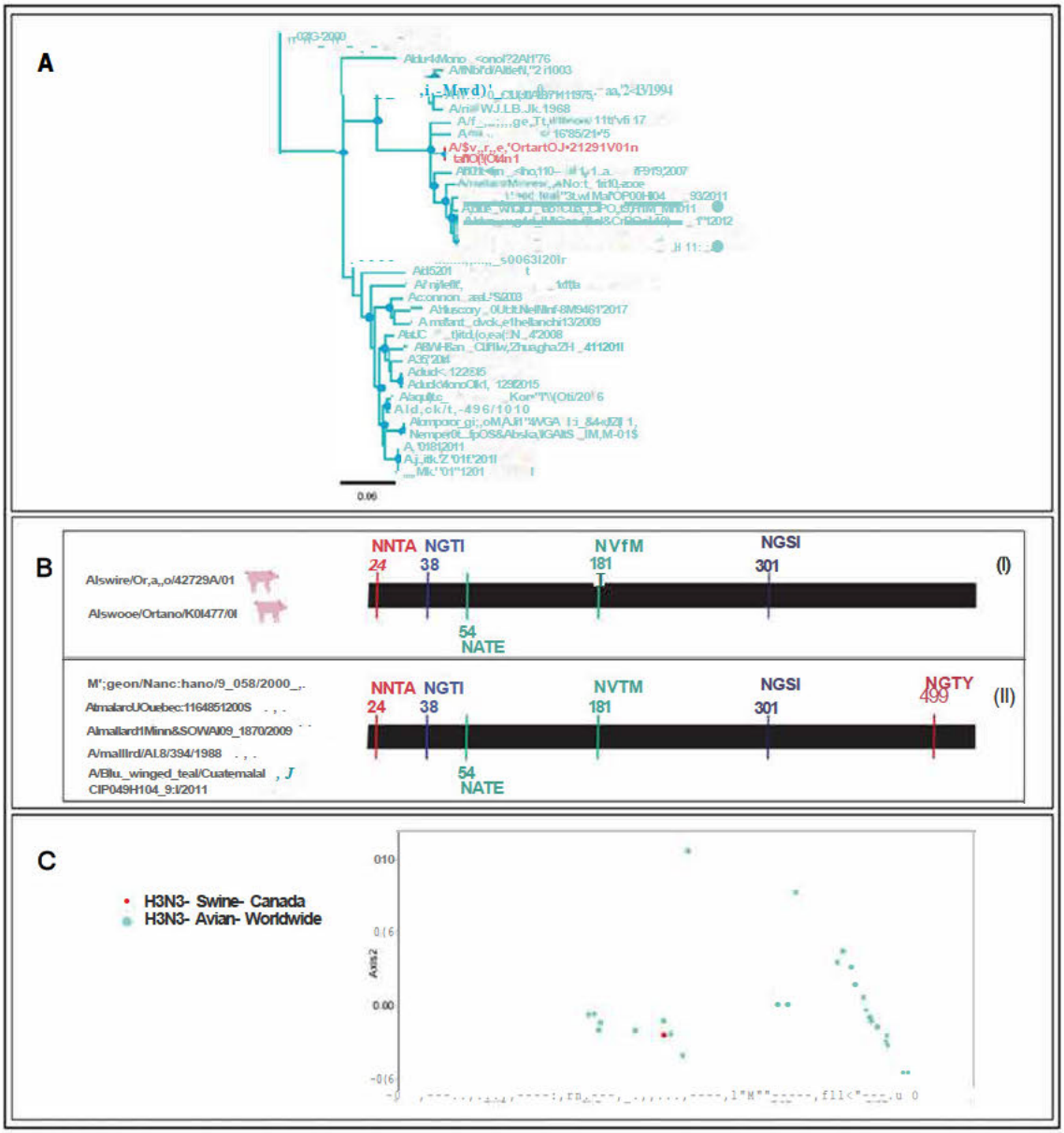

Fig. 4 A Neighbour-joining tree of 36 full-length HA nucleotide sequences of avian and swine H3N3 viruses determined the wild bird to swine transmission in Ontario. The blue dots at the nodes represented > 90% bootstrap support. B 1be Joss of glycosylation at amino acid residue 499 (NGTY) appeared to have transmitted H3N3 virus to swine; however, it should be noted that the availability of a limited number of avian and swine H3N3 virus sequences restricted our ability to infer avian to swine spillover. C 1be PCA plot illustrated the occurrence of swine and avian H3N3 viruses globally

the identical glycosylation sites in H4N8 viruses obtained from swine and ducks in China as well as ducks in South Africa (Fig. 5B) along with shared phylogenetic clustering suggested a long-distance intercontinental dissemination of H4N8 viruses. These findings were concerning regarding the existing risk of avian to swine transmission of H4N8 viruses in South Africa. The global transmission of H4N8 viruses in avian species and swine was further depicted using a PCA plot (Fig. 5C).

H6N6 virus

The neighbour-joining tree offull-length HA gene sequences of swine H6N6 (n = 2) and avian H6N6 (n= 419) viruses suggested two separate events of avian to swine transmission in China (Fig. 6A). One event suggested a pigeon to swine transmission of H6N6 virus in China, while the other event suggested a duck to swine transmission. The identical glycosylation pattern (Fig. 6B) suggested a common origin and widespread occurrence of H6N6 viruses in Chinese avian

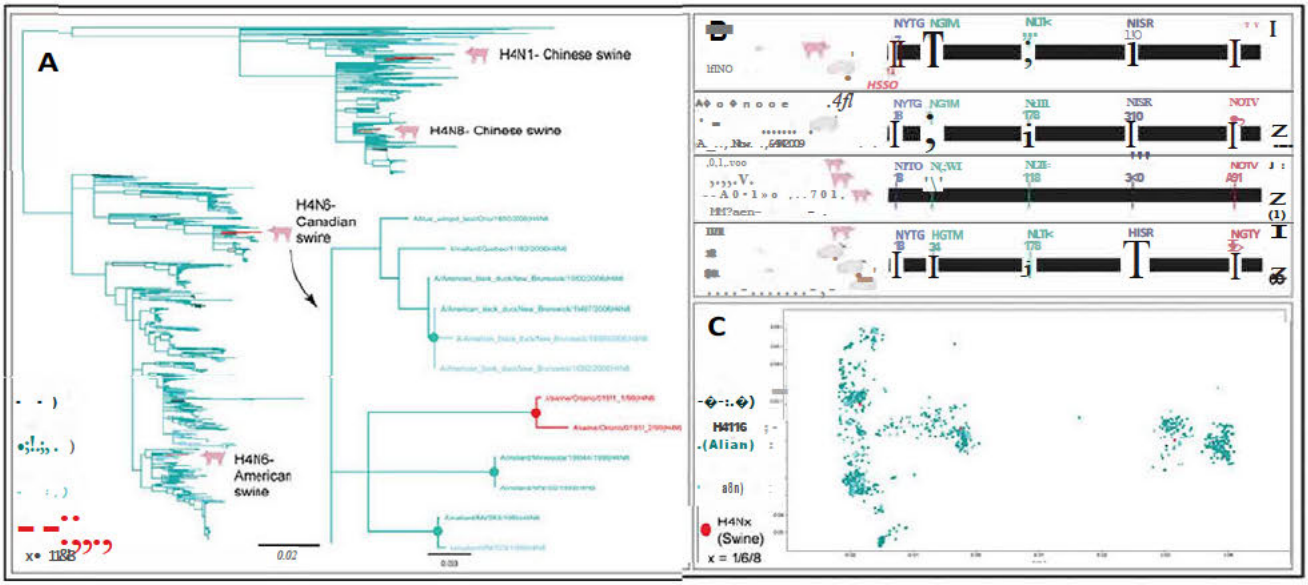

Fig. 5 A Neighbour-joining tree of avian and swine H4Nl, H4N6, and H4N8 virus HA nucleotide sequences depicted duck to swine transmission of H4Nl and H4N8 viruses in China and mallard to swine transmission of H4N6 viruses in North America. The blue and red dots at the nodes in the subtree represented> 9()% bootstrap support. B The identical N-linked glycosylation patterns of swine and duck H4Nl and H4N8 viruses supported the phylogenetic clustering. The glycosylation at amino acid position 14 (NSSQ) appeared crucial for duck to swine spillover of H4Nl virus. The identical glycosylation sites of swine and closely related mallard H4N6 viruses suggested mallard to swine transmission of H4N6 virus in North America. C The PCA plot represented the occurrence of swine and avian H4 subtypes which supported the phylogenetic analyses

species. The PCA analysis confirmed two independent transmission events of H6N6 viruses in Chinese swine (Fig. 6C).

H10NS virus

The neighbour-joining phylogenetic tree of 90 avian and one swine full-length HA sequence of H10N5 viruses suggested chicken to swine transmission of the H10N5 virus in China. It was interesting to note that these closely related chicken and swine H10N5 virus sequences were not contemporary, suggesting a circulation of H10N5 viruses in various domestic and wild avian species in China and Southeast Asia (Fig. 7A) which may be circulating at a low frequency and hence remained undetected. The identical glycosylation patterns of closely related swine and various avian H10N5 virus HA proteins suggested a common origin of H10N5 viruses in avian species in China (Fig. 7B). The PCA plot depicted the transmission of H10N5 viruses in swine and avian species (Fig. 7C). TheH10N5 virus appeared to be in circulation in avian species in China and the Southeast Asia at a low frequency.

In summary, the HA gene sequences of eleven AIV subtypes, including highly pathogenic H5Nl and H7N9, and low pathogenic H3N3, H4Nl, H4N6, H4N8, H5N2, H6N6, H7N2, H9N2, and H10N5 viruses, have been reported in swine until 31 August 2020. The neighbour-joining phylogenetic trees followed by N-linked glycosylation and PCA analyses determined multiple spillover events for highly pathogenic H5Nl viruses in Chinese and Indonesian swine. There were also numerous spillover events for low pathogenic H9N2 viruses in Chinese swine; however, a limited spillover occurred for other AIV subtypes.

Adaptation of avian-origin IAVs in swine

Several amino acid substitutions in HA glycoproteins of IAV subtypes have been reported to determine their preference of host receptor binding, which as a result, may facilitate interspecies transmission and adaptation of avian IAVs in a mammalian host, including swine. Therefore, we analysed potential mammalian adaptation markers in mature HA proteins of swine IAV subtypes (Fig. 8).

The presence of 226L and 228S (H3 numbering) in the HA protein of H4N6 viruses suggested that swine H4N6 viruses have already adapted for mammalian transmission, since these are the key markers associated with increased a-2,6- SA receptor binding ability facilitating mammalian adaptation and transmission [6]. The other swine H4 subtypes, including H4Nl and H4N8 viruses, had 226Q and 228G, which prefer a-2,3-SA receptor binding and therefore do not appear to be adapted in swine for efficient mammalian transmission. Intriguingly, swine H5Nl viruses, despite lacking the above key adaptation markers, have acquired other potential markers, including S137A, 1155T, N158D,

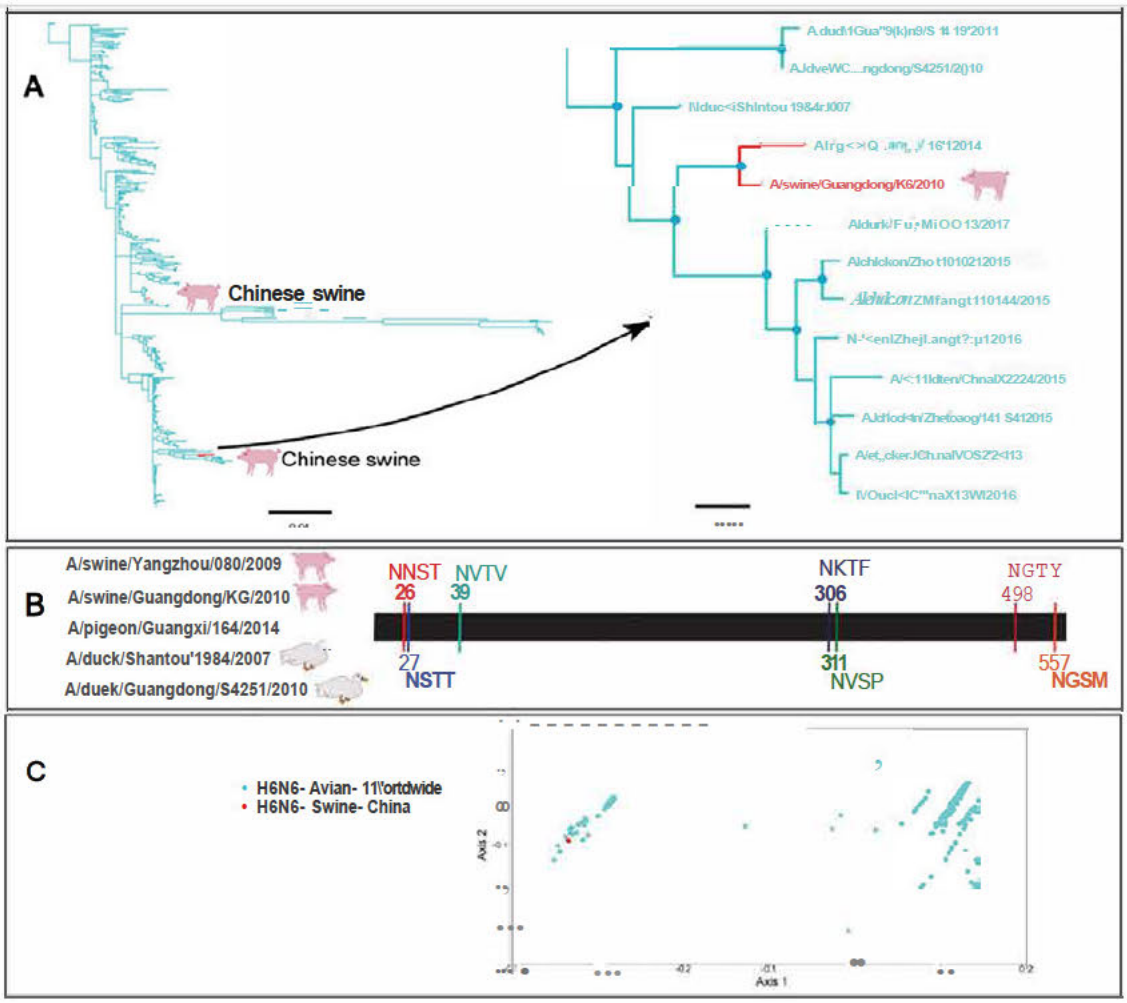

Fig. 6 A Neighbour-joining tree of 421 full-length HA nucleotide sequences of swine and avian H6N6 viruses represented two separate events of avian to swine transmission in China highlighted with red nodes. The blue dots at the nodes in the subtree represented> 90% bootstrap support. B The identical glycosylation patterns of pigeon, duck, and swine H6N6 viruses in China suggested a broad circulation of H6N6 viruses in avian species and their sporadic spillover to swine. C The PCA plot confirmed the occurrence of two independent spillover events of H6N6 viruses in Chinese swine

T160A, T192I, and K193R. The T160A and T192I substitutions were also observed in some of the swine HSN2 viruses. Since these amino acid substitutions prefer a-2,6-SA receptor binding, the probability of swine-to-swine transmission of these swine HS viruses remains feasible.

Interestingly, the T160A substitution was also observed in other swine viruses, including H7N2, H7N9, and H9N2. From these observations, it appears that the Thr to Ala substitution at amino acid position 160 would be essential for swine adaptation of avian-origin HS, H7, and H9 viruses; however, it warrants further investigation using swine model. Interestingly, swine H7N9 and some of the swine H9N2 viruses also had a critical mammalian adaptation marker, Q226L. In addition to this, all swine H9N2 viruses (n= 22) had the D22SG substitution, which in a previous study was suggested to be required for swine adaptation of H9N2 viruses [32]. One of the two swine H6N6 viruses has acquired A222V and G228S substitutions, which prefer a-2,6-SA receptor binding while retaining 226Q, which prefers a-2,3-SA receptor binding. The other swine viruses, including H3N3 (n = 2) and H IONS (n= 1), did not appear to carry potential mammalian adaptation markers in their HA proteins.

Overall, in our analysis, the presence of Q226L and G228S substitutions in swine H4N6 viruses suggested the possibility of their adaptation in swine. The presence of T160A and other potential substitutions in some of the swine HSNl and HSN2 viruses suggested the possibility of their adaptation in swine. The presence of T 160A and Q226L substitutions in swine H7N9 and some of the swine H9N2 viruses along with D22SG substitution in

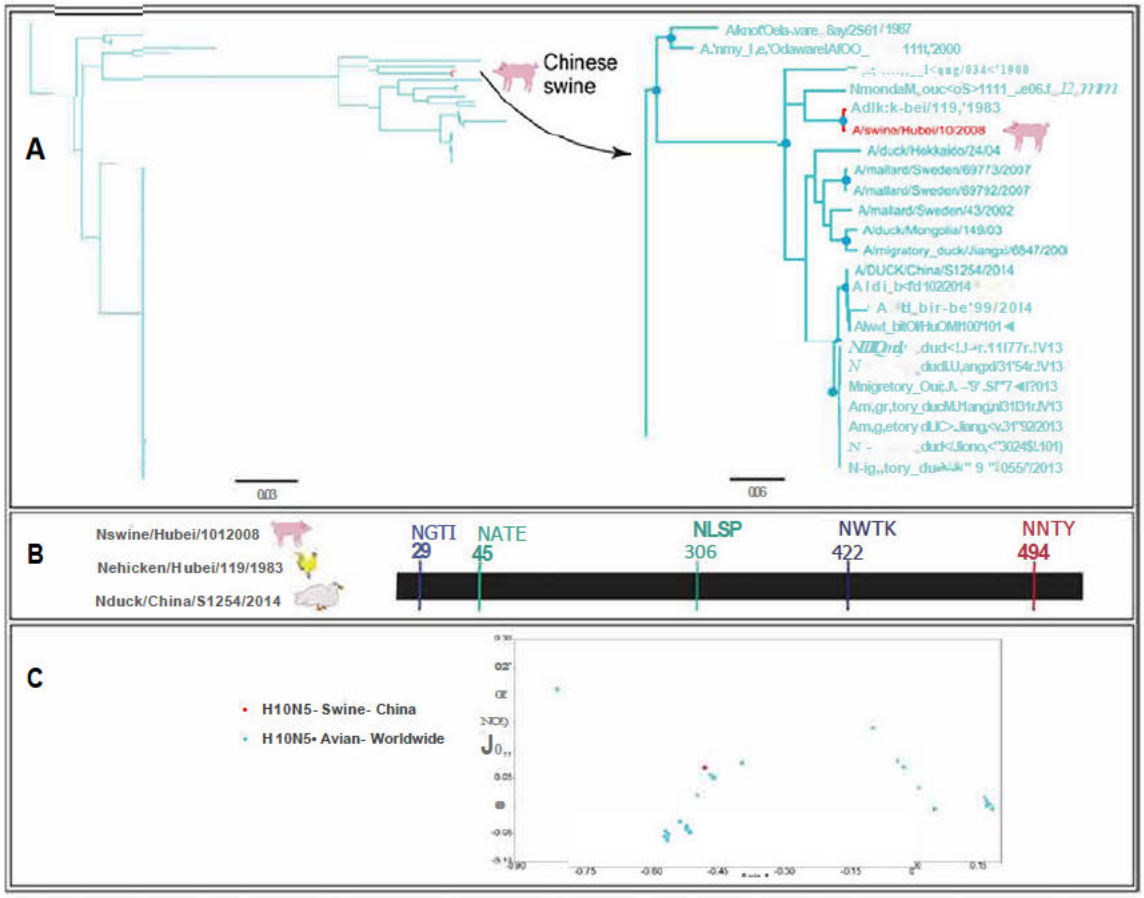

Fig. 7 A Neighbour-joining tree of 91 full-length HA nucleotide sequences of HION5 viruses reported from avian species and swine determined one spillover event from chicken to swine in China. The closely related chicken and swine HI0N5 virus sequences were not contemporary suggesting a circulation of these viruses in avian species at a low frequency in the region. The blue dots at the nodes in the subtree represented> 90% bootstrap support. B The identical glycosylation pattern of chicken and duck HION5 viruses suggested a common origin of circulating HION5 viruses in China with one spillover event to swine. C The PCA plot represented the global transmission pattern of avian and swine HI0N5 viruses

swine H9N2 viruses suggested a strong possibility of their adaptation in swine.

Discussion

In a recent systematic review, we reported the prevalence of multiple AIV subtypes in swine populations [7] which warranted further investigation into the transmission dynamics and possible spillover events of these viruses. In the present study, we used phylogenetic analysis followed by N-linked glycosylation and PCA analyses to unravel the events of avian to swine transmission of various AIV subtypes. Intriguingly, highly pathogenic H5Nl viruses had multiple sporadic transmissions from avian to swine in China and Indonesia. Similarly, numerous sporadic events of avian to swine transmission of low pathogenic H9N2 viruses were also identified in China, with limited avian to swine transmission observed for the other AIV subtypes. Since China is considered an epicentre of avian influenza viruses, multiple reports of avian H5Nl and H9N2 viruses from China were not surprising. However, the occurrence of numerous independent events of avian to swine transmission of H5Nl viruses in Indonesian swine suggested a broad intracontinental circulation of highly pathogenic H5Nl viruses in Southeast Asia. Mine et al. [35] reported that the migratory flyways of wild birds for inter-and intracontinental dissemination of IAVs appear to be responsible for higher H5Nl and H9N2 virus circulation in Southeast Asia [35]. To our surprise, we found two groups of identical HA nucleotide sequences of H5Nl viruses obtained from seven Indonesian swine, which strongly suggested the possibility of swine-to-swine transmission belonging to two HS clades (2.1.3 and 2.1.3.3). Since IAV RNA polymerases have a low fidelity with a substitution rate of 2 x 10-5 per nucleotide [39, 43, 53), the probability

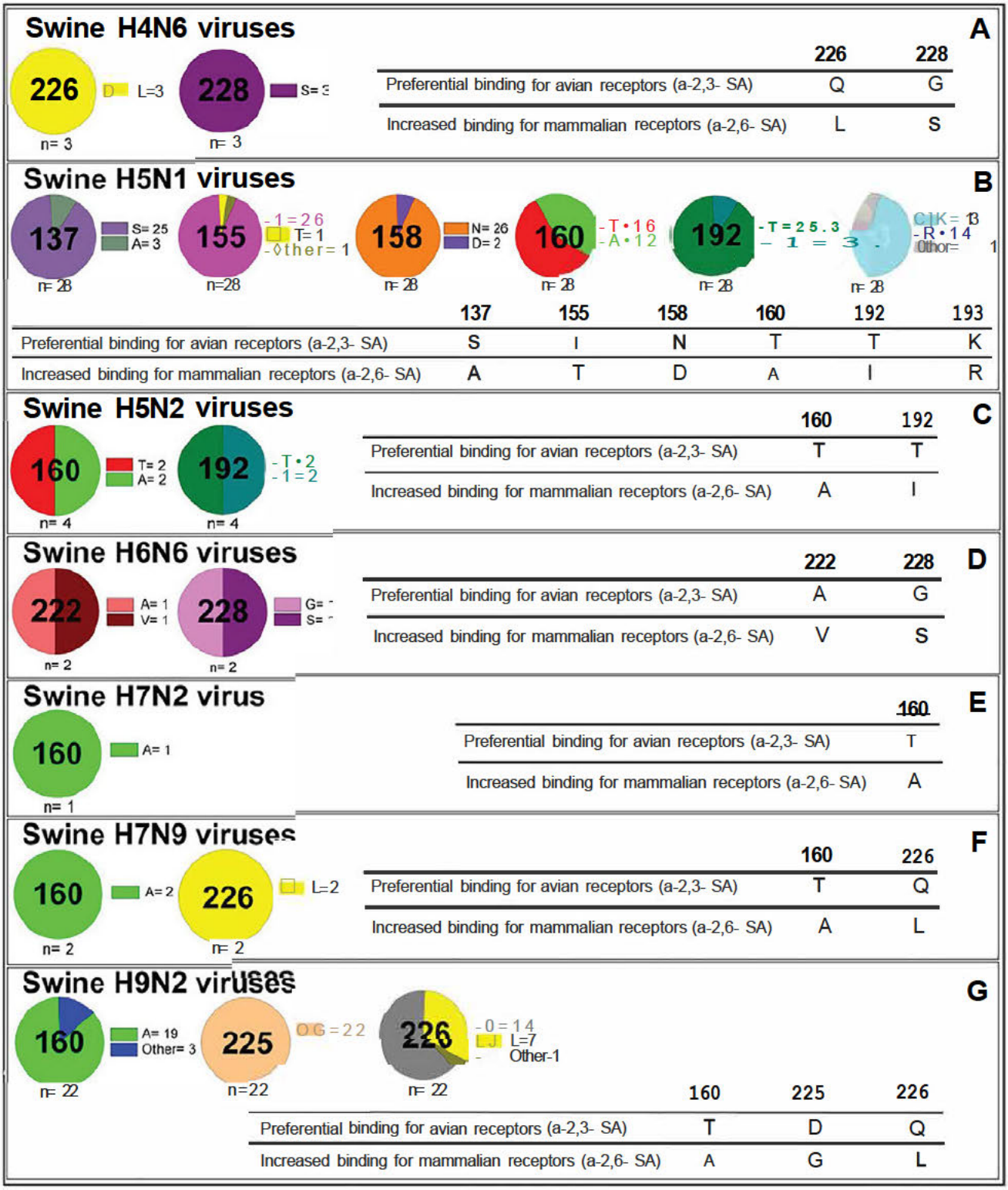

Fig. 8 A-G Schematic representation of potential mammalian adaptation markers (H3 numbering) [6] in mature HA proteins of swine IAVs. The analysis was conducted using the Sequence Variation (SNP) tool of the Influenza Research Database [65]. A key amino acid substitution for mammalian adaptation, **Q226L**, was observed in swine H4N6, H7N9 and some of the H9N2 viruses, while another potential mammalian adaptation marker, T160A, was present in several swine HSN1, HSN2, H7N2, H7N9 and H9N2 viruses. A potential swine adaptation marker, **02250** [32], was present in all of the swine H9N2 viruses

of unrelated H5N1 viruses having identical nucleotide sequences is low.

We also found that those swine and avian IAVs that were closely related phylogenetically, appeared to have an additional glycosylation site in the HA protein. An exception was seen for H3N3 viruses, where the loss of a glycosylation site at position 499 (NGTY) appeared to have triggered the transmission from avian to swine. The highest variation in the pattern of the glycosylation sites was observed in the highly pathogenic H5N1 viruses. Seven different glycosylation patterns were observed for H5N1 viruses which suggested multiple sporadic transmission events of H5N1 viruses from avian reservoirs to swine. Similarly, the glycosylation sites at positions 145 (NGTS), 218 (NRTF or NKTF), and 313 (NCSK) were unstable in swine and avian H9N2 viruses, having four patterns of glycosylation which suggested multiple origins of swine H9N2 viruses as well.

The N-linked glycosylation deploys multiple functions to promote IAV fitness inside the host cell and hence plays a vital role in IAV evolution and adaptation in the host. Kim et al. [24] suggested that analyses of N-linked glycosylation patterns of HA protein may provide signatures for the identification of ecological spillover and adaptation of AIVs in swine [24]. Interestingly, the occurrence of identical glycosylation patterns in HA proteins of H4N8 viruses in Chinese ducks and swine and South African ducks and wild birds suggested a broad intercontinental bird migration pattern with an existing risk of avian to swine spillover of the H4N8 virus in South Africa.

While there have been reports of sporadic cases of highly pathogenic H5N1 virus transmission directly from avian (poultry) to human within family clusters in Indonesia in 2006 and Turkey during December 2015 to January 2016 [61], there has been limited human-to-human transmission. As a result, these infections have not yet been able to trigger an epidemic. However, the adaptation of highly pathogenic H5N1 virus in swine, either through mutation(s) or selective pressure [41], or swine-swine transmission, may facilitate a broader mammalian or human-to-human transmission.

The amino acid substitutions that confer increased binding affinity for the α-2,6-SA receptors have been identified as key mammalian adaptation markers that could facilitate mammalian or human-to-human transmission. We observed that swine H4N6 viruses had two key mammalian adaptation markers, 226L and 228S (H3 numbering) in the HA protein. A previous study reported that strains with the 226L mutation acquired binding affinity for α-2,6-SA receptors and induced higher infectivity in primary swine respiratory epithelial cells compared to H4N6 virus with 226Q [2]. Multiple passages of wildtype and mutant H4N6 viruses in swine respiratory epithelial cells revealed that the Q226L substitution was primarily related to swine adaptation of the H4N6 virus [2]. Interestingly, G228S did not appear to be essential for the adaptation of H4N6 virus in swine respiratory epithelial cells [2]. Despite the presence of 226L and 228S in swine H4N6 viruses, they had a limited replication in swine upper respiratory tract [1]. The presence of lesions and the detection of H4N6 virus in the lungs of experimentally inoculated piglets were reported with a limited transmissibility [1] which suggested a low risk of H4N6 virus transmission in the field setting.

In the present study, six amino acid substitutions: S137A, I155T, N158D, T160A, T192I, and K193R (H3 numbering), were observed in some of the swine H5N1 viruses which have been previously associated with enhanced affinity for α-2,6-SA receptor binding [13, 18, 59, 62] which suggests that these six amino acid substitutions could have facilitated the adaptation of H5N1 virus in swine. Additionally, T160A and N158D substitutions were found to be associated with the loss of glycosylation in the HA protein and H5N1 transmission in guinea pigs and ferrets [13, 18]. It was interesting to note that swine H5N1 viruses did not show any of the key adaptation markers, Q226L and G228S. Intriguingly, in our study, we observed dual substitutions, T160A and K193R, in six swine H5N1 viruses, which were indicative of their potential for increased α-2,6-SA receptor binding and adaptation in swine.

Lipatov et al. [17] showed that avian H5N1 virus in swine could be detected in nasal swabs of intranasally inoculated piglets up to 5 days post inoculation (dpi). While they did not cause any behavioural and food consumption changes in the swine [31], the infection was limited to the respiratory tract, mainly the lungs causing alveolitis and bronchiolitis [31]. Similarly, the intranasal inoculation of swine with a low pathogenic avian H5N2 virus also resulted in subclinical infections; however, the virus could be detected in nasal swabs up to 6 dpi [11] with virus more frequently isolated from the swine upper respiratory tract [11]. Taken together, these findings support the possibility of replication of H5N1 and H5N2 viruses in swine. It is possible that our observation of the T160A and T192I substitutions in some of the swine H5N2 viruses could be related to its replication in swine which warrants further investigation.

Further, we observed A222V and G228S substitutions in one of the swine H6N6 viruses. Interestingly, G228S but not the A222V substitution was previously identified to be associated with increased binding affinity to guinea pig erythrocytes [47]. While the authors showed that virus could be detected in nasal wash fluids of inoculated ferrets up to 5 dpi, the infection was subclinical. Interestingly, only one of the three ferrets that were in a direct contact with the virus inoculated ferret had the seroconversion; however, no virus shedding could be detected in direct contact ferrets suggesting a limited transmissibility of the H6N6 virus with A222V and G228S dual substitutions [47]. This suggested that

swine H6N6 virus with A222V and G228S substitutions may have a limited transmissibility in swine and is yet to be adapted in swine to acquire critical substitutions for efficient mammalian transmission.

It was interesting to note that the four HA mutations reported by Xu et al. [60] (G63C, P65L, R66L, and M67I) acquired after the serial passage of an avian H7N9 virus in swine were different from the T160A and Q226L mutations observed in the naturally infected swine H7N9 viruses analysed in the present study. The passaged viruses could be detected in nasal and throat swabs of inoculated swine up to four dpi with focal mild interstitial pneumonia and lung infection at day four; however, the infection was always subclinical [60]. Intriguingly, despite the absence of the key Q226L substitution, the avian H7N9 virus was reported to rapidly adapt in the swine [60], which suggested that various amino acid substitutions, including T160A and Q226L, observed in the present study, may facilitate the adaptation of avian H7N9 virus in swine.

Neumann et al. [32] reported that avian H9N2 viruses with the 226L mutation were able to replicate in the upper respiratory tract of swine, with virus shedding in nasal swabs up to eight dpi [32]. They also reported an additional mutation, D225G, that was observed after four virus passages in swine. The passaged H9N2 virus with dual mutations, 225G and 226L, replicated and transmitted more efficiently in swine compared to the parental H9N2 virus with 225D and 226L [32]. Also, the H9N2 virus became contact transmissible after it acquired 225G, although the infections were always subclinical [32]. In our study, we observed that D225G was present in all swine H9N2 viruses ($n = 22$), while dual mutations (D225G + Q226L) were present in seven naturally infected swine. This suggested that swine H9N2 virus would be able to replicate and transmit between swine via contact in a natural setting. In addition, T160A was observed in most of the swine H9N2 viruses ($n = 19$). This mutation was also consistently present in several H5 and H7 viruses, suggesting that it had facilitated swine adaptation of these avian viruses.

Overall, the present study provided evidence of multiple spillover events of avian-origin H5N1 and H9N2 viruses in swine populations. The occurrence of certain putative mammalian adaptation markers, especially in swine H5N1, H7N9, and H9N2 viruses, suggested that these viruses have already adapted in swine, with supporting reports of these strains being able to replicate in the upper respiratory tract of swine. The replication and adaptation of these avian viruses in swine may facilitate the emergence of a pandemic influenza virus strain through reassortment with endemic swine viruses and circulating human IAVs in swine.

Conclusion

The interaction of swine with domestic and wild avian species may facilitate the interspecies transmission of avian IAVs to swine. Avian to swine spillover of AIVs, especially H5N1, H7N9, and H9N2 viruses, which have acquired some of the amino acid substitutions associated with mammalian adaptation, poses a considerable threat regarding the potential for the emergence of a pandemic influenza virus strain. Because the largest number of available full-length HA nucleotide sequences from swine belonged to either highly pathogenic H5N1 ($n = 28$) or low pathogenic H9N2 viruses ($n = 22$), this allowed us to use these two subtypes to understand their circulation and adaptation in swine. Numerous spillover events of H5N1 and H9N2 viruses from birds to swine suggest a high frequency of these viruses in avian reservoirs. In light of these risks, we recommend that swine farmers implement adequate biosecurity measures to minimize avian–swine interactions and that active IAV surveillance to monitor virus evolution in swine populations is imperative in order to combat the emergence of an influenza strain with pandemic potential.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The influenza virus sequences analysed in this study were downloaded from the Influenza Research Database (https://www.fludb.org/). This research is part of a research project which has obtained full approval from the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban; Reference: AREC/041/019D. Additionally, the authors have permission in terms of section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) from the Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa; Reference: 12/11/1/5/4 (1425).

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CHAPTER 7

No evidence of influenza A virus RNA in South African backyard swine in KwaZulu-Natal province

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Abstract

Influenza A virus (IAV) is the most widely reported influenza virus type in swine populations worldwide. In this study, we, for the first time, conducted molecular surveillance for detecting IAV prevalence in backyard swine in the KwaZulu-Natal province of South Africa. We collected swine saliva samples (n=102) from three backyard farms located in the uMgungundlovu district in March 2021. Total RNA was used to detect IAV using a one-step real-time RT-PCR assay with matrix gene-specific oligonucleotide primers and a TaqMan probe. None of the samples amplified the IAV matrix gene suggesting that the swine under investigation were free from IAV active infection.

Keywords: Influenza A virus, South African backyard swine, qRT-PCR, IAV matrix gene.

Introduction

Influenza A virus (IAV) is the most widely occurring influenza virus type reported from swine populations worldwide (Chauhan and Gordon, 2020). The occurrence of error-prone RNA-dependent RNA polymerase (Cheung et al., 2014) and a high frequency of recombination make IAV a constantly evolving virus pathogen with an imminent threat to public health (Nelson and Holmes, 2007). In addition, due to the occurrence of α-2,3 and α-2,6 sialic acid receptors in the trachea, swine serves as an 'intermediate host' or the 'mixing vessel' for IAV reassortment, evolution, and transmission (Ma et al., 2008). Most recently, the emergence of the 2009 swine-flu pandemic in Mexican swine (Mena et al., 2016; Smith et al., 2009) reiterates the significance of active IAV surveillance in swine populations to combat future influenza pandemics.

Interaction of swine with other species, for example, chicken, duck, and cattle raised in the backyard poses a significant risk factor for IAV transmission among backyard animals (Bravo-Vasquez et al., 2020; Bravo-Vasquez et al., 2016; Gcumisa et al., 2016). In addition, the lack of suitable biosecurity measures poses an increased risk of IAV transmission from wild birds to the backyard poultry and swine (Hamilton-West et al., 2012; Hill et al., 2019). For example, the reports of avian-origin highly pathogenic H5N1 viruses in backyard swine populations in Nigeria (Meseko et al., 2018) and Indonesia in 2018 (Mahardika et al., 2018) provide the evidence of IAV transmission from avian species to the backyard swine. We recently reported avian to swine transmission of eleven IAV subtypes in various countries (Chauhan and Gordon 2021). In addition, the occurrence of human-origin pandemic strains of A(H1N1)pdm09 virus in backyard swine populations in Guatemala in 2017 (Gonzalez-Reiche et al., 2017), Peru in 2016 (Tinoco et al., 2016), Cameroon in 2014 (Larison et al., 2014), and the USA in 2013 (Weller et al., 2013) provide evidence of reverse-zoonotic transmission of IAV from human to swine. Similarly, the reports of the H1N2 virus in

backyard swine in Chile in 2017 (Bravo-Vasquez et al., 2017) and 2016 (Bravo-Vasquez et al., 2016) further signify the importance of active IAV surveillance in backyard swine populations to safeguard backyard swine farming and public health.

A recent study established that the intercontinental wild bird migration has contributed to the dissemination and spread of IAV in the African continent (Fusaro et al., 2019). The climate and geographical location of South Africa are unique in terms of avian migration for over-wintering and breeding (Bussière et al., 2015; Szép et al., 2006). As a result, several wild bird species visit South African territories each year, serving as carriers for exotic virus pathogens, including IAV (Cumming et al., 2011). The past outbreaks of avian-origin H5N2, H7N1, and H7N7 viruses in Ostrich farms in South Africa signify the risk of interspecies transmission of IAV from wild birds (Abolnik et al., 2016). Furthermore, the presence of various IAV subtypes in avian species in South Africa, including H5N8 (Abolnik, 2020), H6N2 (Abolnik et al., 2019), and H5N2 viruses (Venter et al., 2017), poses a constant threat for its dissemination to the backyard swine populations in the country. However, due to the lack of active influenza surveillance, a persistent knowledge gap occurs regarding IAV prevalence and circulation within South African backyard swine populations. Therefore, we, for the first time, conducted molecular surveillance to identify IAV prevalence in backyard swine populations in the KwaZulu-Natal province of South Africa.

Materials and Methods

Sample collection

Informed consent was obtained from backyard swine farmers, who agreed to participate voluntarily in the study. Data was collected regarding swine health, vaccination status, general awareness of the backyard farm owners regarding virus pathogens in swine, biosecurity, routine swine handling, and related practices on a questionnaire to assess IAV

transmission and circulation risk at the backyard farms under investigation. The questionnaire data were used to evaluate the farmers' understanding of the swine diseases and preparedness to avoid the transmission of IAV and other zoonotic viruses. We collected swine saliva samples (n=102) from three backyard swine farms located within the uMgungundlovu District of KwaZulu-Natal province in South Africa (Figure 1) in March 2021 to conduct passive surveillance for detecting IAV molecular prevalence.

The backyard swine farms were identified in coordination with the State Veterinary Department of the uMgungundlovu district. Three backyard farms that voluntarily agreed to participate in the study were selected, including a small, medium, and large backyard farm. Samples were collected using a three-strand twisted 100% cotton rope following a standard non-invasive protocol detailed and reviewed previously (Henao-Diaz et al., 2020; Prickett et al., 2008). An Animal Health Technician (AHT) from the State Veterinary Department of uMgungundlovu district assisted in sampling and identifying the disease symptoms in backyard swine under investigation. Saliva samples were collected from the symptomatic as well as apparently healthy swine in various age groups, from one-month-old piglets up to three-year-old sows.

The targeted sample size was estimated using the following formula:

$N = Z_{1-\alpha/2}{}^2 P (1 - P)/d^2$ (Where, N = sample size, $Z_{1-\alpha/2}$ = Standard normal variate at 5% type 1 error ($P<0.05$); its value is 1.96. P = Expected proportion in population based on previous studies, d = Absolute error or precision). A report on IAV prevalence in pig populations within the African continent reported an 8% prevalence in Nigeria. Since there was no report on IAV prevalence in pigs in South Africa at the time of the study design, the reported prevalence from Nigeria was used as an expected prevalence within the continent. Therefore, the proposed sample size in the current study would be:

$N = (1.96)^2 \times 0.08 (1 - 0.08)/ (0.05)^2$

N = 3.8416 x 0.0736/0.0025

N = 113.09 or 113.

Each saliva sample that originated from the swine in the age group of 1- 5 months (n=48) represented a pooled sample because the rope was chewed by multiple piglets or growers of the same age that were confined within the pens. The saliva samples that were collected from adult swine in the age group of 1- 3 years (n=54) represented an individual sample because the rope was chewed only by one swine at a time. At least one milliliter (ml) of the saliva was taken for each sample, while most of the samples had 1.5- 2.5 ml of saliva. The swine saliva samples were immediately transferred to sterile 15 ml Falcon tubes after collection and transported to the laboratory on dry ice. In the laboratory, each sample was aliquoted into three replicates using 2 ml sterile cryovials to avoid freeze-thawing, under aseptic conditions inside a class II biological safety cabinet, properly labeled, and stored in a -80°C freezer for downstream processing.

RNA Extraction

Total RNA was extracted from swine saliva samples using QIAamp viral RNA Mini Kit (QIAGEN) as per the manufacturer's protocol. Briefly, the frozen saliva samples were thawed on ice and then vortexed. The samples were centrifuged at 1,500 g for 10 min at 4℃ to eliminate the cells and other contaminants present in the swine saliva. A total of 140 µl of the supernatant was used for RNA extraction. The RNA was eluted in nuclease-free water to assess the concentration of extracted RNA samples using a Nanodrop 2000c Spectrophotometer. Further, the quantity and integrity of a subset of the randomly selected RNA samples were verified using Qubit Fluorometer and PerkinElmer's 'LabChip' equipment, respectively. Finally, the RNA samples were appropriately labeled and stored in a -80°C freezer for downstream processing.

122 **Real-time RT-PCR**

123 The total RNA extracted from swine saliva samples was used to detect IAV using the
124 SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen). A conserved sequence of the
125 IAV matrix gene was amplified using the protocol recommended by the Centre for Disease
126 Control and Prevention (CDC), Atlanta, GA, USA (CDC, 2009). Briefly, the oligonucleotide
127 primers, including IAV-Forward: 5'- GAC CRA TCC TGT CAC CTC TGA C -3' and IAV-
128 Reverse: 5'- AGG GCA TTY TGG ACA AAK CGT CTA- 3' in the presence of a TaqMan
129 probe 5'- TGC AGT CCT CGC TCA CTG GGC ACG- 3' were used in the assay (CDC,
130 2009). The TaqMan probe had a reporter molecule, 6-carboxyfluorescein (FAM), at the 5'
131 end, while the 3'end was labeled with a quencher (Black Hole Quencher 1 or BHQ-1) as
132 recommended by the CDC, Atlanta, GA, USA (CDC, 2009). While the RT-PCR assay is not
133 approved by the Department of Agriculture, Land Reform and Rural Development
134 (DALRRD) for the diagnosis of IAV in pigs in South Africa, it is widely used for the
135 molecular detection of IAV in swine internationally, as reviewed in a previous study
136 (Chauhan and Gordon, 2020).

137 Initially, the one-step real-time RT-PCR assay was optimized and validated using the known
138 concentrations of an IAV positive control template procured from the Integrated DNA
139 Technologies (IDT). Nuclease-free water was used as a negative control template in the
140 assays. One-step real-time RT-PCR assays were conducted in a 'LightCycler 480 II' (Roche)
141 instrument in a 25 µl reaction (WHO, 2014). The real-time RT-PCR assay included one cycle
142 of reverse transcription at 50℃ for 30 min, one cycle of Taq activation at 95℃ for 2 min, and
143 45 cycles of PCR amplification at 95℃ for 15 sec and 55℃ for 30 sec (CDC, 2009; WHO,
144 2014). The FAM data was collected at the 55℃ steps during PCR amplification (CDC, 2009;
145 WHO, 2014). We considered a cycle threshold (Ct) value of ≤ 38 to identify IAV positive

146 samples (WHO, 2014). The real-time RT-PCR assay had two replicates of positive and
147 negative control templates to validate the assay.

148 **Results**

149 Saliva samples were taken from swine in various age groups from three backyard farms in the
150 uMgungundlovu District of KwaZulu-Natal (Table 1). Data related to backyard swine
151 farming were collected to assess the risk of IAV transmission within the backyard. The
152 backyard farmers were asked about their understanding and awareness of various virus
153 pathogens that may infect swine. In addition, questions regarding the vaccination status,
154 presence of poultry, cattle, and wild birds, any previous disease outbreaks in swine, and day-
155 to-day backyard operations were asked (Table 2) to assess the risk of IAV transmission and
156 circulation within the backyard. While the participating backyard swine farmers were aware
157 of the swine influenza virus disease, they acknowledged the interactions of the swine with
158 poultry and wild birds, which indicated the lack of biosecurity at the backyard farms under
159 investigation. Information on the current health status of swine under study was also
160 collected. An AHT from the State Veterinary Department of the uMgungundlovu district
161 assisted us in identifying any apparent disease symptoms in the backyard swine. Saliva
162 samples were collected from the apparently healthy as well as symptomatic swine from each
163 backyard farm under investigation (Table 3).

164 Initially, the Nanodrop 2000c Spectrophotometer assessed all the extracted total RNA
165 samples (n=102). Most of the samples had RNA concentrations in the range of 100- 200
166 ng/µl, with only a few exceptions when RNA concentrations were either below or above this
167 range. The proportion of RNA-positive samples at the three backyard swine farms is
168 illustrated in Figure 2. Further, the RNA quantity and integrity of a subset of the randomly
169 selected RNA samples (n=10) were verified using Qubit Fluorometer and PerkinElmer's
170 'LabChip' instruments, respectively. The Qubit Fluorometer determined that the RNA

concentrations in samples were in the range of 62.7 to 156 ng/µl, with two exceptions when RNA concentrations were 220 and 268 ng/µl. Additionally, the 'LabChip' instrument identified the sharp viral RNA peaks in the swine RNA samples in the range of approximately 300 to 1500 nucleotides (Supplementary Figure 1). These findings confirmed the quantity and integrity of viral RNA in the backyard swine samples under investigation. The real-time RT-PCR assay was validated using the known concentration of a positive control template which was used to make the serial dilutions up to 0.01 nM/L, which yielded the Ct value of 27. This suggests that the real-time RT-PCR assay was sensitive to detecting the small quantities of the viral RNA in swine saliva samples under investigation. The RNA concentrations in swine saliva samples were independently confirmed. For the validation of the real-time RT-PCR assay, we chose the serial dilution of 0.1 nM/L which yielded the Ct value of 16. Interestingly, none of the swine RNA samples (n=102) amplified the IAV matrix gene using a one-step real-time RT-PCR assay, suggesting that the samples under study were negative for active IAV infection.

Discussion

For the first time, the present study attempted to investigate the prevalence of active IAV infection in backyard swine in the KwaZulu-Natal province of South Africa. Henao-Diaz et al. (2020) suggested that collecting oral fluids or saliva using the hanging rope method is a non-invasive and a preferred method for RT-PCR based detection of IAV in pigs because it represents the pooled saliva samples since the rope is chewed by multiple piglets or growers simultaneously, that are confined together within the pens. The abundance of α-2,3 and α-2,6 sialic acid receptors in the swine trachea and bronchi triggers the shedding of IAV in the swine's upper respiratory tract, resulting in the shedding of the IAV in the pharyngeal region and, therefore, in the oral fluid or saliva. However, certain other sample types, such as tracheal washes or bronchial lavages, can also be used for the IAV detection but collecting

these samples from the pigs would be challenging as it requires sufficiently trained personnel who can perform the invasive technique to collect the sample. Therefore, we collected the swine saliva samples in various age groups from three backyard farms that were distantly located within the uMgungundlovu District. We extracted and quantified the viral RNA from swine saliva samples which were then screened for IAV using a matrix gene-specific one-step real-time RT-PCR assay. The current investigation did not identify any active IAV infection in the swine populations under study. It should be noted that this study was conducted during the COVID-19 lockdown. Since movement-related measures were in place in South Africa, we could only interact with the backyard swine farmers during a narrow time frame, forcing us to limit the duration of the backyard swine sampling in the field. It should be noted that the absence of IAV in a subset of backyard swine populations investigated in the present study in the South African province of KwaZulu-Natal does not necessarily rule out the possibility of IAV prevalence at other backyard swine holdings in the country.

It should also be noted that during the ongoing COVID-19 pandemic, a decreased influenza activity was reported in the 2020 flu season in several countries, especially in the Southern Hemisphere, including South Africa (Olsen et al., 2020). As per the CDC report, only six influenza-positive human specimens were reported in South Africa out of 2,098 human specimens that were tested during the flu season from April-July 2020 (Olsen et al., 2020). Only 51 human samples were collectively identified influenza-positive out of 83,307 that were tested in April- July 2020 flu season in three countries in the Southern Hemisphere, including Australia, Chile, and South Africa. In contrast, 24,512 influenza-positive human specimens were reported out of 178,690 tested during April- July 2017- 2019 in these countries (Olsen et al., 2020). These data represented a significant decrease in influenza activity in the Southern Hemisphere, including South Africa, during the ongoing COVID-19 pandemic. This substantial decrease in influenza circulation may be influenced by increased

221 alertness and behavioural changes in people for hygiene and using a face mask for day-to-day
222 operations in public places to mitigate the transmission of the Severe acute respiratory
223 syndrome coronavirus 2 (SARS-CoV2) (Olsen et al., 2020). Most likely, the protective
224 behaviour of people could significantly impact the transmission of IAV, provided that the
225 SARS-CoV2 and IAV share the same transmission route (Cowling et al., 2020). Our study
226 noticed that the backyard swine farmers and the household members except one were
227 wearing the face mask during our visits for backyard swine sampling. Such practices would
228 limit the transmission of IAV between humans and swine.

229 It is also likely that the virus-virus interactions and competition between the SARS-CoV2 and
230 IAV could be a factor in the sharp decrease in influenza activity in the flu season
231 (Nickbakhsh et al., 2019; Zipfel and Bansal, 2020). The SARS-CoV2 is reported to have a
232 higher potential for pathogenicity in the human respiratory tract than IAV (Piroth et al.,
233 2021). It is concerning that domestic swine (*Sus scrofa domestics*) has been susceptible to
234 SARS-CoV2 experimental infections with flu-like symptoms, including ocular and nasal
235 discharge that appeared immediately after experimental inoculations (Pickering et al., 2021).
236 However, the reports on natural infections of swine with SARS-CoV2 are not available yet; a
237 recent report has expressed the concern that a significantly high transmission rate along with
238 a high viral load of SARS-CoV2 among human populations make the domestic swine
239 susceptible to natural spillover, and thus has suggested for close monitoring of the swine
240 populations (Opriessnig and Huang, 2020).

241 While in the present study, we did not detect IAV active infection in the backyard swine
242 under investigation, several other studies have reported active IAV infection in backyard
243 swine populations in various countries, including Chile (Bravo-Vasquez et al., 2016), Nigeria
244 (Meseko et al., 2018), and Indonesia (Mahardika et al., 2018) reporting interspecies
245 transmission of IAV from avian species to the backyard swine.

Interestingly, during sampling, we noticed that a few piglets at the second and third backyard farms were coughing and sneezing, while a few others appeared to be lethargic and inactive. In addition to this, diarrhoea, discoloration of the skin, and the symptoms of pneumonia were reported by the farmers in some of the swine in previous months. We noticed that some of the piglets at the second backyard farm were able to exit the pens because the gates were not kept in good condition. In addition to this, the presence of birds was also noticed at this farm. In this study, all the backyard swine farmers mentioned the visits of wild birds to the backyards and their interactions with the swine. Two of the backyard farms kept the swine confined within the pens, while the third backyard farmer mentioned that their swine were free-roaming and kept within the pens. This was the same backyard farm where we noticed a small herd of sheep that was openly grazing beside the backyard. We noticed that a few adult swine at this backyard farm roamed freely outside the pens and approached the sheep herd. Interestingly, some of the piglets and grower swine at this backyard farm had redness in the eyes and red patches on the skin. These observations indicate a considerable risk that exists in terms of disease transmission between interacting animal species.

In another study which included the deep sequencing of the RNA fraction of the selected saliva samples (n = 3), one from each backyard swine farm, for determining the diversity of RNA viruses, we did not detect the IAV genome. This confirms that the subset of swine saliva samples used for the deep sequencing was free from active IAV infection.

The inadequate biosecurity standards at the South African backyard swine farms put the backyard swine farming at a high risk of disease transmission. Furthermore, in the light of the existing reports on the occurrence of multiple IAV subtypes in avian species in South Africa, a significant challenge exists to backyard swine farming in the country. We, therefore, recommend periodic active surveillance of IAV in South African backyard swine populations to safeguard the backyard swine farming practices and public health.

Conclusion

Despite the absence of active IAV infection in backyard swine populations under investigation, we were able to quantify viral RNA in swine samples under study (Supplementary Figure 1), which needs to be verified further to determine if RNA viruses other than IAV are present in these samples. However, the present study was aimed at detecting active IAV prevalence in South African backyard swine populations in KwaZulu-Natal province; we are intrigued to extend it to identify the RNA virus(es) present in the backyard swine saliva samples collected under the present study.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Author contributions

R.P.C. and M.L.G. conceived and designed the study and collected the samples. R.P.C. performed the experimental analysis, analyzed the data, and wrote the first draft of the manuscript. R.P.C. and M.L.G. reviewed and revised the manuscript.

Ethical considerations

This study had the full approval of the research protocol from the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban; AREC Reference# AREC/041/019D. Additionally, we obtained the required permit in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) from the Department of Agriculture, Land Reform and Rural Development (DALRRD), previously known as the Department of Agriculture, Forestry and Fisheries (DAFF) of the Republic of South Africa; DAFF Reference# 12/11/1/5/4 (1425).

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Data availability

Supplementary material for this article is available online.

Disclaimer

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Table 1: Backyard swine samples included in the current study

Swine age groups	**Backyard Farm 1**	**Backyard Farm 2**	**Backyard Farm 3**	**Total**
1- 3 months**	7	14	10	31
4- 5 months**	0	0	17	17
1 year*	13	4	1	18
2 years*	0	0	28	28
3 years*	0	6	2	8
Total	**20**	**24**	**58**	**102**

*Individual samples

**Pooled samples

'0' represents the unavailability of swine in particular age group

462 **Table 2:** Data related to IAV disease awareness and day-to-day swine farming practices on
463 backyard farms in uMgungundlovu district of KwaZulu-Natal

Data*	**Backyard Farm 1**	**Backyard Farm 2**	**Backyard Farm 3**
Awareness about the swine influenza virus	Yes	Yes	Yes
Awareness about other swine viruses	Yes	No	No
Influenza vaccination of backyard farm owners	No	No	No
Swine roaming status	Confined within pens	Confined within pens	Free-roaming, as well as confined within pens
Interaction of chicken with backyard swine	No	Yes	Yes
Presence of duck or other poultry in backyard	No	No	No
Presence of cattle in backyard	No	Yes	Yes
Interaction of cattle and swine in backyard	No	No	No

Interaction of wild birds with backyard swine	Yes	Yes	Yes
Use of face mask while working with swine	Yes	No	Yes
Any sickness in swine in the recent past (reported by the backyard farmers)	One piglet appeared lethargic and inactive about one week before sampling	Pneumonia and discoloration of skin in some of the swine about six months before sampling	Diarrhea in some of the swine about one month before sampling
Swine mortality due to sickness in the past	Yes	No	Yes
Slaughtering of swine within the backyard	No	Yes	No

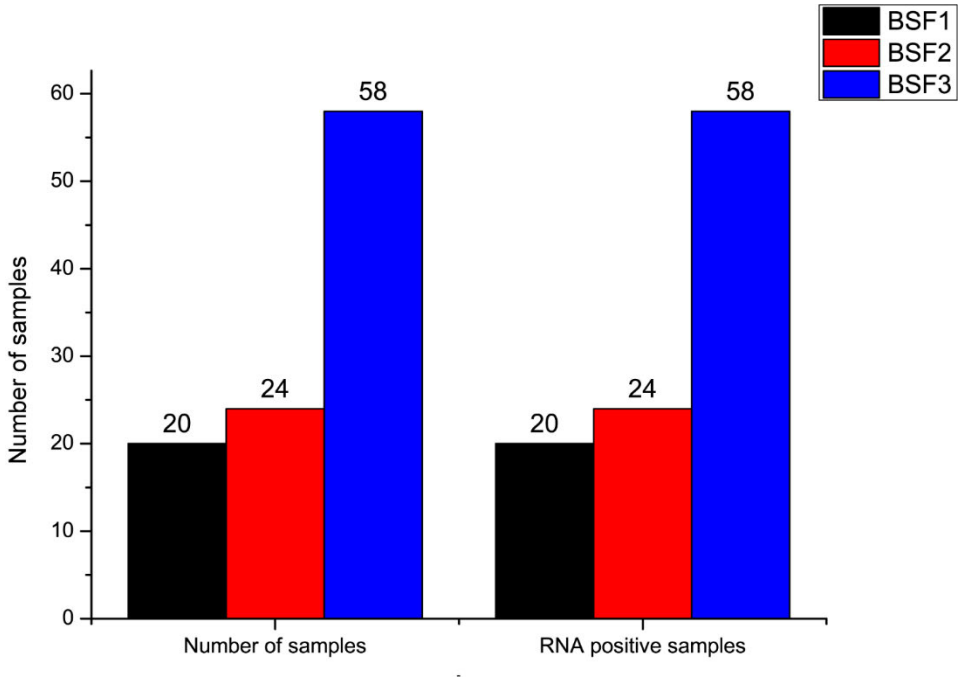
*Reported by the backyard farmers

472 **Table 3:** Health status of backyard swine observed during sampling

Backyard Farm ID	**Swine age groups**	**Health status of swine during sampling***
Backyard Farm 1	1- 3 months	Apparently healthy
	1- year	Apparently healthy
Backyard Farm 2	1- 3 months	Apparently healthy as well as symptomatic. Five piglets were sneezing; two piglets were coughing and sneezing, three piglets were lethargic and inactive
	1- year	Apparently healthy
	3- years	Apparently healthy
Backyard Farm 3	1- 3 months	Apparently healthy as well as symptomatic. Three piglets had red patches on the skin; two piglets had red eyes
	4- 5 months	Apparently healthy as well as symptomatic. One swine had red patches on the skin; one swine had red eyes, and one swine appeared lethargic and inactive
	1- year	Apparently healthy
	2- years	Apparently healthy
	3- years	Apparently healthy

473 *An Animal Health Technician from State Veterinary Department visually identified the
474 disease symptoms in backyard swine under investigation

Figure 1: Backyard swine sampling sites highlighted with red drops on the map are located within the uMgungundlovu District in the KwaZulu-Natal province of South Africa. These sites were identified in coordination with the State Veterinary Department in Pietermaritzburg. The map was generated online using Google Maps (https://www.google.co.za/maps).

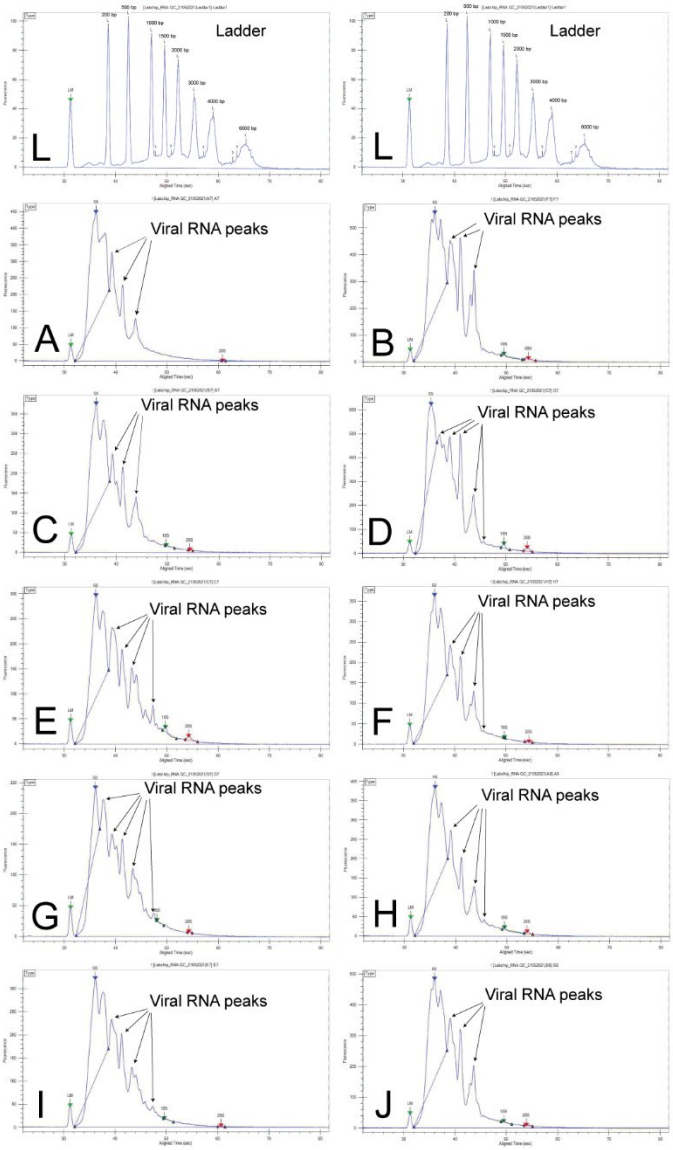


490

491 **Figure 2:** Proportion of RNA positive samples at the three backyard swine farms.

492

493



494

495 **Supplementary Figure 1:** Figure 'L' represents 'Ladder' ranging between 200 to 6000
496 nucleotides. Figures 'A-J' represent viral RNA peaks for the backyard swine RNA samples
497 under investigation. The viral RNA peaks in swine samples ranged between approximately
498 300 to 1500 nucleotides in length. Sharp peaks in combination with strong fluorescence
499 signals on the y-axis confirmed the presence of significant quantities of unidentified viral
500 RNA in swine samples under study. The negligible quantities of 18s and 28s ribosomal
501 RNAs verified the elimination of cells and contaminants during extraction following the
502 centrifugation of the swine saliva at 1,500 g for 10 min at 4°C as per the manufacturer's
503 protocol.

CHAPTER 8

Metagenomic analysis of RNA fraction reveals the diversity of swine oral virome on South African backyard swine farms in the uMgungundlovu District of KwaZulu-Natal province

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Abstract

Backyard swine farming is common in many developing countries, including South Africa. Due to the lack of active disease surveillance, a persistent knowledge gap exists regarding the diversity of RNA viruses in South African backyard swine populations. We conducted a metagenomic analysis of three backyard swine oral secretions (saliva) collected from three distantly located backyard swine farms (BSFs) in the uMgungundlovu District, KwaZulu-Natal province, South Africa. The RNA fraction was used to generate approximately 21 million reads for each of the saliva samples obtained from BSF1 and BSF2, and 8 million reads for the saliva obtained from BSF3 using the Illumina HiSeq X. Contigs were assembled using the Genome Detective tool. The sequence analyses identified high diversity of swine enteric viruses in the saliva samples obtained from the BSF2 and BSF3, while only a few viruses were identified in the saliva obtained from the BSF1. The swine enteric viruses belonged to various animal virus families; however, a few plant viruses and five species of unclassified RNA viruses were also identified. Specifically, viruses of the family *Astroviridae*, according to the number of reads, were the most prevalent. Interestingly, the occurrence of Porcine rotavirus A and Rotavirus C at the BSF2 as well as Rotavirus C and Hepatitis E virus (HEV) at the BSF3 were concerning because these viruses may be transmitted to exposed human populations. The presence of these viruses in the backyard swine makes it imperative to implement hygiene and biosecurity to prevent their zoonotic transmission from swine to humans.

Keywords: Backyard swine, deep sequencing, Illumina HiSeq X, phylogenetic analysis, RNA viruses, South African backyard farms, swine oral virome.

Introduction

Backyard swine (*Sus scrofa domesticus*) farming adds to the economy and food security of the rural and semi-urban South African households [1]. Lack of knowledge and resources coupled with inadequate biosecurity pose major risk factors that are known to facilitate virus disease outbreaks in backyard swine populations in developing countries [2]. In addition, zoonotic transmission of virus pathogens poses a public health risk [3]. The emergence of the 2009 swine flu pandemic in Mexico is an example of how zoonotic viruses may evolve and trigger the outbreak causing a pandemic and may claim innumerable human lives and decimates swine farming. Apart from IAV, there are several other viruses that may transmit

from swine to humans and cause disease outbreaks. For example, the zoonotic transmission of the Nipah vims (NiV) [4] from the swine to humans at the swine faims in Malaysia resulted in the death of 105 swine fai·m workers during 1998-1999 [5,6]. The occmTence of NiV antibodies in swine populations in Australia [7], Bangladesh [8], Ghana [9], and more recently in Uganda in 2019 [10] suggested a continued circulation and, therefore, a constantly growing risk ofNiV disease outbreak, threatening public safety.

Numerous other notifiable RNA vimses, including Porcine epidemic dianhoea vims (PEDV), an emerging porcine coronavims [11-13], causes clinical signs such as vomiting and dianhoea in neonatal piglets and have been repo1ted to inflict 90-100% piglet mortality, leading to substantial economic losses to the swine fanners [11,14]. Interestingly, PEDV was identified as the cause of death for over eight million neonatal piglets in a single yeai· after its emergence in the United States in 2013 [14]. The Classical swine fever virus (CSFV) [15,16], Porcine reproductive and respirato1y syndrome vims (PRRSV) [17-19], Foot and mouth disease vims (FMDV) [20] and Swine vesicular disease vims (SVDV) [21,22] are other controlled RNA vimses which have caused serious diseases in swine populations in different countries in the recent past.

Rota.vims is another RNA vims that may cause diaiThoea, weight loss, and mo1tality in piglets [23]. Rotavims infection in piglets in South Africa was first repo1ted in 1977 [24]. Later, a pilot study in 1993 repo1ted the yeai·-round occmTence of Rotavims A in dianhoeic piglets at a swine fa1m located in No1thern Transvaal [25]. Subsequently, the Rota.virus types A, B, and C were reported in swine populations from vai-ious South African provinces in 1996 [26]. Furthe1more, FMDV-associated m01tality in commercial swine was first reported in September 2000 in the Camperdown district of KwaZulu-Natal province [27]. The first repo1ted CSFV disease outbreak occmTed in South African swine in June 2005 at a commercial pigge1y in the Western Cape province and repo1ted a high mortality rate [28]; the CSFV outbreak led to the culling of over 335,000 swine resulting in a significant production loss to the swine industry in South Africa [29]. Recently, the Hepatitis E vims (HEV) was repo1ted in commercial and communal swine faims located in Chris Hani and Amathole Disti·ict Municipalities of the Eastern Cape province [30]. Owing to the risk of zoonotic ti·ansmission ofHEV to the exposed human populations, maintenance of high hygienic standards at these swine faims and proper cooking of pork meat before consumption were recommended [30].

It is noteworthy that while the above virus pathogens were reported from the commercial swine populations in South Africa, to our knowledge, there is no study of viruses in backyard swine populations in the country. Since some of these virus pathogens may inflict severe disease in the swine, unraveling their prevalence and diversity in South African backyard swine is crucial to mitigate the risk of disease outbreaks in resource-scarce rural communities. Therefore, we conducted a study to determine the diversity of the swine oral RNA virome at three distantly located backyard swine farms (BSFs) in the uMgungundlovu District of KwaZulu-Natal province of South Africa. This study enabled us to review the biosecurity standards employed at these backyard swine farms and will serve as a preliminary reference for the stakeholders to evaluate the disease outbreak risk at these backyard swine farms. This information, therefore, would assist the stakeholders in preparing a strategy to mitigate the interspecies transmission of the currently occurring virus pathogens in South African backyard swine to ensure public safety and sustainable backyard farming.

Materials and Methods

Sample processing

We used three of the backyard swine oral secretion (saliva) samples initially collected for influenza A virus (IAV) detection in March 2021 from three distantly located backyard swine farms in the uMgungundlovu District of KwaZulu-Natal province, South Africa. Our strategy was to obtain the saliva samples primarily from those swine that had clinical signs of influenza-like illness or any other apparent disease symptoms. In cases where the swine under investigation did not exhibit any signs of illness and therefore appeared healthy, we sampled them as well.

The backyard swine under investigation were visually screened with the assistance of an Animal Health Technician (AHT) from the State Veterinary Department, uMgungundlovu District. The first backyard swine farm (BSF1) had only been established a year prior to the commencement of this study and had apparently healthy sows and piglets with no clinical sign of disease. The second backyard swine farm (BSF2) was more than four years old and had healthy sows, although some of the piglets exhibited influenza-like illness, including coughing and sneezing. The third backyard swine farm (BSF3) was also more than four years old and had healthy sows and piglets, although some of the grower pigs had red patches on their skin.

For deep sequencing, where available, we chose pooled saliva samples obtained from the piglets and growers confined in multiple numbers within barns with clinical signs of illness to get a better assessment of the diversity of their oral RNA virome. However, the saliva sample chosen from the BSF1 belonged to one of the healthy adult pigs kept in separate pens, as there was insufficient volume of saliva available from the pooled piglet samples.

For metagenomic analysis, the saliva samples were centrifuged at 1500 g for 10 min at 4℃ to eliminate any feed contaminants and stored at -80^{0}C. The saliva samples were shipped on dry ice to the Biotechnology Platform Laboratory of Agricultural Research Council (ARC), Onderstepoort, Pretoria, South Africa, for deep sequencing using the Illumina HiSeq X platform.

Genome assembly

The fastq files containing 125 bp long paired-end raw sequencing reads were analysed using Genome Detective v 1.135 (www.genomedetective.com), with default settings, for quality check and assembly. The Genome Detective v 1.135 runs a *de novo* assembly and verifies the assembled contigs using reference genomes available in the NCBI database to determine percent genome coverage and percent nucleotide and amino acid identities. A total of 21,376,716 raw reads were generated for the first saliva sample; 487,604 (2%) reads containing adapters and low-quality reads were removed. This resulted in 20,889,112 reads processed for viral genome contig assembly, out of which 20,820,604 (99%) reads did not match viral sequences available in the NCBI database and were removed. The remaining 68,508 reads were assembled into viral contigs using *de novo* assembly. The second saliva sample generated 21,036,832 raw reads, out of which 403,012 (1%) reads containing adapters and low-quality reads were removed. Total 20, 633,820 reads were processed for viral contig assembly; a total of 20,245,000 (98%) reads did not match viral sequences in the NCBI database; hence were removed. The remaining 388,820 reads were assembled into viral contigs using *de novo* assembly. The third saliva sample generated 7,953,568 raw reads, out of which 156,5304 (19%) reads containing adapters and low-quality reads were removed. Total 6,388,264 reads were processed for viral contig assembly; 6,212,686 (97%) reads that did not match viral sequences and were removed. The remaining 175,578 reads were processed for virus contig *de novo* assembly. We then manually performed nucleotide BLAST analysis to verify the virus contigs obtained in the stud. Later, we manually analysed all the virus contigs in 'Geneious Prime 2021.2.2' software for the determination of insertions and deletions compared to the reference genome.

Phylogenetic analysis

Nucleotide sequences were aligned using the MUSCLE algorithm in MEGA-X software [31] to find the best model for the phylogenetic analysis. The GTR+G+I model was used to construct Maximum Likelihood (ML) trees using PhyML [32] in 'Geneious Prime 2021.2.2'. Bootstrap analysis was performed using 1000 bootstrap replications. Phylogenetic analyses were conducted for the virus genomes with complete or near-complete coding sequences.

Results

The RNA fraction of the backyard swine saliva samples (n=3) generated either near full-length (Table 1) or partial genomes, which mostly belonged to swine enteric viruses; however, Hepatitis E virus (HEV), three species of plant viruses, two species of fungal viruses, and five species of unclassified viruses were also identified (**Fig. 1**).

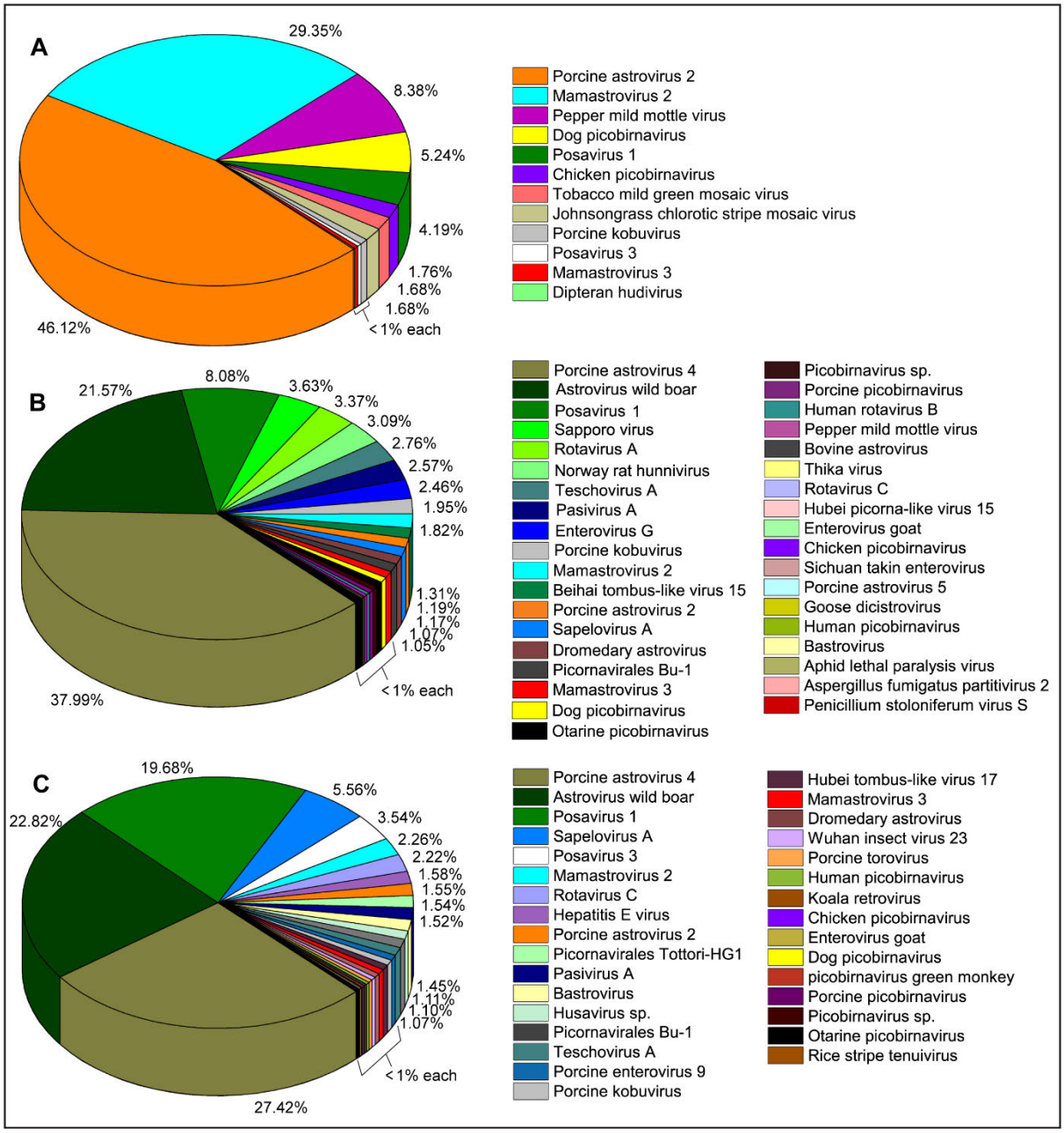

Fig 1: Prevalence (# reads) of RNA viruses in backyard swine saliva samples. Viruses of the family *Astroviridae* were prevalent in the backyard swine saliva samples. (**A**) A higher prevalence of Porcine astrovirus type-2 and Mamastrovirus type-2 was detected in a healthy adult swine at the BSF1. (**B**) There was a higher prevalence of Porcine astrovirus type-4 (PAstV-4) in piglets with clinical signs of coughing and sneezing at the BSF2. (**C**) A higher prevalence of PAstV-4 was identified in grower pigs that had red patches on the skin at the BSF3. Higher diversity of RNA viruses was determined in saliva obtained from piglets and growers with clinical signs of disease at BSF2 and BSF3, respectively. In contrast, only a few RNA viruses were identified in a saliva sample obtained from a healthy adult swine at BSF1.

Table 1: Near full-length genomes retrieved from backyard swine saliva samples

Virus ID	Near full-length genomes retrieved- percent coverage		
	BSF1	**BSF2**	**BSF3**
Hepatitis E virus	-	-	97.7
Mamastrovirus 2	-	-	98.4
Mamastrovirus 3	-	97.9	-
Norway rat hunnivirus	-	97.6	-
Pasivirus A	-	99.3	96.3
Picornavirales Tottori-HG1	-	-	99.2
Pepper mild mottle virus	98.0	-	-
Porcine astrovirus 2	99.3	-	-
Porcine bastrovirus	-	-	97.3
Porcine enterovirus 9	-	99.3	-
Porcine enteric sapovirus	-	99.5	-
Posavirus 1	-	99.2	99.2
Posavirus 3	-	-	99.5
Sapelovirus A	-	98.9	98.9
Teschovirus A	-	98.3	-

BSF= Backyard swine farm

Identification and characterization of swine viruses

Family *Astroviridae*

Viruses of the family *Astroviridae* were prevalent in all three backyard swine saliva samples. While Porcine astrovirus type-2 (PAstV-2) generated the maximum number of reads (46.12%) in a saliva sample obtained from BSF1, the reads of Porcine astrovirus type-4 (PAstV-4) were prevalent in the other two saliva samples obtained from BSF2 (37.99%) and BSF3 (27.42%). Two contigs of PAstV-2 were generated from BSF1, one of which generated

a complete coding sequence (99.3% genome coverage; 6364 nt). In addition, partial coding sequences of PAstV-2 were also identified in BSF2 (82.1% genome coverage; 5185 nt) and BSF3 (70% genome coverage; 4421nt). Complete coding sequences for ORF1ab and ORF1a and partial coding sequences of ORF2 of the PAstV-4 were generated from BSF2 (96.2% genome coverage; 6384 nt) and BSF3 (77% genome coverage; 5111 nt). PAstV-4 was not identified at the BSF1. While the maximum likelihood (ML) phylogenetic tree determined the independent origins of PAstV-2 sequences obtained from the BSF2 and BSF3, the PAstV-2 sequences from BSF1 clustered with Asian sequences reported from Japan and China. Similarly, the genome sequences of PAstV-4 obtained from the BSF2 and BSF3 were determined to be independently originated; however, they appeared to be more closely related to the Chinese sequences than the North American sequences (**Fig. 2**).

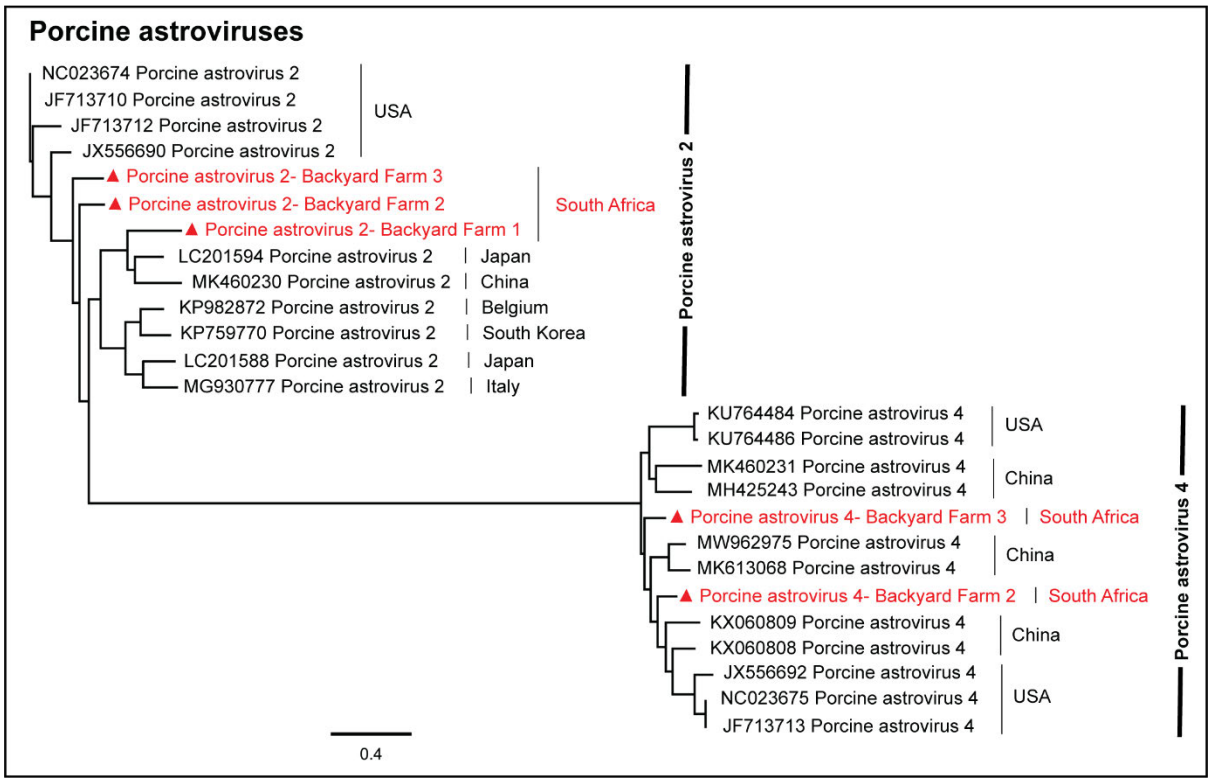

Fig 2: The maximum likelihood phylogenetic tree of Porcine astroviruses. PAstV-2 genome sequences retrieved from South African backyard swine clustered with the Eurasian sequences, while the PAstV-4 sequences were more closely related to the PAstV-4 sequences reported from the swine in China.

While a complete coding sequence of Mamastrovirus 3 (97.9% genome coverage; 6567 nt) was generated from the BSF2, only partial sequences of Mamastrovirus 2 could be generated

from BSF1 (54.1% genome coverage; 3432 nt) and BSF2 (77.5% genome coverage; 4920 nt). A complete coding sequence of Porcine bastrovirus (97.4% genome coverage; 5854 nt) was obtained from the BSF3. While Mamastrovirus 2 sequences obtained from the BSF3 appeared to be more closely related to the African sequences reported from the Kenyan swine, the Mamastrovirus 3 sequences obtained from the BSF2 appeared to be more closely related to the Chinese sequences (**Fig. 3**).

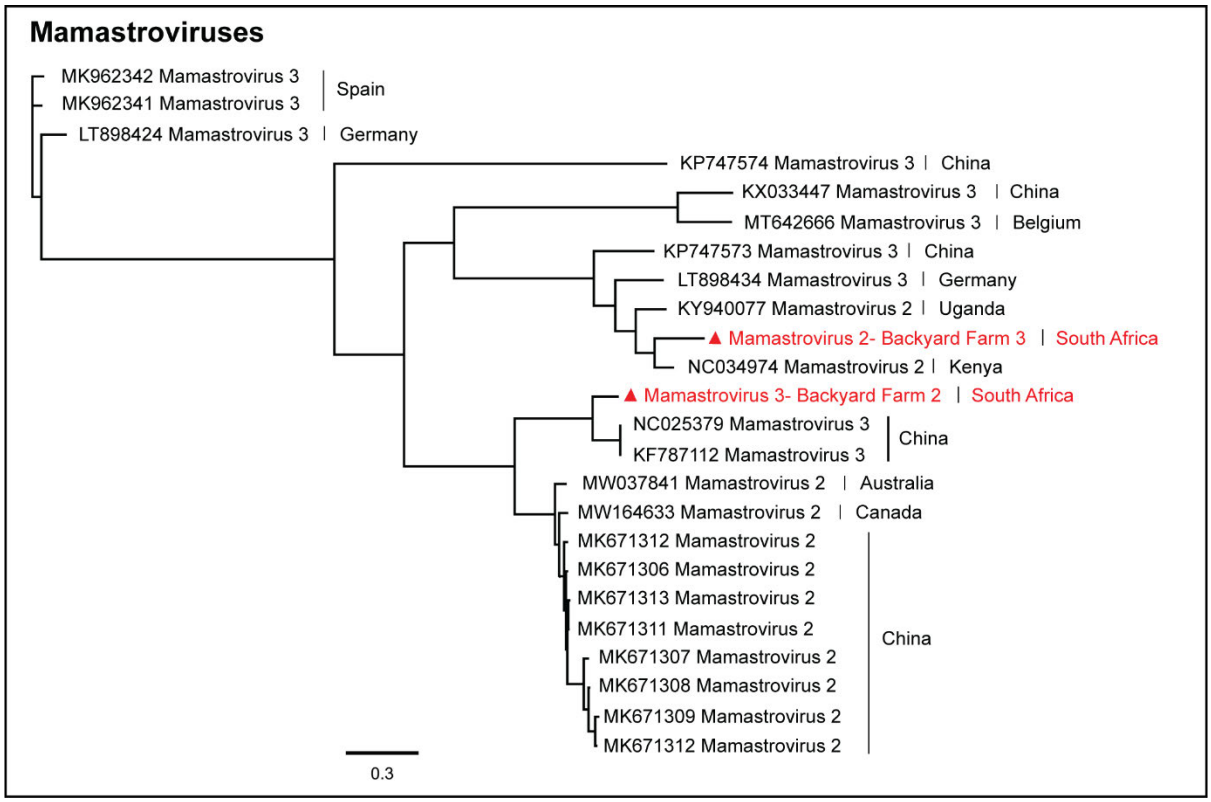

Fig 3: The maximum likelihood phylogenetic tree of Mamastroviruses. The Mamastrovirus 2 sequences obtained from the BSF3 appeared to be more closely related to the Mamastrovirus 2 sequences reported from swine in Kenya. Mamastrovirus 3 sequences obtained from the BSF2 appeared to be more closely related to the Chinese sequences.

One near full-length genome of Porcine bastrovirus (97.4% genome coverage; 5854 nt) was generated from a saliva sample obtained from the BSF3. The South African genome sequences of Porcine bastrovirus appeared to be closely related to the Japanese Porcine bastrovirus sequences (**Fig. 4**).

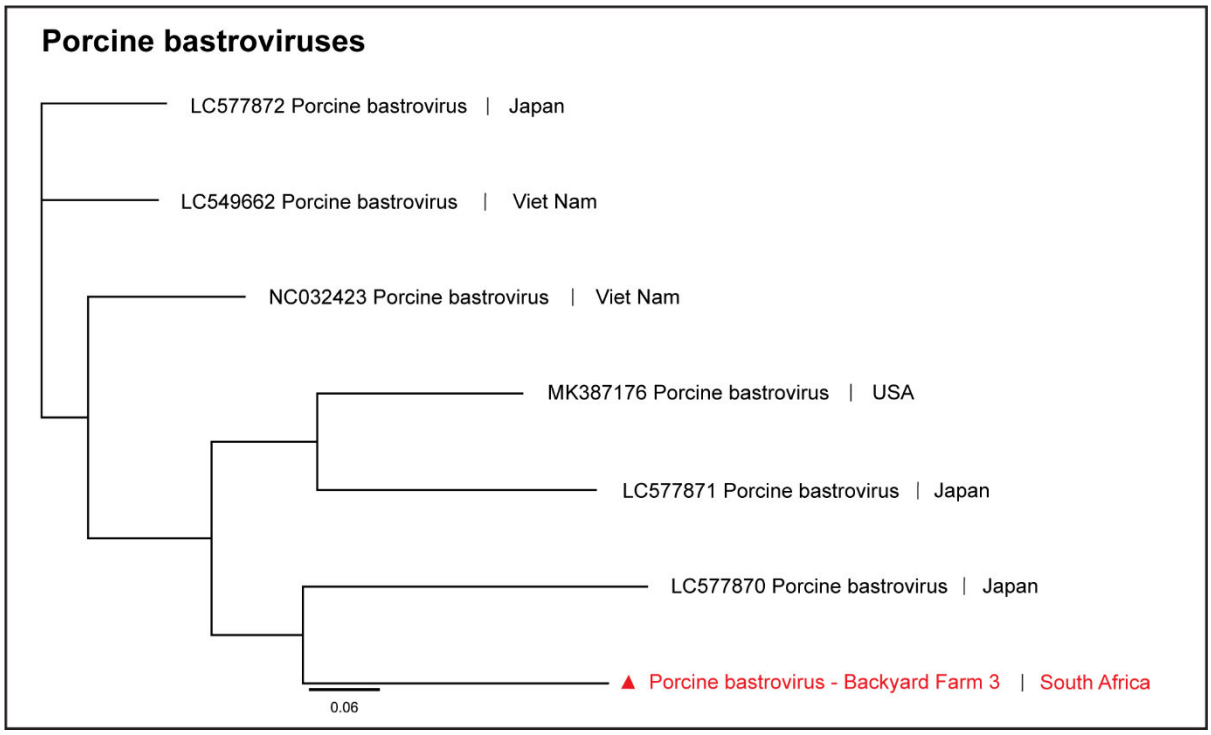

Fig 4: The maximum likelihood phylogenetic tree of Porcine bastroviruses. Porcine bastrovirus sequences generated from a saliva sample of South African backyard swine at the BSF3 clustered with the Japanese Porcine bastrovirus sequence.

In addition, complete coding regions for ORF1ab and ORF1b and the partial coding region for ORF2 of Astrovirus wild boar were generated from BSF2 (92.7% genome coverage; 6219 nt) and BSF3 (87% genome coverage; 5833 nt). Partial genome sequences of Dromedary astrovirus (89.7% genome coverage; 5674 nt) and Bovine astrovirus (29.3% genome coverage; 1829 nt) in a saliva sample obtained from BSF2 and Dromedary astrovirus in a saliva sample obtained from BSF3 (47.1% genome coverage; 2979 nt) were also generated.

Family *Hepeviridae*

A near full-length genome sequence (97.7% genome coverage; 7011 nt) of a Hepatitis E virus (HEV) was obtained from the BSF3. This genome had a complete coding sequence for hypothetical protein and capsid protein and a partial coding sequence for the nonstructural protein. The HEV genome obtained from the South African backyard swine clustered with other HEV genomes reported from Asia and North America (**Fig. 5**).

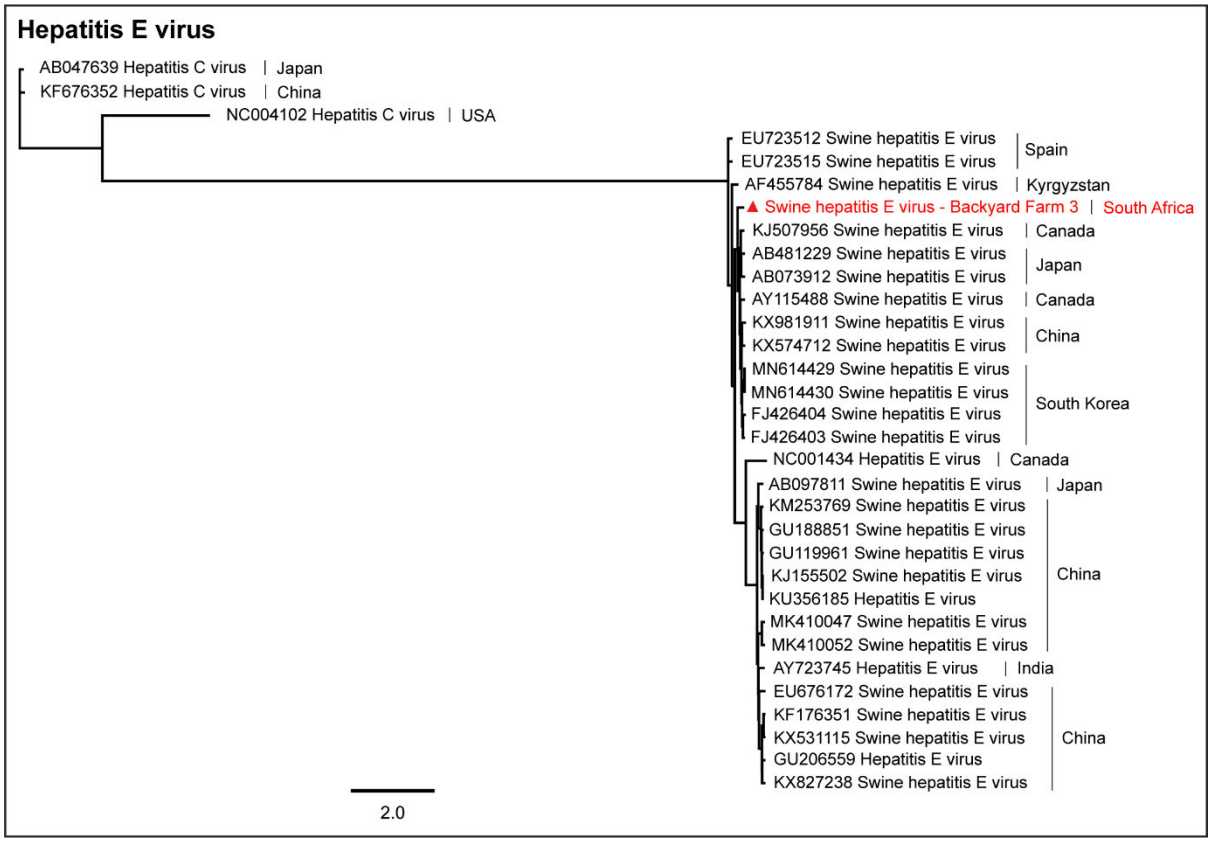

Fig 5: The maximum likelihood phylogenetic analysis of the Hepatitis E virus genome obtained from the backyard swine at the BSF3 in South Africa.

Family *Picornaviridae*

A complete coding sequence of Porcine kobuvirus (92.8% genome coverage; 7618 nt) was obtained from the South African backyard swine at BSF2, which appeared to have a Chinese origin. In addition, partial coding sequences of Porcine kobuvirus (25.8% – 54.8% genome coverage) were generated from the BSF1 and BSF3 (**Fig. 6A**). While the complete coding sequence of Pasivirus A (99.4% genome coverage; 6872 nt) was generated from the BSF2, a near-complete coding sequence of Pasivirus A (96.3% genome coverage; 6663 nt) was generated from the BSF3. These sequences were determined to be related to the Pasivirus A sequences reported from Germany (**Fig. 6B**). The partial coding sequence of Porcine enterovirus 9 (87.8% genome coverage; 6485 nt) was obtained from the BSF3, a complete coding sequence (99.4% genome coverage; 7347 nt) was obtained from the BSF2, which appeared to be evolved from the Chinese sequences (**Fig. 6C**). The Partial coding sequences of Enterovirus goat (31.6% genome coverage; 2354 nt and 37.2% genome coverage; 2779 nt) were generated from the BSF2 and BSF3, respectively. Partial coding sequences of Sichuan

takin enterovirus (32.3% genome coverage; 2104 nt and 43.5% genome coverage; 2838 nt) were generated from the BSF2 and BSF3, respectively. While the complete coding sequence of Porcine teschovirus A (98.4% genome coverage; 7001 nt) was generated from BSF2, partial sequences (97.3% genome coverage; 6922 nt) were obtained from the BSF3. These sequences appeared to have evolved independently of each other and were grouped into separate clusters (**Fig. 6D**). The complete coding sequence of Sapelovirus A (99.0% genome coverage; 7418 nt and 99.1% genome coverage; 7422 nt) was generated from the BSF2 and BSF3, respectively. These sequences were grouped into a cluster with the sequences reported from Germany and Zambia (**Fig. 6E**). The complete coding sequence of Norway rat hunnivirus (97.8% genome coverage; 7333 nt) was generated from the BSF2. It was intriguing to note that the Norway rat hunnivirus genome obtained from the South African backyard swine had significantly low nucleotide (50.5%) and amino acid (39.9%) identities with the reference genome in the complete coding sequence. Additionally, we observed an unusually high frequency of insertions and deletions in the Norway rat hunnivirus genome which requires further investigation for its full characterization to determine if this is a potentially novel virus. The maximum likelihood phylogenetic analysis determined that the South African genome of the Norway rat hunnivirus diverged from the Chinese genome (**Fig. 6F**).

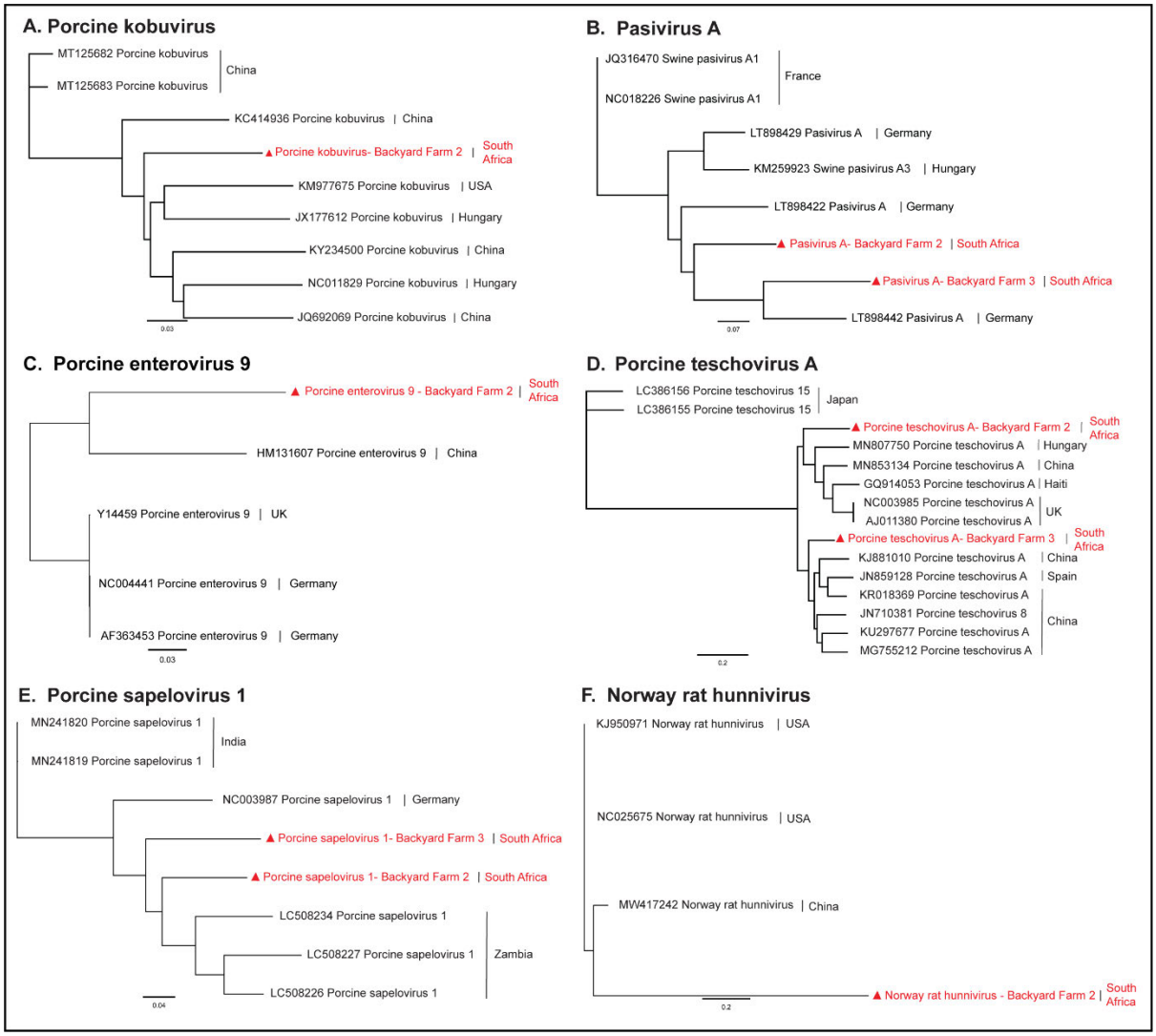

Fig 6: Maximum likelihood phylogenetic analysis of viruses of the family *Picornaviridae*. (**A**) South African sequences of Porcine kobuvirus appeared to have evolved independently. (**B**) South African sequences of Pasivirus A appeared to be closely related to the sequences reported from Germany. (**C**) South African Porcine enterovirus 9 was observed to be related to the Chinese sequences. (**D**) Porcine teschovirus A sequences obtained from South African backyard swine at the BSF2 and BSF3 clustered into different groups suggesting independent origins. (**E**) South African sequences of Porcine sapelovirus 1 grouped into the cluster of the Sapelovirus 1 sequences reported from Germany and Zambia. (**F**) Norway rat hunnivirus sequences obtained from the South African backyard swine appeared to have diverged from the Chinese sequences.

Order *Picornavirales*

Either partial or complete coding sequences of Posavirus 1 (96.1% – 99.2% genome coverage) and Posavirus 3 (21.4% – 99.6% genome coverage) were obtained from all the three BSFs. While the South African Posavirus 1 sequences obtained from the BSF2 and BSF3 appeared to have evolved from the sequences reported from the USA, the Posavirus 1 sequences obtained from the BSF1 appeared to have evolved from the Chinese sequences. South African Posavirus 3 sequences obtained from the BSF3 appeared to have evolved independently (**Fig. 7A**). Partial coding sequences of Picornavirales Bu-1 (93.9% genome coverage; 8658 nt and 93.1% genome coverage; 8584 nt) were obtained from the BSF2 and BSF3, respectively. The complete coding sequence of Picornavirales Tottori-HG1 (99.2% genome coverage; 9788 nt) was generated from the BSF3. Both the Picornavirales Bu-1 and Picornavirales Tottori-HG1 sequences obtained from the South African backyard swine appeared to have evolved from the sequences reported from Japan (**Fig. 7B**).

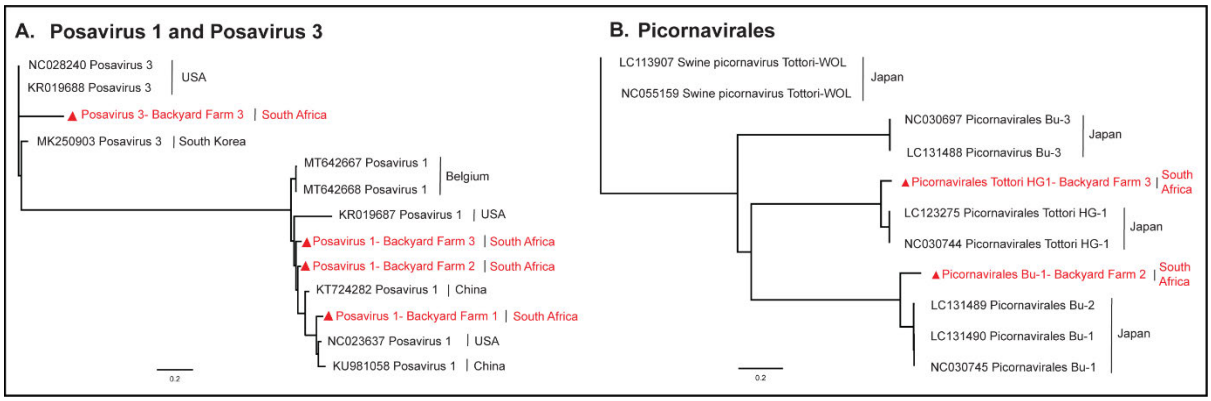

Fig 7: Maximum likelihood phylogenetic analysis of Picornavirales. (**A**) Posavirus 1 sequences from the South African backyard swine appeared to have evolved from North American and Chinese sequences. (**B**) Picornavirales Bu-1 Picornavirales Tottori-HG1 sequences obtained from the South African backyard swine appeared to have evolved from the Japanese sequences.

Family *Reoviridae*

Either partial or complete coding sequences (37.3% – 98.5% genome coverage) of all the eleven genome segments of Porcine rotavirus A were generated from the BSF2. In addition, partial sequences of a few segments of Rotavirus C (19.8% – 50.6% genome coverage) and Human Rotavirus B (35.5% – 75.5% genome coverage) were also generated from the BSF2.

The Porcine rotavirus A sequences obtained from the South African BSF2 appeared to be related to the Porcine rotavirus A sequences reported from Mozambique (**Fig. 8**).

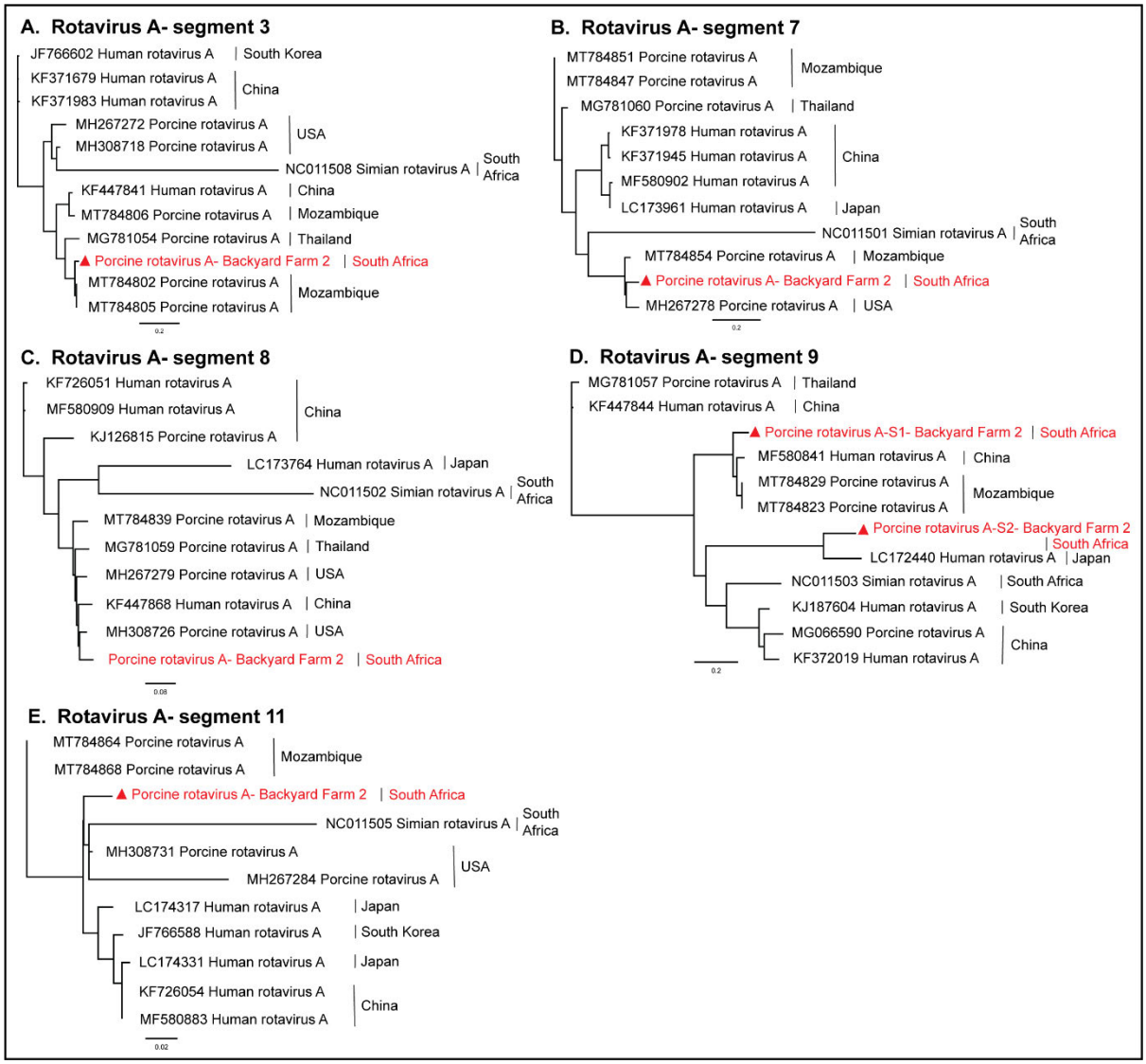

Fig 8: Maximum likelihood phylogenetic analysis of Porcine rotavirus A. (**A-E**) The genome segments of South African Porcine rotavirus A appeared to be related to the Porcine rotavirus A sequences reported from Mozambique.

While Porcine rotavirus A sequences were not identified at the BSF3, either partial or complete coding sequences (42.3% – 99.1% genome coverage) of all the eleven segments of Rotavirus C were generated from the BSF3. While Rotavirus C segments one and four appeared to be related to the sequences reported from the United Kingdom, segment ten appeared to have evolved independently, while segment 11 appeared to be related to the Japanese sequences (**Fig. 9**). This might be due to the presence of multiple strains of

Rotavirus C or the reassortment in the Rotavirus C genome in the backyard swine, which requires further investigation.

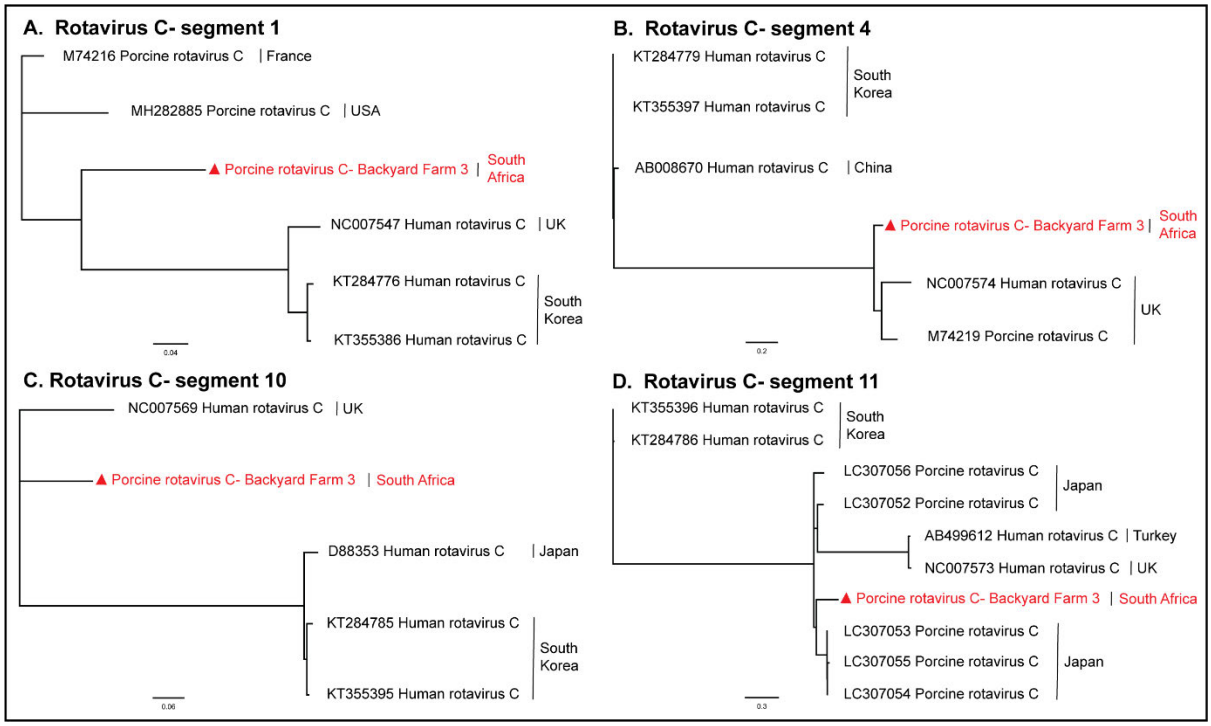

Fig 9: Maximum likelihood phylogenetic analysis of Rotavirus C. (**A-D**) Rotavirus C genome segments 1, 4, 10, and 11 appeared to have different evolutionary trajectories. This might be either due to the circulation of multiple Rotavirus C strains in the backyard swine population or reassortment in Rotavirus C genome in the swine.

Family *Picobirnaviridae*

Either partial or complete coding sequences of Dog picobirnavirus segment 2 (57.5% – 97.2% genome coverage) and Chicken picobirnavirus segment 2 (32.8% – 85.4% genome coverage) were generated from all the three BSFs. The complete coding sequence of Otarine picobirnavirus segment 2 (97.7%) was obtained from the BSF2. Partial or near-complete coding sequences of Roe deer picobirnavirus segment 2 (28.7% – 97.1% genome coverage) were obtained from the BSF2 and BSF3. Partial or complete coding sequences of Porcine picobirnavirus segment S (38.3% – 96.8% genome coverage), partial coding sequences of Green monkey picobirnavirus (32.2% – 84.9% genome coverage), and Human picobirnavirus segment 2 (32.7% – 79.9% genome coverage) were obtained from the BSF2 and BSF3 (**Fig. 10**). The presence of these Picobirnaviruses, especially Dog picobirnavirus, Chicken picobirnavirus, Green monkey picobirnavirus, Roe deer picobirnavirus, and Feline (Cat)

picobirnavirus in saliva samples of South African backyard swine suggested the possible interaction of the backyard swine with the hosts of these Picobirnavirus sp.

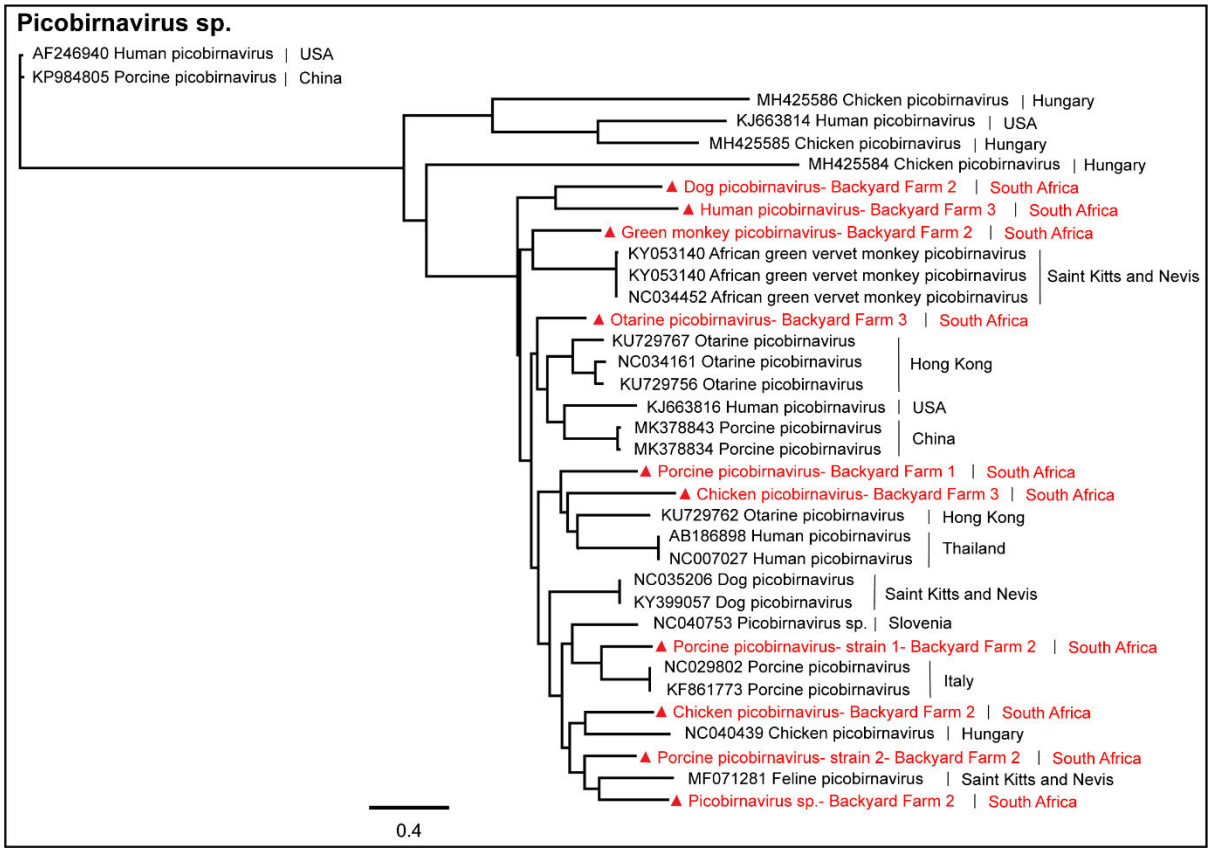

Fig 10: Maximum likelihood phylogenetic analysis of Picobirnavirus sp. The presence of several Picobirnaviruses, including, Porcine picobirnavirus, Dog picobirnavirus, Human picobirnavirus, Chicken picobirnavirus, Green monkey picobirnavirus, Otarine picobirnavirus, and Feline (Cat) picobirnavirus in saliva samples of South African backyard swine suggested active circulation of these viruses in the backyard swine farms.

Family *Caliciviridae*

A near-complete coding sequence of Porcine enteric sapovirus (99.5% genome coverage; 7284 nt) was obtained from the BSF2 (**Fig. 11**). Porcine enteric sapovirus sequences were not observed in other backyard swine samples under investigation.

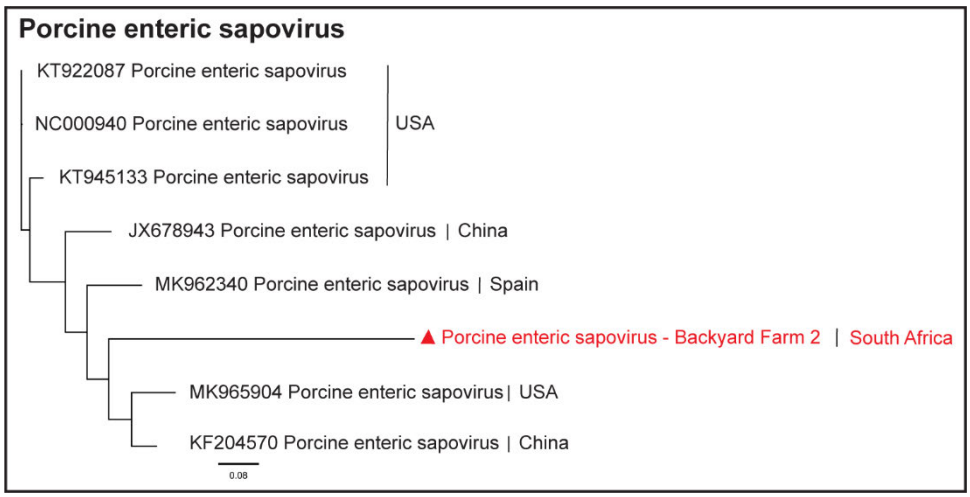

Fig 11: Maximum likelihood tree of Porcine enteric sapovirus suggested that the South African sequence obtained from the backyard swine might have evolved from the European sequences.

Family *Virgaviridae*

Genome sequences of plant viruses were also obtained from the BSF1 and BSF2. Near-complete coding sequences of Pepper mild mottle virus (98.0% genome coverage; 6233 nt and 88.6% genome coverage; 5632 nt) were obtained from the BSF1 and BSF2, respectively. A single contig of Pepper mild mottle virus obtained from the saliva of the backyard swine at the BSF1 appeared to be related to the sequences reported from China (**Fig. 12**). In addition, partial coding sequences of the Tobacco mild green mosaic virus (80.4% genome coverage; 5108 nt and 17.1% genome coverage; 1084 nt) were generated from the BSF1 and BSF2, respectively.

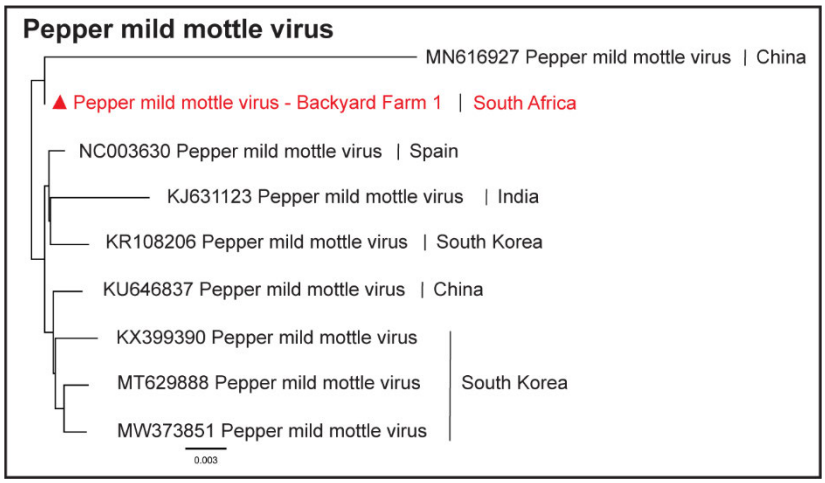

Fig 12: Maximum likelihood phylogenetic tree of Pepper mild mottle virus suggested that South African sequences obtained from the saliva sample of the backyard swine at the BSF1 were related to the Chinese sequences.

Family *Phenuiviridae*

A partial coding sequence of Dipteran hudivirus segment 3 (47.9% genome coverage; 592 nt) was generated from the BSF1. A partial coding sequence of Rice stripe tenuivirus (32.9% genome coverage; 709 nt) was obtained from the BSF3.

Family *Dicistroviridae*

The partial coding sequence of Goose dicistrovirus (39.8% genome coverage; 3636 nt) and Aphid lethal paralysis virus (20.5% genome coverage; 2007 nt) were obtained from the BSF2.

Family *Partitiviridae*

The partial coding sequences of Aspergillus fumigatus partitivirus 2 (55.6% genome coverage; 1013 nt) and Penicillium stoloniferum virus S (20.3% genome coverage; 356 nt) were generated from the BSF2.

Family *Tobaniviridae*

A partial coding sequence of Porcine torovirus (33.7% genome coverage; 9528 nt) was obtained from the BSF3.

Family *Retroviridae*

A partial coding sequence of Koala retrovirus (22.2% genome coverage; 1869 nt) was obtained from the BSF3.

Family *Tombusviridae*

The partial coding sequence of Johnsongrass chlorotic stripe mosaic virus (93.0% genome coverage; 4112 nt) was obtained from the BSF1.

Unclassified RNA viruses

The partial coding sequences of five unclassified RNA viruses, including Beihai tombus-like virus 15 (40.9% genome coverage; 1361 nt), Thika virus (39.6% genome coverage; 3603 nt), and Hubei picorna-like virus 15 (39.6% genome coverage; 3926 nt) were generated from the

BSF2. Partial coding sequences of Hubei tombus-like virus 17 (49.9% genome coverage; 1854 nt) and Wuhan insect virus 23 (26.2% genome coverage; 387 nt) were obtained from the BSF3. Overall, the present study identified high diversity of swine oral virome in backyard swine saliva samples under investigation (**Table 2**).

Accession numbers

The virus genome sequences generated in this study have been deposited to GenBank. The accession numbers are the following:

OM104029, OM104030, OM104031, OM104032, OM104033, OM104034, OM104035, OM104036, OM104037, OM104038, OM104039, OM104040, OM104986, OM104987, OM104988, OM104989, OM104990, OM104991, OM104992, OM104993, OM104994, OM104995, OM104996, OM104997, OM104998, OM104999, OM105000, OM105001, OM105002, OM105003, OM105004, OM105005, OM105006, OM105007, OM105008, OM105009, OM105010, OM105011, OM105012, OM105013, OM105014, OM105015, OM105016, OM105017, OM105018, OM105019, OM105020, OM105021, OM105022, OM105023, OM105024, OM105025, OM105026, OM105027, OM105028, OM105029, OM105030, OM105031, OM105032, OM105033, OM105034, OM105035, OM105036, OM105037, OM105038, OM105039, OM105040, OM105041, OM105042, OM105043, OM105044, OM105045, OM105046, OM105047, OM105048, OM105049, OM966657, OM966722, OM966723

427 **Table 2:** Overview of virus genome sequences generated from backyard swine saliva samples

Virus ID (Backyard Farm ID)	**Genome characterization**						
	#Contigs	**Percent coverage**	**Reference genome**	**Percent identity**	**#Nucleotides (annotation)**	**Insertion (amino acid position)**	**Deletion (amino acid position)**
Family *Astroviridae*							
Porcine astrovirus type-2- contig 1 (BSF1)	1	99.3	NC023674	nt (70.1) aa (75.6)	6364 nt (complete coding regions for ORF1ab, ORF1a, ORF2)	**ORF1a:** K (396) **ORF2:** Q (8), S (286), I (315), N (366), S (401), Y (422), HG (482), GNGY (539), DVVT (626), E (639), PPPSEE (653), EASNFYDFDSDL (665)	**ORF1a:** I (626) **ORF2:** S (36), R (42), A (43), G (435), L (489)
Porcine astrovirus type-2- contig 2 (BSF1)	1	65.4	NC023674	nt (80.8) aa (88.9)	4130 nt (complete coding regions for ORF 1ab and ORF 1a, partial coding region for ORF2)	None	**ORF1ab:** L (1281), A (1282)
Porcine astrovirus type-2 (BSF2)	7	82.1	NC023674	nt (79.1) aa (85.9)	5185 nt (complete coding region for ORF1ab, partial coding regions for ORF1a, ORF2)	**ORF2:** V (535), ECS (540), VS (629), PPPED (653), 665 (E),	None
Porcine astrovirus type-4 (BSF2)	4	96.2	NC023675	nt (80.9) aa (84.0)	6384 nt (complete coding regions for ORF1ab and ORF1a, partial coding region for ORF2)	None	**ORF1a:** T (603)

Astrovirus wild boar (BSF2)	7	92.7	NC016896	nt (81.5) aa (81.5)	6219 nt (complete coding regions for ORF1a and ORF1b, partial coding region for ORF2)	**ORF1a:** LV (226) **ORF2:** E (756)	**ORF1a:** H (214), R (233), R (234) G (235), G (236) **ORF1b:** T (398) **ORF2:** G (718), A (721)
Dromedary astrovirus (BSF2)	2	89.7	NC027711	nt (74.0) aa (80.4)	5674 nt (complete coding regions for ORF1a and ORFX, partial coding regions for ORF1ab and ORF2)	**ORF2:** P (9), T (401), Q (491), M (505), HNNN (542), E (554), GT (625), INA (638), PEEET (671)	**ORF2:** T (426)
Porcine astrovirus type-4 (BSF3)	4	77.0	NC023675	nt (85.2) aa (87.3)	5111 nt (complete coding regions for ORF1ab and ORF1a, partial coding region for ORF2)	None	None
Porcine astrovirus type-2 (BSF3)	8	70.0	NC023674	nt (80.7) aa (85.5)	4421 nt (complete coding region for ORF1ab, partial coding regions for ORF1a, ORF2)	ORF2: NNYS (540)	None
Astrovirus wild boar (BSF3)	4	87.0	NC016896	nt (83.5) aa (84.5)	5833 nt (complete coding regions for ORF1a and ORF1b, partial coding region for ORF2)	**ORF1a:** LM (226),	**ORF1a:** R (233), R (234) G (235), G (236) **ORF1b:** T (398) **ORF2:** L (708)
Dromedary astrovirus (BSF3)	2	47.1	NC027711	nt (79.4) aa (85.7)	2979 nt (complete coding region for ORF1a, partial coding region for ORF1ab)	None	None

Bovine astrovirus (BSF2)	1	29.3	NC024297	nt (62.2) aa (58.8)	1829 nt (partial coding sequence for capsid protein precursor)	**Capsid protein:** S (304), T (422), ADTNH (437), RADG (655), SFGC (665), L (679)	**Capsid protein:** P (387)
Mamastrovirus 2 (BSF1)	1	54.1	NC034974	nt (75.1) aa (74.5)	3432 nt (complete coding sequence for capsid protein, partial coding sequence for RdRp)	**Capsid protein:** N (365), K (454), S (515), TNSS (547), NT (601)	**Capsid protein:** S (10), R (42), T (43), P (319), A (342), V (374), Q (375), T (408), P (409), P (410), G (411), N (449), G (458), L (564), Y (565), G (619), L (620), T (642), T (643), L (644), R (645), V (646), G (647)
Mamastrovirus 2 (BSF2)	4	77.5	NC034974	nt (80.4) aa (84.1)	4920 nt (complete coding sequence for RdRp, partial coding sequence for capsid and nonstructural proteins)	None	**Capsid protein:** T (43)
Mamastrovirus 3 (BSF2)	1	97.9	NC025379	nt (84.6) aa (91.4)	6567 nt (complete coding sequence)	**Capsid protein:** D (643)	**Capsid protein:** A (662), S (663)
Porcine bastrovirus (BSF3)	1	97.4	NC032423	nt (81.6) aa (91.8)	5854 nt (complete coding sequence)	**Structural polyprotein:** KQWN (464)	**Structural polyprotein:** I (433), P (434), S (643), A (651), G (652), G (653)
Family *Hepeviridae*							

Hepatitis E virus (BSF3)	1	97.7	NC001434	nt (74.3) aa (84.8)	7011 nt (complete coding sequence for hypothetical protein and capsid protein, partial coding sequence for nonstructural protein)	**Nonstructural protein:** IWV (739), VDVV (748), PSLA (759)	**Nonstructural protein:** A (775) **Hypothetical protein:** R (4)
Family *Reoviridae*							
Rotavirus A (BSF2)	3	96.1	NC011507	nt (78.9) aa (90.0)	Segment 1 (VP1): 3173 nt- near complete coding sequence	None	None
	2	78.5	NC011506	nt (80.3) aa (91.4)	Segment 2 (VP2): 2114 nt- partial coding sequence	DVTMES (29), KE (340),	None
	1	98.5	NC011508	nt (76.4) aa (83.5)	Segment 3 (VP3): 2552 nt- complete coding sequence	None	None
	3	62.7	NC011510	nt (73.5) aa (79.8)	Segment 4 (Outer capsid spike): 1482 nt- partial coding sequence	None	S (603), S (604), V (605)
	1	37.3	NC011500	nt (64.4) aa (52.0)	Segment 5 (NSP1): 602 nt- partial coding sequence	None	E (152)
	2	75.1	NC011509	nt (79.9) aa (90.9)	Segment 6 (VP6): 1018 nt- partial coding sequence	None	None
	1	95.5	NC011501	nt (75.0) aa (77.9)	Segment 7 (NSP3): 1055 nt- near complete coding sequence	None	G (313), C (314)

	1	93.5	NC011502	nt (79.1) aa (86.5)	Segment 8 (NSP2): 990 nt- complete coding sequence	None	None
	1	96.5	NC011503	nt (78.7) aa (85.9)	Segment 9 (VP7): 1024 nt- complete coding sequence	None	Y (175)
	1	64.6	NC011504	nt (78.4) aa (81.4)	Segment 10 (NSP4): 485 nt- partial coding sequence	None	None
	1	88.2	NC011505	nt (88.9) aa (90.9)	Segment 11 (NSP5 and NSP6): 588 nt- complete coding sequence for NSP6, near-complete coding sequence for NSP5	None	NSP5: N (140)
Rotavirus C (BSF2)	2	38.6	NC007547	nt (85.9) aa (93.4)	Segment 1 (VP1): 1277 nt- partial coding sequence	None	None
	2	19.8	NC007546	nt (85.8) aa (95.9)	Segment 2 (VP2): 542 nt- partial coding sequence	None	None
	2	50.6	NC007574	nt (82.4) aa (85.8)	Segment 4 (VP3): 1095 nt- partial coding sequence	None	None
	2	44.7	NC007543	nt (75.8) aa (72.5)	Segment 6 (NSP3): 603 nt- partial coding sequence	None	None

	1	23.8	NC007544	nt (67.9) aa (61.4)	Segment 7 (NSP1): 302 nt- partial coding sequence	None	None
	1	27.7	NC007571	nt (83.7) aa (83.7)	Segment 8 (VP7): 294 nt- partial coding sequence	None	None
Rotavirus C (BSF3)	1	99.1	NC007547	nt (85.1) aa (90.6)	Segment 1 (VP1): 3279 nt- near complete coding sequence	None	None
	2	79.3	NC007546	nt (82.1) aa (90.9)	Segment 2 (VP2): 2170 nt- partial coding sequence	None	None
	2	82.4	NC007572	nt (71.7) aa (71.1)	Segment 3 (VP4): 1882 nt- partial coding sequence	QY (142)	N (132), N (133), P (209), G (210), I (211), N (212), S (253), K (254), L (255), G (256), D (257), S (390), S (392),
	1	97.1	NC007574	nt (81.8) aa (85.6)	Segment 4 (VP3): 2104 nt- near complete coding sequence	None	None
	2	69.0	NC007570	nt (83.7) aa (90.4)	Segment 5 (VP6): 933 nt- partial coding sequence	None	None
	1	69.4	NC007543	nt (78.5) aa (80.8)	Segment 6 (NSP3): 937 nt- partial coding sequence	None	None
	1	80.6	NC007544	nt (65.5)	Segment 7 (NSP1): 1023 nt- partial coding sequence	None	None

				aa (59.2)			
	2	61.0	NC007571	nt (74.2) aa (76.5)	Segment 8 (VP7): 648 nt-partial coding sequence	SSTL (244)	None
	1	42.3	NC007545	nt (80.4) aa (80.3)	Segment 9 (NSP2): 439 nt-partial coding sequence	None	None
	1	85.1	NC007569	nt (74.1) aa (67.8)	Segment 10 (NS26): 621 nt-partial coding sequence	None	T (131), E (132)
	1	70.5	NC007573	nt (65.7) aa (56.9)	Segment 11 (NSP4): 432 nt-near complete coding sequence	None	None
Human rotavirus B (BSF 2)	6	75.5	NC021541	nt (70.7) aa (76.8)	Segment 1 (VP1): 2652 nt-partial coding sequence	None	None
	3	61.5	NC021545	nt (73.1) aa (79.9)	Segment 2 (VP2): 1752 nt-partial coding sequence	R (67)	R (612)
	3	61.3	NC021551	nt (69.6) aa (74.0)	Segment 3 (VP3): 1434 nt-partial coding sequence	None	None
	2	35.5	NC021543	nt (67.2) aa (71.7)	Segment 4 (VP4): 819 nt-partial coding sequence	None	S (315)
	1	59.7	NC021542	nt (61.5) aa (55.3)	Segment 9 (VP7): 486 nt-partial coding sequence	None	H (35)

	1	58.3	NC021550	nt (66.0) aa (69.2)	Segment 10 (NSP4): 438 nt-partial coding sequence	None	None
Family *Picobirnaviridae*							
Dog picobirnavirus (Segment 2)-Contig 1 (BSF1)	1	94.4	NC035206	nt (62.9) aa (66.3)	Segment 2 (RdRp): 1594 nt-near complete coding sequence	E (178), A (531)	T (514), E (515)
Dog picobirnavirus (Segment 2)-Contig 2 (BSF1)	3	57.5	NC035206	nt (68.8) aa (70.1)	Segment 2 (RdRp): 972 nt-partial coding sequence	L (98)	None
Dog picobirnavirus (Segment 2)-Contig 1 (BSF2)	1	97.2	NC035206	nt (67.1) aa (68.8)	Segment 2 (RdRp): 1642 nt-complete coding sequence	GG (174), G (439)	None
Dog picobirnavirus (Segment 2)-Contig 1 (BSF3)	2	75.7	NC035206	nt (67.8) aa (69.8)	Segment 2 (RdRp): 1278 nt-partial coding sequence	KR (178)	E (168), V (169), V (170), N (171), E (385)
Chicken picobirnavirus (Segment 2)-Contig 1 (BSF1)	1	85.4	NC040439	nt (57.4) aa (57.8)	Segment 2 (RdRp): 1451 nt-partial coding sequence	MEF (169), KAIFL (317), ELDRT (443), NAPR (515)	K (177), Q (511),

Chicken picobirnavirus (Segment 2)-Contig 2 (BSF1)	2	32.8	NC040439	nt (69.6) aa (72.3)	Segment 2 (RdRp): 557 nt-partial coding sequence	K (177), G (181)	None
Chicken picobirnavirus (Segment 2)-Contig 1 (BSF2)	3	69.1	NC040439	nt (63.6) aa (69.1)	Segment 2 (RdRp): 1175 nt-partial coding sequence	PTAS (162), DV (179)	None
Chicken picobirnavirus (Segment 2)-Contig 1 (BSF3)	1	73.5	NC040439	nt (60.8) aa (63.8)	Segment 2 (RdRp): 1249 nt-partial coding sequence	NNGSSEAARI (171)	Y (165)
Chicken picobirnavirus (Segment 2)-Contig 2 (BSF3)	1	63.6	NC040439	nt (66.9) aa (71.3)	Segment 2 (RdRp): 1082 nt-partial coding sequence	HA (177), G (440)	K (289), D (290)
Otarine picobirnavirus (Segment 2)-(BSF2)	1	97.7	NC034161	nt (68.7) aa (70.0)	Segment 2 (RdRp): 1650 nt-complete coding sequence	H (41)	None
Roe deer picobirnavirus	1	97.1	NC040753	nt (64.3) aa (64.2)	Segment 2 (RdRp): 1671 nt-near complete coding sequence	T (172)	V (10), I (11), A (19), R (534)

(Segment 2)-Contig 1- (BSF2)							
Roe deer picobirnavirus (Segment 2)-Contig 2- (BSF2)	2	43.8	NC040753	nt (61.7) aa (61.4)	Segment 2 (RdRp): 753 partial coding sequence	None	V (10), I (11), K (21), D (335)
Roe deer picobirnavirus (Segment 2)-Contig 3- (BSF2)	2	28.7	NC040753	nt (66.6) aa (70.1)	Segment 2 (RdRp): 494 partial coding sequence	None	None
Roe deer picobirnavirus (Segment 2)-Contig 1 (BSF3)	2	39.0	NC040753	nt (63.7) aa (59.1)	Segment 2 (RdRp): 671 partial coding sequence	YYNYWTLD (18)	None
Roe deer picobirnavirus (Segment 2)-Contig 2- (BSF3)	3	46.8	NC040753	nt (65.5) aa (67.9)	Segment 2 (RdRp): 805 partial coding sequence	None	None
Porcine picobirnavirus (Segment S)-Contig 1 (BSF2)	1	96.8	NC029802	nt (72.1) aa (73.7)	Segment S (RdRp): 1675 nt-complete coding sequence	Q (536)	R (9), S (10), R (11), D (12),

Porcine picobirnavirus (Segment S)-Contig 2 (BSF2)	2	61.1	NC029802	nt (66.1) aa (72.9)	Segment S (RdRp): 1057 nt- partial coding sequence	GNGAS (167)	None
Porcine picobirnavirus (Segment S)-Contig 3 (BSF2)	3	38.3	NC029802	nt (68.7) aa (76.7)	Segment S (RdRp): 748 nt- partial coding sequence	None	None
Porcine picobirnavirus (Segment S)-Contig 1 (BSF3)	1	43.2	NC029802	nt (68.5) aa (76.8)	Segment S (RdRp): 663 nt- partial coding sequence	None	None
Porcine picobirnavirus (Segment S)-Contig 2 (BSF3)	1	40.3	NC029802	nt (70.8) aa (76.0)	Segment S (RdRp): 697 nt- partial coding sequence	None	None
Green monkey picobirnavirus (BSF2)	5	84.9	NC034452	nt (65.1) aa (63.7)	RdRp: 1449 nt- partial coding sequence	None	R (518), R (519), E (520), R (538)
Green monkey picobirnavirus-Contig 1 (BSF3)	2	63.9	NC034452	nt (66.4) aa (65.1)	RdRp: 1091 nt- partial coding sequence	None	A (13), R (14), K (15), F (16)

Green monkey picobirnavirus-Contig 2 (BSF3)	2	61.5	NC034452	nt (65.0) aa (64.2)	RdRp: 1050 nt- partial coding sequence	None	T (294), P (295), S (331)
Green monkey picobirnavirus-Contig 3 (BSF3)	2	32.2	NC034452	nt (65.6) aa (63.6)	RdRp: 550 nt- partial coding sequence		
Human picobirnavirus (Segment 2)-(BSF2)	9	64.9	NC007027	nt (63.8) aa (66.7)	Segment 2 (RdRp): 1133 nt-partial coding sequence	None	None
Human picobirnavirus (Segment 2)-Contig 1 (BSF3)	1	79.9	NC007027	nt (56.9) aa (57.3)	Segment 2 (RdRp): 1395 nt-partial coding sequence	ETNKEKL (41), DKRGVVAIQ (170), D (286), A (314)	W (8), Y (146), I (158), P (159), C (160), A (307)
Human picobirnavirus (Segment 2)-Contig 2 (BSF3)	2	68.7	NC007027	nt (67.1) aa (69.2)	Segment 2 (RdRp): 1199 nt-partial coding sequence	V (288)	None
Human picobirnavirus (Segment 2)-Contig 3 (BSF3)	1	32.7	NC007027	nt (66.2) aa (70.2)	Segment 2 (RdRp): 571 nt-partial coding sequence	T (292)	None
Family *Picornaviridae*							

Porcine kobuvirus (BSF1)	6	25.8	NC016769	nt (89.7) aa (96.3)	2117 nt (partial coding sequence for polyprotein)	None	T (407), Q (408), D (409)
Porcine kobuvirus (BSF2)	1	41.1	NC011829	nt (89.1) aa (96.6)	3375 nt (partial coding sequence for polyprotein)	None	None
Porcine kobuvirus (BSF3)	4	33.0	NC011829	nt (90.6) aa (96.9)	2706 nt (partial coding sequence for polyprotein)	None	None
Porcine kobuvirus (BSF3)	6	54.8	NC016769	nt (88.0) aa (94.5)	4501 nt (partial coding sequence for polyprotein)	None	None
Pasivirus A (BSF2)	1	99.4	NC018226	nt (81.4) aa (88.8)	6872 nt (complete coding sequence)	EG (688)	T (350)
Pasivirus A (BSF3)	1	96.3	NC018226	nt (77.1) aa (81.7)	6663 nt (near complete coding sequence)	HG (688)	P (354)
Enterovirus G (BSF 2)	1	99.4	NC004441	nt (79.6) aa (89.3)	7347 nt (complete coding sequence for polyprotein)	None	None
Enterovirus G (BSF 3)	27	87.8	NC004441	nt (77.7) aa (88.2)	6485 nt (partial coding sequence for polyprotein)	None	None
Enterovirus goat (BSF 2)	4	31.6	NC034267	nt (78.0) aa (90.0)	2354 nt (partial coding sequence for polyprotein)	None	T (496), G (497)

Enterovirus goat (BSF3)	4	37.2	NC034267	nt (73.4) aa (82.2)	2779 nt (partial coding sequence for polyprotein)	None	None
Sichuan takin enterovirus (BSF 2)	5	32.3	NC037654	nt (75.0) aa (84.0)	2104 nt (partial coding sequence for polyprotein)	None	None
Sichuan takin enterovirus (BSF 3)	5	43.5	NC037654	nt (74.0) aa (83.2)	2838 nt (partial coding sequence for polyprotein)	F (837)	E (500), Y (501)
Teschovirus A-Contig 1 (BSF2)	1	98.4	NC003985	nt (83.0) aa (89.7)	7001 nt (complete coding sequence for polyprotein)	P (332)	Q (956)
Teschovirus A-Contig 2 (BSF2)	3	92.0	NC003985	nt (80.8) aa (88.4)	6550 nt (partial coding sequence for polyprotein)	V (328), GT (654), LK (919)	None
Teschovirus A-(BSF3)	5	97.3	NC003985	nt (82.5) aa (90.2)	6922 nt (partial coding sequence for polyprotein)	D (333), M (919)	None
Sapelovirus A (BSF 2)	1	99.0	NC003987	nt (84.4) aa (93.2)	7418 nt (complete coding sequence for polyprotein)	GSTA (132), ASAEQLGPFYPAI (892), A (2081)	None
Sapelovirus A (BSF 3)	1	99.1	NC003987	nt (85.4) aa (94.9)	7422 nt (complete coding sequence for polyprotein)	STAG (133), A (2081)	None

Norway rat hunnivirus (BSF2)	1	97.8	NC025675	nt (50.5) aa (39.9)	7333 nt (complete coding sequence for polyprotein)	Highly diverged	Highly diverged
Order *Picornavirales*							
Posavirus 1 (BSF1)	2	96.1	NC023637	nt (94.8) aa (95.6)	9453 nt (partial coding sequence for polyprotein)	None	S (2610), S (2611), E (2612), K (2613), V (2614), N (2615), G (2616), N (2617), Y (2618), V (2619)
Posavirus 1 (BSF 2)	1	99.2	NC023637	nt (88.6) aa (89.2)	9766 nt (complete coding sequence for polyprotein)	G (2428)	N (2906)
Posavirus 1 (BSF 3)	1	99.2	NC023637	nt (88.6) aa (89.1)	9759 nt (complete coding sequence for polyprotein)	G (2428)	N (2906)
Posavirus 3 (BSF1)	8	24.1	NC028240	nt (80.8) aa (85.8)	2138 nt (partial coding sequence for polyprotein)	None	None
Posavirus 3 (BSF2)	7	21.4	NC028240	nt (80.2) aa (86.6)	1906 nt (partial coding sequence for polyprotein)	None	None
Posavirus 3 (BSF3)	1	99.6	NC028240	nt (81.5) aa (86.9)	8851 nt (near complete coding sequence for polyprotein)	PD (178), M (1107)	S (155), P (156), A (157), S (158), E (159)
Picornavirales Bu-1 (BSF2)	3	93.9	NC030745	nt (87.2) aa (89.9)	8658 nt (partial coding sequence for polyprotein)	H (1160), T (2577)	Q (91)

Picornavirales Bu-1 (BSF3)	3	93.1	NC030745	nt (87.4) aa (90.6)	8584 nt (partial coding sequence for polyprotein)	N (1160)	None
Picornavirales Tottori-HG1 (BSF 3)	1	99.2	NC030744	nt (92.0) aa (95.6)	9788 nt (complete coding sequence for polyprotein)	None	None
Family *Caliciviridae*							
Porcine enteric sapovirus (BSF2)	1	99.5	NC000940	nt (76.8) aa (85.0)	7284 nt (near complete coding sequence for polyprotein and complete coding sequence for small basic protein)	**Polyprotein:** PN (30), R (50)	**Polyprotein:** L (41)
Family *Phenuiviridae*							
Dipteran hudivirus (Segment 3)-BSF1	2	47.9	NC032279	nt (70.4) aa (73.1)	592 nt (partial coding sequence for putative nucleoprotein)	None	None
Rice stripe tenuivirus (BSF 3)	2	32.9	NC003753	nt (73.6) aa (83.5)	709 nt (partial coding sequence)		
Family *Dicistroviridae*							
Goose dicistrovirus (BSF2)	9	39.8	NC029052	nt (83.9) aa (87.5)	3636 nt (partial coding sequences for nonstructural	**Capsid protein precursor:** SSSVK (520)	**Capsid protein precursor:** T (535), A (536)

					polyprotein and capsid protein precursor)		
Aphid lethal paralysis virus (BSF2)	7	20.5	NC004365	nt (97.7) aa (98.0)	2007 nt (partial coding sequences for nonstructural polyprotein and capsid protein precursor)	None	None
Family *Partitiviridae*							
Aspergillus fumigatus partitivirus 2 (BSF2)	3	55.6	NC040757	nt (77.7) aa (83.7)	Segment 1 (RdRp): 1013 nt (partial coding sequence)	None	D (72), R (73), K (74), G (420)
Penicillium stoloniferum virus S (BSF 2)	1	20.3	NC005976	nt (72.2) aa (70.1)	Segment 1 (RdRp): 356 nt (partial coding sequence)	None	None
Family *Tobaniviridae*							
Porcine torovirus (BSF3)	24	33.7	NC022787	nt (87.4) aa (87.1)	9528 nt (partial coding sequence)	None	None
Family *Retroviridae*							
Koala retrovirus (BSF3)	2	22.2	NC039228	nt (69.8) aa (77.6)	1869 nt (partial coding sequence for gag and polymerase proteins)	**Polymerase protein:** E (595)	None
Family *Virgaviridae*							

Pepper mild mottle virus (BSF1)	1	98.0	NC003630	nt (99.7) aa (99.8)	6233 nt (near complete coding sequence)	None	None
Pepper mild mottle virus (BSF2)	7	88.6	NC003630	nt (99.6) aa (99.5)	5632 nt (near complete coding sequence)	None	None
Tobacco mild green mosaic virus (BSF1)	5	80.4	NC001556	nt (98.2) aa (98.3)	5108 nt (partial coding sequence)	gp1: P (1332)	None
Tobacco mild green mosaic virus (BSF2)	3	17.1	NC001556	nt (96.6) aa (96.0)	1084 nt (partial coding sequence)	None	None
Family *Tombusviridae*							
Johnsongrass chlorotic stripe mosaic virus (BSF1)	3	93.0	NC005287	nt (75.5) aa (76.9)	4112 nt (partial coding sequence)	**Coat protein:** VG (24), AG (41), G (274)	**Coat protein:** N (37)
Unclassified RNA viruses							
Beihai tombus-like virus 15 (BSF2)	3	40.9	NC033202	nt (58.1) aa (54.5)	1361 nt (partial coding sequence)	**Hypothetical protein 1:** LSQ (538)	**Hypothetical protein 1:** N (525), D (526), E (531)

Thika virus (BSF2)	8	39.6	NC027127	nt (65.8) aa (67.2)	3603 nt (partial coding sequence)	L (2119)	P (2090), G (2091)
Hubei picorna-like virus 15 (BSF2)	11	39.6	NC032757	nt (90.2) aa (94.2)	3926 nt (partial coding sequence)	None	None
Hubei tombus-like virus 17 (BSF3)	3	49.9	NC032813	nt (63.4) aa (61.6)	1854 nt (partial coding sequence)	None	**Hypothetical protein 2:** T (98) **Hypothetical protein 3:** Q (233)
Wuhan insect virus 23 (BSF3)	1	26.2	NC032146	nt (69.5) aa (73.6)	387 nt (partial coding sequence)	None	None

428 BSF= Backyard swine farm, nt= nucleotides, aa= amino acids

429

Discussion

In a previous molecular study to determine the prevalence of influenza A virus (IAV) in backyard swine populations in the uMgungundlovu District of the KwaZulu-Natal province of South Africa, we did not find IAV RNA in samples under investigation. However, some of the swine did show disease symptoms such as coughing, sneezing, lethargy, and red patches on the skin, which warranted further analysis of a subset of the swine saliva samples to explore their oral virome. Therefore, metagenomic analysis of the RNA fraction was performed on one saliva sample from each of the three backyard farms under investigation. While the healthy swine saliva had only a few RNA viruses, the saliva taken from the other two pooled swine samples having clinical signs of disease generated a wide range of RNA viruses (**Fig 1**). Interestingly, viruses of the family *Astroviridae* were prevalent, according to the number of reads; genomes of PAstV-2, PAstV-4, Astrovirus wild boar, Porcine bastrovirus, Mamastrovirus 2, and Mamastrovirus 3 were identified. Most of these enteroviruses are endemic to swine populations worldwide and are associated with diarrhoea in swine, including neonatal piglets [33,34]; however, they have also been detected in healthy swine in all age groups [35,36]. Particularly the PAstV-2 and PAstV-4 have been frequently reported in swine populations in Asia [37,38]. These viruses have a single-stranded, positive-sense RNA genome comprising three open reading frames (ORFs); ORF1a, ORF1b, and ORF2 [39]. While ORF1a encodes the nonstructural protein, the ORF1b encodes RNA-dependent RNA polymerase (RdRp) and the ORF2 encodes the capsid protein, which is highly divergent due to the immune pressure from the host [39].

Intriguingly, in a recent study, PAstV-4 was associated with acute respiratory illness in the piglets in Oklahoma, USA [40]. Whether this explains our observation of the piglets at the BSF2 with clinical signs of coughing and sneezing that were found infected with PAstV-4 in this study requires further investigation. Interestingly, PAstV-4 sequences were also detected in grower swine at the BSF3, which did not exhibit any clinical signs of respiratory illness; instead, these swine had red patches on the skin, which may be associated with other sources of infection and therefore requires further investigation.

Genome sequences of several members of the family *Picornaviridae* were obtained in this study, including, Porcine kobuvirus, Porcine teschovirus A, Porcine sapelovirus 1, Porcine enterovirus 9, Pasivirus A, and Norway rat hunnivirus. These viruses have a monopartite, positive-sense, single-stranded RNA genome and have been associated with swine

gastrointestinal, reproductive, respirato1y, and neurologic diseases; however, the infections have also been reported to be subclinical [41]. Another enteric virus, Porcine enteric sapovirns, was also detected in this study. This vims has previously been detected in clinically healthy as well as dianhoeic pigs in various age groups [42], including 15-day-old neonatal piglets [43].

Genome sequences of several virnses from the Order Picornavirales were also obtained, including Posavirns 1, Posavirus 3, Picornavirales Bu-1, and Picornavirales Tottori-HG1. These are porcine stool-associated RNA virnses with a positive-sense single-stranded RNA genome [44]. They have been reported in swine populations in various age groups causing dianhoea; however, infections may also be subclinical [45-47].

Several species of Picobirnavirnses that were identified from either paiiial or complete coding sequences of the Rd.Rp gene (RNA segment 2) of Picobirnavirnses included Porcine picobimavirns, Dog picobimavirns, Human picobirnavirns, Chicken picobimavirns, Green monkey picobirnavirns, Otarine picobimavirns, Roe deer picobirnavirns, and Feline (Cat) picobirnavirns. These ai·e enteric virnses and have been detected in the faeces of various mammalian species and humans with or without dianhoeic diseases [48,49]. Primarily these are oppo1iunistic enteric pathogens found in birds and mammals [49-51], and the avian host may serve as the caiTier of these Picobirnavirnses [52,53]. Picobirnavirnses have a bi-segmented double-stranded RNA genome [54] in which RNA segment 1 encodes the capsid protein, and segment 2 encodes the Rd.Rp gene [48]. The presence of different species of Picobirnavirnses in backyai·d swine suggested a widespread circulation of these virnses in animals and birds, which may have transmitted to the swine possibly through direct contact on the backyard faims. A high prevalence and diversity of Picobimavirnses were also recently repo1ied in swine populations on the island of Saint Kitts in the Cai·ibbean [51] and in Hong Kong [55]. Our observation of Otai·ine picobimavirns in backyai·d swine was intri$_{g\,u}$ing since the prima1y host of the Otarine picobimavirns is an aquatic mainmal, the California sea lion, from which the first genome sequence of Otarine picobimavirns was retrieved in Hong Kong in 2012 [56]. Due to limited info1mation on the Otai·ine picobimavirns, the transinission dynainics remain obscure. Therefore, the possible route of transinission of Otai·ine picobimavirns to the backyard swine in the KwaZulu-Natal province of South Africa could not be ascertained. Interestingly, in a recent study, we repo1ied on the possible dissemination of avian IAV subtypes to swine populations worldwide [57] suggesting the potential role of migrato1y birds in the dissemination of these RNA virnses

[58,59]. It should be noted that the present study was conducted in a coastal area of South Africa where aquatic birds are frequently seen; therefore, if the occurrence of Otarine picobirnavirus in the backyard swine was related to the migration of aquatic avian species, this remains a question for further investigation?

Taken together, the presence of swine enteric viruses, such as Astroviruses, Picornaviruses, Picobirnaviruses, Porcine rotavirus A, Rotavirus C, and Human rotavirus B in the backyard swine saliva suggest that these swine are at risk of developing diarrhoeic diseases [33,37]. In addition, the possibility of zoonotic transmission of Porcine rotavirus A and Rotavirus C from swine to the exposed household members or workers also poses a risk of diarrhoeic disease in these contacts.

Our finding of HEV in the South African backyard swine was not surprising because HEV has been reported in swine populations in various age groups in several countries [60,61], including South Africa [30]. Since the zoonotic transmission of HEV from swine to humans is possible either through contact or the consumption of pork meat [60], adequate precautions should be taken to avoid this. An experimental study determined that the fecal shedding of HEV in swine starts 12.6 days post-infection and may last up to 10.5 days in contact-infected swine [60]; however, the length of HEV fecal shedding was reported to be dependent upon the route as well as the dose of infection, and the coinfection status of the swine with other viral pathogens. When HEV infection was administered orally or through contact infection, the length of HEV fecal shedding in swine was 9.7 days [60,62]. The fecal shedding of HEV on the backyard farms may predispose other species, including humans, to HEV infection.

The presence of plant viruses, including Pepper mild mottle virus, Tobacco mild green mosaic virus, and Johnsongrass chlorotic stripe mosaic virus in backyard swine saliva samples, suggested a possibility of feed-related transmission of these viruses. A plant virus genome was also detected in a recent backyard swine fecal virome study in Mexican swine [63]. The partial sequences of a few other viruses, including fungal and unclassified RNA viruses, suggested a limited transmission of these viruses on the backyard swine farms. Overall, this study reported a high diversity of swine enteric viruses on the backyard swine farms under investigation. While most of these enteric viruses are endemic in swine populations globally, some of the Picobirnaviruses appeared to be transmitted from other animals present on the farm, based on the phylogenetic analysis. Therefore, backyard swine farmers should restrict the interactions of their swine with other animals to avoid further

ecological spillover of these vimses. In addition, maintenance of hygiene on the backyard fanns would benefit and minimize the transmission of these vimses between swine.

In a previous study, we did not detect IAV active infection in swine saliva samples collected from the three backyard swine fanns in the uMgungundlovu District of KwaZulu-Natal province, which prompted us to detennine the RNA vims diversity of selected saliva samples using deep sequencing. Om findings of RNA vims diversity in the selected saliva samples were in agreement of the repo1t, which recently investigated the virome of the backyard swine in mral Mexico [63]. However, the diversity of RNA vimses in selected backyard swine saliva samples in the present study may only be used as a reference for broader countrywide surveillance for RNA vimses in backyard swine holdings in South Africa. Since there is limited info1mation available on vims diseases in South African swine populations, the next-generation sequencing would facilitate exploring the diversity of South African swine virome to dete1mine the prevalence and circulation of ce1tain impo1tant vimses, including contr·olled or notifiable vimses.

Conclusion

A high prevalence of PAstVs and other swine enteric vimses, including rotavimses, may pose a significant risk of dianhoea and other associated diseases in the swine on the backyard swine faims under study. In addition, the presence ofHEV in backyai·d swine saliva at the BSF3 poses the risk of zoonotic tr·ansmission to the exposed humans. We recommend proper hygiene and avoiding direct contact with swine faeces and saliva to avoid the zoonotic tr·ansmission of these vimses.

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Author contributions

Conceptualization: RPC and MLG, Sample collection: RPC and MLG, Preliminary data analysis: RPC and EJS, Comprehensive data analysis: RPC, Preparation of Figures and Tables: RPC, Writing-First draft preparation: RPC, Writing-Reviewing and Editing: RPC and MLG. All authors have read and approved the present version of the manuscript for publication.

Ethical approval

We obtained a full approval for the present study from Animal Research Ethics Committee of the University of KwaZulu-Natal; Reference# AREC/041/019D. We also obtained a Section 20 permit from the Department of Agriculture, Land Reform and Rural Development (DALRRD), South Africa, in terms of Animal Diseases Act, 1984 (Act No. 35 of 1984); Reference# 12/11/1/5/4 (1425 AC) (1).

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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CHAPTER 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Swine may harbor various virus pathogens, including some notifiable viruses [1-4]. Some of these viruses, such as IAV, in the event of co-infections within a swine with multiple IAV subtypes of different origins, such as from humans and birds, among others, may reassort and evolve into a novel IAV strain or subtype which may be capable of immune evasion and thus may inflict severe respiratory illness in the swine [5, 6]. In addition, specific mutations in swine IAV genomes may facilitate the acquisition of mammalian adaptation and transmission and thus threaten public health [7-9]. Therefore, to ensure sustainable swine farming and simultaneously minimize the zoonotic transmission of swine IAVs to safeguard public health, it is necessary to identify the currently circulating IAV subtypes in swine populations and track their molecular evolution. This would also be required for making vaccine-related decisions [10] and assisting the stakeholders in implementing preventive measures to contain the spread of disease in an outbreak.

The systematic reviews, which we conducted to analyze the prevalence and circulation of IAV in swine populations globally [11], suggested that in recent decades great strides in terms of detection of IAV have taken place in commercial swine populations in the countries which have been the front runners in commercial swine production [12, 13]. The figures presented in the reviews are based on the literature cited in the Chapters. The available literature also suggested considerable efforts have been made to investigate the prevalence of other virus pathogens, including notifiable viruses in commercial swine populations in various countries [14-16]. However, backyard swine farming, an integral part of the rural agricultural economy in several developing countries, remains neglected in terms of active surveillance of controlled virus pathogens, including IAV, with only limited information available on the occurrence of virus diseases in backyard swine populations. While limited information on some of the RNA viruses is available from commercial swine populations in South Africa, there is a lack of data on the prevalence of these viruses in backyard swine populations. Various constraints, including limited awareness of virus diseases and their mode of transmission coupled with scarce resources, make the sustainable operation of backyard swine farming challenging for economic profits for the backyard swine farmers and public safety.

We observed that eleven subtypes of avian origin IAV had been reported in swine populations in various countries [7]. The phylogenetics, principal coordinate analysis, N-

linked glycosylation analysis, and the presence of signature mammalian adaptation markers in HA gene sequences of these eleven IAV subtypes suggested avian to swine transmission and adaptation of some of these IAV subtypes in swine. The HPAIV H5N1 and LPAIV H9N2 viruses were more commonly transmitted to swine populations and have already adapted in swine, given the presence of specific mammalian adaptation markers [7]. Previous experimental studies suggested that specific mutations in the HA glycoprotein have facilitated the adaptation of H5N1, H6N6, H7N9, and H9N2 avian influenza viruses in swine [17-20].

Recent studies suggested that the interactions of domestic and wild birds with backyard swine may transmit avian influenza viruses to swine [21, 22]. In the recent past, various high and low pathogenic avian influenza viruses have been reported in domestic poultry [23-25], ostriches [26, 27], and wild birds in South Africa [28, 29]. The presence and circulation of these avian IAV subtypes in wild and domestic birds in South Africa, upon interaction with backyard swine, present a risk of spillover of these avian IAVs to the backyard swine populations in the country.

To the best of our knowledge, no study has been conducted to determine the molecular prevalence of IAV and other virus pathogens in backyard swine populations in South Africa. Therefore, we reported the prevalence of IAV on the South African backyard swine farms located in the uMgungundlovu District of KwaZulu-Natal province for the first time. The study area and the backyard swine farms for participation were identified in consultation with the State Veterinary Department. While we did not identify active IAV infection (viral RNA) in the swine saliva samples under investigation, the observation of clinical signs of the respiratory illness, including coughing and sneezing in some of the piglets at BSF2 and red patches on the skin of some of the grower pigs at BSF3 was intriguing. This study was conducted during the COVID-19 related lockdown in South Africa and could reflect the impact of limited movement, social distancing, sanitization, and the use of face masks by backyard swine farm workers. These protective measures could diminish the transmission of various virus pathogens, including IAV, between backyard swine and the farmworkers or household members. The change in human activity and behaviour during COVID-19 lockdown was reflected in significantly low influenza activity reported in countries located in the Southern Hemisphere, including South Africa [30].

Further metagenomic analysis of selected saliva samples collected from three BSFs under study showed a high diversity of swine enteric viruses in the saliva samples obtained from the

symptomatic backyard swine at the BSF2 and BSF3. In contrast, only a few RNA viruses occurred in the saliva obtained from a healthy adult swine at BSF1. In addition, we also identified HEV genome sequences in the saliva obtained from BSF3. HEV was recently identified in commercial swine populations in the Eastern Cape province of South Africa and is the only other report of HEV in South Africa [31]. Since HEV infection in swine is usually subclinical and may remain undetected, it is possible that the prevalence of HEV is under-estimated nationally. In addition, the persistent circulation of HEV in swine populations would pose a significant risk of zoonotic transmission to the exposed human population [32]. From our investigation, we determined that the clinical signs of respiratory illness of coughing and sneezing, which we primarily suspected to be related to possible IAV infection in piglets at BSF2, may be associated with PAstV-4 disease and not the IAV [33]. While the five genotypes of Porcine astroviruses (PAstV-1 to PAstV-5) are mostly associated with gastroenteritis and diarrhoea [34-36], Lv *et al.* demonstrated that PAstV-4 and PAstV-2 infections in swine were associated with respiratory disease or diarrhoea [37]. Padmanabhan and Hause reported that PAstV-4 infection was related to respiratory illness leading to coughing and sneezing in 10- 21 days-old-piglets [33]. However, these viruses were also detected in swine with no clinical signs of disease [37, 38].

Interestingly, the grower pigs at BSF3 that displayed red patches on the skin did not exhibit coughing and sneezing; however, they were also infected with PAstV-4 among several other swine enteric viruses and HEV. Here it should be noted that the piglets infected with PAstV-4 that had coughing and sneezing illness at BSF2 were about three months old, while the grower pigs at BSF3 were about 5–6 months old. While the present study could not determine whether PAstV-4 infection manifests as clinical respiratory symptoms in piglets and not in the older swine, it remains a question for further investigation to ascertain whether these viruses are incidental findings or are indeed associated with the clinical signs.

A high diversity of the oral RNA virome which was dominated by swine enteric viruses suggested an increased risk of diarrhoeic diseases in swine at the BSF2 and BSF3 compared to BSF1. Several swine enteric viruses have been found to be associated with diarrhoeic diseases in swine in various countries [34, 35, 39-41]; however, rotavirus species, including RVA and RVC, have also been reported to have a zoonotic propensity. For example, Ianiro *et al.* reported zoonotic transmission of RVA from swine to a child in Italy who was hospitalized due to gastroenteritis [42] while another long-term study identified eight RVA strains that were zoonotically transmitted to hospitalized pediatric patients over a 15-year

period in Hungary [43]. In addition, Tacharoenmuang *et al.* reported swine-to-human transmission of RVA in Thailand, which caused severe diarrhoea in the infected human population [44] while the zoonotic transmission of RVC was reported from swine to children causing diarrhoea in Brazil [45].

In addition, the prevalence of HEV has been detected in swine in various age groups in several countries globally [31, 46-51]. Silveira *et al.* have reported that backyard swine may serve as a reservoir for zoonotic transmission of HEV to humans [52]. Numerous other studies have documented zoonotic transmission of HEV from swine to the exposed human populations [53-57]. This substantiates the risk of zoonotic transmission of HEV to the exposed household members in the present study.

In conclusion, a high diversity of swine enteric viruses in backyard swine populations under investigation may pose the risk of diarrhoeic diseases on these backyard farms. In addition, despite the absence of active IAV infection in the backyard swine population, several other RNA viruses such as HEV and rotaviruses pose a significant risk of their zoonotic transmission to the exposed household members. With the persistent direct interaction between backyard swine and the household members without the necessary biosecurity measures in place, this would enhance the disease burden in backyard swine and threaten the health and well-being of the household members. Information on the RNA virome of backyard swine is imperative to assess the risk of disease outbreaks in swine farming and rural communities. Therefore, large-scale countrywide molecular surveillance in swine populations should be implemented to explore the diversity of RNA viruses in other backyard swine holdings in South Africa.

LIMITATIONS

The study area and backyard swine farms investigated in this study were limited and therefore may only be used as a reference for broader countrywide surveillance for RNA viruses in backyard swine holdings. The backyard swine saliva samples were collected during the COVID-19 lockdown. Since movement-related measures were in place in South Africa, we could only interact with the backyard swine farmers during a narrow time frame. However, the serological assays would be useful to detect IAV antibodies in backyard swine saliva samples to determine past infection, the objective of this study was to investigate the

active infection (viral RNA) in the backyard swine; therefore, we did not perform serology to detect antibodies for determining the past infection.

FUTURE RESEARCH PERSPECTIVES AND RECOMMENDATIONS

A countrywide active molecular surveillance is required to gain a comprehensive understanding of currently circulating IAV subtypes in backyard swine populations. In addition, investigation of other zoonotic virus pathogens, such as HEV and rotaviruses, is recommended given the possibility of their zoonotic transmission to the exposed human populations. Our evidence suggests that HEV and rotaviruses may be currently circulating in backyard swine populations remaining undetected due to subclinical infections in swine. Future research should emphasize virus genome sequencing, which would provide helpful information for upgrading molecular detection assays to achieve accurate and reliable detection of RNA viruses in backyard swine populations in South Africa. In addition, genome sequencing would also assist in selecting vaccine candidates for certain swine RNA viruses.

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APPENDICES

APPENDIX I

Informed consent form

I, (name of participant) _____ am voluntarily participating in the study which is aimed to investigate the prevalence of emerging and re-emerging porcine viruses in backyard swine in South African households within the province of KwaZulu-Natal during the years 2020-2021. The participation in this study is voluntary and I have the right to withdraw my consent and participation at any time. I am aware that I have the right to ask questions to satisfy myself regarding the purpose of the study. I am also aware that if there is any suspicion of the controlled diseases including avian influenza, classical swine fever, porcine reproductive and respiratory syndrome, foot and mouth disease, swine vesicular disease, I will have my animals placed under quarantine as further disease investigation will have to take place. If such situation arises, I am willing to cooperate with the State Veterinary department, as and when required. The Investigators of the study have confirmed that my identity, as a participant, will be confidential as per the applicable laws of the Republic of South Africa. I am also aware that I am not liable to any monetary compensation from the investigators towards my participation in the study. I reserve the right to know the outcome of the study. I may contact the investigators at any time during the study to express my concern regarding my participation in the study. I, herewith, provide my consent for the study.

_____

(Signature of the participant)
Date of consent:
Place:
Mobile Phone Number:
E-Mail ID, if available:

Principal Investigator: Mr. Ravendra Pratap Singh Chauhan
Designation: Ph.D. student (Medical Microbiology)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences, University of KwaZulu-Natal
Durban, South Africa
Cell Phone: [redacted]
E-Mail: 218084293@stu.ukzn.ac.za

Co-Principal Investigator: Dr. Michelle L. Gordon
Designation: Senior Lecturer & Infectious Diseases Leader
School of Laboratory Medicine and Medical Sciences
College of Health Sciences, University of KwaZulu-Natal
Durban, South Africa
Phone: 031 260 4498
E-Mail: Tarinm@ukzn.ac.za

APPENDIX II

Questionnaire for the participants in South African households having backyard swine in KwaZulu-Natal province

Site of sampling: _____Date of sampling (dd/mm/yyyy): _____

This study is conducted to assess the current prevalence of influenza A virus and other emerging and re-emerging porcine viruses in backyard swine in South African households within the province of KwaZulu-Natal during 2019-2022. This study is undertaken as the part of a Ph.D. thesis titled "*Prevalence and characterization of Influenza A virus in backyard swine in the province of KwaZulu-Natal of South Africa*" at the University of KwaZulu-Natal, Durban, South Africa. The participants are requested to answer the following questions to assess the risk of virus disease transmission to the households rearing backyard swine.

1. Name of participant:

   _____
2. Gender of participant:
   - ☐ Male
   - ☐ Female
   - ☐ Not answered
3. Marital status of the participant:
   - ☐ Married
   - ☐ Unmarried
   - ☐ Single
   - ☐ Prefer not to disclose
4. The pigs belong to the:
   - ☐ Respondent
   - ☐ Spouse
   - ☐ Someone else
5. Date of Birth of the participant (dd/mm/yyyy):

   _____
6. Highest level of education obtained by the participant:
   - ☐ No education
   - ☐ Primary School
   - ☐ High School
   - ☐ Vocational or professional course
   - ☐ Graduate or post graduate degree
7. Number of the individuals living in the household/family:

   _____
8. How many swine do you have in the backyard?
   - ☐ One
   - ☐ Two

- ☐ Three
- ☐ Four
- ☐ Five
- ☐ More than five

9. Are the other members of the household also involved in rearing of swine, or exposed to the swine within the household?

- ☐ Yes
- ☐ No
- ☐ Not sure

10. Did you suffer from a respiratory illness or seasonal flu in recent past?

- ☐ Yes
- ☐ No
- ☐ Not sure

11. Did you notice anyone within your household with flu-like symptoms in recent past?

- ☐ Yes
- ☐ No
- ☐ Not sure

12. Have you ever sought medical assistance for a seasonal flu illness?

- ☐ Yes
- ☐ No
- ☐ Not sure

13. Are you aware of the swine influenza virus?

- ☐ Yes
- ☐ No
- ☐ Not sure

14. Are you aware of other viruses that may infect swine?

- ☐ Yes
- ☐ No
- ☐ Not sure

15. How do you keep swine in the backyard?

- ☐ Free- roaming
- ☐ Restricted/ within barns
- ☐ Other (_____)

16. Do you vaccinate your backyard swine for influenza virus?

- ☐ Yes
- ☐ No
- ☐ Not sure

17. Do you vaccinate your backyard swine for any other virus?

- ☐ Yes
- ☐ No
- ☐ Not sure

18. Do you receive vaccination for seasonal influenza virus?

- ☐ Yes
- ☐ No
- ☐ Not sure

19. Are you aware of any active surveillance for swine diseases in your area?

- ☐ Yes
- ☐ No
- ☐ Not sure

20. Do you rear chickens in your household/backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

21. Do you rear ducks in your household/backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

22. Do you have any other poultry in your household/backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

23. Do you notice wild birds visiting your backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

24. Do you notice any interaction of wild birds with your backyard swine?

- ☐ Yes
- ☐ No
- ☐ Not sure

25. Do you notice any interaction of poultry with your backyard swine?

- ☐ Yes
- ☐ No
- ☐ Not sure

26. Do you have cattle in your household/backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

27. Do you notice any interaction between cattle and swine in your backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

28. Do you use gloves while attending the pigs?

- ☐ Yes
- ☐ No
- ☐ Not sure

29. Do you use a face mask while attending the pigs?

- ☐ Yes
- ☐ No
- ☐ Not sure

30. Do you use dedicated boots and clothing while working with pigs in the backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

31. Do you seek assistance from your local Veterinarian if your pigs get sick?

- ☐ Yes
- ☐ No
- ☐ Not sure

32. Have you ever noticed any sickness in your pigs?

- ☐ Yes; most recently during _____
- ☐ No
- ☐ Not sure

33. Have your pigs ever died due to disease outbreak or sickness?

- ☐ Yes
- ☐ No
- ☐ Not sure

34. For how many years have you been rearing the pigs in the backyard?

- ☐ Up to 1 year
- ☐ Up to 2 years
- ☐ Up to 3 years
- ☐ Up to 4 years
- ☐ More than 4 years

35. Do you slaughter your pigs by yourself?

- ☐ Yes
- ☐ No
- ☐ Not sure

36. Do you sell your pigs to a slaughterhouse?

- ☐ Yes
- ☐ No
- ☐ Not sure

37. Do you slaughter poultry within your backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

38. Do you slaughter any other wildlife within your backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

39. Do you buy the piglets for rearing in your backyard?

- ☐ Yes; I buy the piglets from _____
- ☐ No
- ☐ Do not want to disclose

40. Do you breed the swine within your household for rearing in your backyard?

- ☐ Yes
- ☐ No
- ☐ Not sure

APPENDIX III

ACKNOWLEDGMENT OF MANUSCRIPT SUBMISSION

Virology Journal - Receipt of Manuscript 'Metagenomic analysis of...'

Virology Journal <beverly.mapua@springernature.com>

Sun 20-Mar-22 4:01 PM

To: [redacted] <[redacted]>

Ref Submission ID 595db81a-fd9b-4b8d-9e5f-66eb6a1672f4

Dear Dr Chauhan,

Please note that you are listed as a co-author on the manuscript "Metagenomic analysis of RNA fraction reveals the diversity of swine oral virome on South African backyard swine farms in the uMgungundlovu District of KwaZulu-Natal province", which was submitted to Virology Journal on 20 March 2022 UTC.

If you have any queries related to this manuscript please contact the corresponding author, who is solely responsible for communicating with the journal.

Kind regards,

Editorial Assistant
Virology Journal

Acknowledgement of the Manuscript BF-1514 entitled No evidence of influenza A virOus RNA in South African backyard swine in the uMgungundlovu District of the KwaZulu-Natal province

ARCC Journals <contact@arccjou ma ls.com>

Sun 06-Mar-22 8:38 PM

To: [redacted] <[redacted]>

@ 1 attachments (14 KB)

Attachments-1624877074842-6089603e9087340b9bdcbbb 1.docx;

Dear Dr. Ravendra P. Chauhan,

Greetings!!

Thank you for submitting the article. It is to acknowledge the receipt of the manuscript for **"Indian Journal of Animal Research"** entitled **"No evidence of influenza A virOus RNA in South African backyard swine in the uMgungundlovu District of the KwaZulu-Natal province".** For any correspondence, please mention Reference Number **BF-1514** for this article in your all emaill.

Submit the attached certificate that this article or its data has not been/will not be sent to any other journal for publication. Also, inform the subject area of specialization of the article to send it the right area of expert for Review Process.

Acknowledge the receipt of this email and submit the attached certificate duly filled with for further processing of the article. Should you require any further clarification, feel free to contact. Check the article status any time at bitp://arccjournals.com/.

--

Best Regards
Managing Editor
Agricultural Research Communication Centre I
294, Narsi Village Part II, Sector 33 I Kamal - 132001 I Haryana (INDIA) I
Web: www.arccjournals.com I E-mail: edrtoc@arcciourna.Js.com /contact@arccjourna.Js.com

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02 March 2020

Mr Ravendra Pratap Singh Chauhan (218084293)
School of Laboratory Medicine & Medical Sciences
Medical School

Dear Mr Chauhan,

Protocol reference number: AREC/041/019D
Project title: Prevalence and characterisation of Influenza A virus in backyard swine in the province of KwaZulu-Natal of South Africa.

Full Approval - Research Application

With regards to your revised application received on 20 January 2020. The documents submitted have been accepted by the Animal Research Ethics Committee and FULL APPROVAL for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its Implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 01 March 2021.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

Itake this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Dalene Vosloo, PhD
Deputy Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Dr Michelle Gordon

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

05 May 2021

Mr Ravendra Pratap Singh Chauhan (218084293)
School of Laboratory Medicine & Medical Sciences
Medical School

Dear Mr Chauhan,

Protocol reference number: AREC/041/019D

Old Project title: Prevalence and characterisation of Influenza A virus in backyard swine in the province of KwaZulu-Natal of South Africa.

New Project title: "Investigating the prevalence of *Influenza A virus* and other RNA viruses in saliva samples of backyard swine in KwaZulu-Natal province of South Africa"

Approval – Amendment Application

With regard to your second amendment request received on 05 May 2021, the Animal Research Ethics Committee has accepted the documents submitted, and **FULL APPROVAL** for the protocol is granted.

We note your request to amend your protocol title and to add the identification of RNA viruses present in South African backyard swine saliva samples using deep sequencing as a new objective.

Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times. Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

Any alteration/s to the approved research protocol, i.e. Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for 5 years.

The ethical clearance certificate is only valid for one year from the date of issue. Renewal for the study must be applied for before 01 March 2021.

Please note: the study renewal in 2022 must be uploaded to the RIG online system as a new application.

Attached to the Approval letter is a template of the Progress Report required at the end of the study or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health/wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee
/kr

cc Supervisor: Dr Michelle L Gordon

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

07 May 2021

Mr Ravendra Pratap Singh Chauhan (218084293)
School of Laboratory Medicine & Medical Sciences
Medical School

Dear Mr Chauhan,

Protocol reference number: AREC/041/019D
Project title: "Investigating the prevalence of *Influenza A virus* and other RNA viruses in saliva samples of backyard swine in KwaZulu-Natal province of South Africa"

Full Approval - Renewal Application

With regards to your renewal application received on 07 May 2021. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 06 May 2022.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, BVSc, MS, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Dr Michelle L Gordon

Animal Research Ethics Committee
Telephone: 031 2608850
Email: animalethics@ukzn.ac.za
University Road
Chiltern Hills
Westville
3629
South Africa

1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE IN KWAZULU-NATAL

Founding Campuses:
Edgewood
Howord College
Medical School
Pietermoritzburg
Westville

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Ilit Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: [redacted]
Reference: 12/11/1/5/4 (1425)

Mr Ravendra Pratap Singh Chauhan

KRISP, K-RITH building

Nelson Mandela School of Medicine

Durban

Tel: 031 260 4498

E-mail: [redacted], Tarinm@ukzn.ac.za;

Keith.Perrett@kzndard.gov.za

Dear Mr Chauhan,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent with the email on 24 January 2020 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form received on 24 January 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to [redacted]
3. Sampling of backyard pigs may only be conducted in the Umgungundlovu district of KwaZulu-Natal for which a State Veterinary letter of no restriction has been received. It is the researcher's responsibility to remain in contact with the responsible State Veterinarian regarding the disease status of the area from which pigs will be sampled. Records must be kept for five years for auditing purposes;
4. Sufficient information needs to be collected from sampling points to ensure that follow up investigations can be attempted if required. Any suspicion or confirmation of swine origin Influenza A or avian origin Influenza A viruses must be reported to the responsible State Veterinarian;

5. PCR analysis and Influenza A subtyping of swine saliva samples may be conducted in the Medical Microbiology BSL-2 laboratory as indicated. Genome sequencing may be conducted at the KwaZulu-Natal Research and Innovation Sequencing Platform of the University of KwaZulu-Natal if positive samples are found. These facilities may be subject to an inspection by the Directorate Animal Heath, should this be deemed necessary;
6. Any suspicion or detection of Influenza A viruses in the pig saliva samples must be verified and confirmed by the Avian Influenza Reference Laboratory at the ARC-OVR, at the cost of the researcher. This should include H and N sub-typing and sequencing to determine the most likely specie-origin of the virus;
7. Feedback that will be sent to the owners of the backyard pigs has to be approved by the Director: Animal Health before it is shared with participants;
8. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified waste contractor. Records must be kept for five years for auditing purposes;
9. Any draft reports, thesis or articles of this study must be sent to the Director: Animal Health for approval before it may be published in any form;
10. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Titled research/study: ***"Prevalence and characterization of Influenza A virus in backyard swine in the province of KwaZulu-Natal of South Africa".***

Researcher: Mr Ravendra Pratap Singh Chauhan

Institution: Medical Microbiology BSL-2 laboratory, Doris Duke Medical Research Institute, Nelson Mandela School of Medicine, Durban

Our ref Number: 12/11/1/5/4 (1425)

Your ref: AREC/041/019D

Expiry date: May 2021

Kind regards,

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date: 2020 -02- 25

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

culture, land reform
& rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001
Enquiries: Ms Mama Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/514 (1425 AC) (1)

Mr Ravendra Pratap Singh Chauhan
KRISP, K-RITH building
Nelson Mandela School of Medicine
Durban
Tel: 031 260 4498
E-mail: ravendrachauhan@hotmail.com ; Tarinm@ukzn.ac.za ; Keith.Perrett@kzndard.gov.za

Dear Mr Chauhan,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "PREVALENCE AND CHARACTERIZATION OF INFLUENZA A VIRUS IN BACKYARD SWINE IN THE PROVINCE OF KWAZULU-NATAL OF SOUTH AFRICA"

An amendment is hereby granted on the Section 20 permit that was issued for the above mentioned study on 25 February 2020:

i) The expiry date is extended to 31 December 2022;
ii) Deep sequencing of RNA extracted from three pig saliva samples may be conducted at the Biotechnology Platform Laboratory of the ARC-OVR;
iii) The detection or suspicion of any controlled or notifiable animal diseases on deep sequencing must be reported to the responsible state veterinarian and epidemiology@dalrrd.gov.za;
iv) Swine saliva samples collected as part of this study and extracted RNA may be stored under access control at -80°C in the HIV Pathogenesis Program Laboratory, K-RITH building, UKZN Medical School campus (Nelson Mandela School of Medicine). Storage is allowed for a maximum of 12 months from the date of signature of this letter, where after the samples have to be destroyed (and proof of destruction provided) if a new Section 20 application has not been successfully made to utilise these samples;
v) Stored samples may not be used for further research or be outsourced without prior written approval from the Director: Animal Health;

vi) All other conditions as specified in the Section 20 permit of 25 February 2020 remain in full effect.

Kind regards,

D

DIRECTOR OF ANIMAL HEALTH

Date:

2021 -08- 17

SUBJECT: *RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "PREVALENCE AND CHARACTERIZATION OF INFLUENZA A VIRUS IN BACKYARD SWINE IN THE PROVINCE OF KWAZULU-NATAL OF SOUTH AFRICA•*

APPENDIXV

APPROVAL FOR PUBLICATION OF THE RESEARCH ARTICLES

culture, land reform & rural development

Department
Agriculture, Land Reform and Rural Development
REPUBLIC Of SOUnt AFRICA

Directaate Aninal Health, Department of Agricuture. Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Mama Laing• Tel: +2712 319 7442 • Fax: +2712 319 7470 • E-mail: MamaL@dalm:l.gov.za
Ret.rence: 12/11/1/514 (1425)

Mr Ravendra Pratap Singh Chauhan
KRISP, K-RITii building
Nelson Mandela School of Medicine
Durban
Tel: 031 260 4498
E-mail: ravendrachauhan@hotmail.com ; Tarinm@ukzn.ac.za

Dear Mr Chauhan,

NO OBJECTION TO PUBLISHIIIG OF SUBMITTED DRAFT ARTICLE ON RESEARCH CONDUCTED UNDER SECTION 20 OF THE ANIMAL DISEASES ACT 1984 (ACT NO 35 OF84)

This office has no objection to the publication of the folowing article as submitted for evaluation under the relevant permissions of Section 20 of the Animal Diseases Act, 1984 (Act no. 35 of 84):

Draft article title: "No evidence of Influenza A virus RNA in South African backyard swine in KwaZulu-Natal province•.
Section 20 study title: "Prevalence and characterization of Influenza A virus in backyard swine in the province of KwaZulu-Natal of South Africa".
Section 20 permit ref no.: 12/11/1/5/4 (1425)
Section 20 permit date: 25 February 2020

This approval indicates that the DAH has no objection to the publication of this artide from a regulatory animal disease control point of view and does not necessarily imply any agreement with any findings or statements made in the article.

Regards,

/IA$tai<1.

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date: 2071 -Di- 2 8

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Mama Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/5/4 (1425 AC) (1)

Mr Ravendra Pratap Singh Chauhan
KRISP, K-RITH building
Nelson Mandela School of Medicine
Durban
Tel: 031 260 4498
E-mail: ravendrachauhan@hotmail.com ; Tarinm@ukzn.ac.za

Dear Mr Chauhan,

NO OBJECTION TO PUBLISHING OF SUBMITTED DRAFT ARTICLE ON RESEARCH CONDUCTED UNDER SECTION 20 OF THE ANIMAL DISEASES ACT 1984 (ACT NO 35 OF 84)

This office has no objection to the publication of the following article as submitted for evaluation under the relevant permissions of Section 20 of the Animal Diseases Act, 1984 (Act no. 35 of 84):

Draft article title: "Metagenomic analysis of RNA fraction reveals the diversity of swine oral virome on South African backyard swine farms in the uMgungundlovu District of KwaZulu-Natal province".
Section 20 study title: "Prevalence and characterization of Influenza A virus in backyard swine in the province of KwaZulu-Natal of South Africa".
Section 20 permit ref no.: 12/11/1/5/4 (1425) and 12/11/1/5/4 (1425 AC) (1)
Section 20 permit date: 25 February 2020 and 17 August 2021.

This approval indicates that the DAH has no objection to the publication of this article from a regulatory animal disease control point of view and does not necessarily imply any agreement with any findings or statements made in the article.

Regards,

[redacted]
Name:
Reason:
Date: 2021.12.08 11:53:12 CAT

Dr Mpho Maja

DIRECTOR: ANIMAL HEALTH

Date: _____

APPENDIX- VI

DALRRD APPROVAL FOR SUBMISSION OF THE Ph.D. THESIS

kulture, land reform
& rural development

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Enqull'lee: Mr.Mamatang• let..Z712.3197442 • Fax:%27123197470• E-mall:Mamataclalml.gov.za
Reltrenc&: 1211111514 (1d25 AC)(1)

Mr Ravendra Pra1ap Singh Chauhan
KRISP, K-RITH ♦iJding
Nelson Mandela School of Medicine
Durban
Tel: 0312604498
E-mail: [redacted]

Dear tl r Chauhan.

NO OBJECTION TO PUBUSHING OF SUBMITTED DRAFT ARTICLE ON RESEARCH CONDUCTED UNDER SECTION 20 OF THE ANIMAL DISEASES ACT 19841 (ACT NO 35 OF 84)

This office has rc; objection to the pu>lic-ation of the following Ph.D. Thesis JS submitted for evaluation under the relevant permissions of Section 20 of the Animal Diseases Act, 1984 (Act no. 35 of 84):

Draft Ph.D. Thesis title: "Invesligating lhe prevalence of inHuenza A virus and other RNA viruses in saliva samples of bactyard swine in KwaZulu-Natal province of Soulh Africa".
Section 20 study title: "Prevale-nce and characterization of Influenza A \irus in backyard swine in Ule province of KwaZulu-Natal of South Africa".
Section 20 permit ♦ f no.: 12/11111514 (1425} and 12/11/1(514 (1425 AC) (1}
Section 20 permit ,date: 25 February 2020 and 17 August 2021.

This approval mdicates tha, the DAH has no objection to the publication of this Ph.D. Thests from a regulatory animal disease con♦! point of view and does not necessarity imply any agreement with any findings o, sta:ements made in the article.

Regards.

[redacted]
Name:
Reason:
Date: 2022.01.17 16:30:22
--6A1'-----11•

OrMphoM.a,jil
DIRECTOR: ANIMAL HEALTH
Date: _ _ _ _ _ _ _ _