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No Evidence of Influenza A Virus RNA in South African Backyard Swine in the uMgungundlovu District of the KwaZulu-Natal Province

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ABSTRACT

Background: Influenza A virus (IAV) is the most widely reported influenza type in swine populations worldwide. Since no information was available on IAV prevalence in backyard swine populations in South Africa, we, for the first time, performed a molecular surveillance for detecting IAV prevalence in backyard swine in the uMgungundlovu District of the KwaZulu-Natal province of South Africa.

Methods: Swine oral secretion (saliva) samples (n=102) were collected from three backyard farms distantly 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.

Result: None of the samples amplified the IAV matrix gene suggesting that the swine under investigation were free from IAV active infection; however, the quantified viral RNA of swine saliva samples needs further investigation to determine the presence of other RNA viruses. The present study was conducted during COVID-19 related lockdown restrictions in South Africa, during which a significantly decreased influenza activity was reported in the Southern Hemisphere. Increased alertness and behavioural changes in people, such as maintaining hygiene and using a face mask for day-to-day operations, might have limited the transmission of IAV between humans and swine.

Key words: Backyard swine, IAV matrix gene, IAV surveillance, Influenza A virus, Real-time RT-PCR, Swine influenza.

INTRODUCTION

Swine or domestic pigs (*Sus scrofa domesticus*) are the reservoirs of several viruses, including but not limited to the influenza A virus (IAV) (Chauhan and Gordon 2022a; Li *et al*. 2016; Patel *et al*. 2021; Rout *et al*. 2017). Interestingly, IAV is the most widely occurring influenza type reported in swine populations worldwide (Chauhan and Gordon 2020). The occurrence of α-2,3 and α-2,6 sialic acid receptors in the swine trachea make them an intermediate host for IAV transmission (Ma *et al*. 2008; Chauhan and Gordon 2022b). Most recently, the emergence of the 2009 swine-flu pandemic (Mena *et al*. 2016) reiterates the need for active IAV surveillance in swine populations.

Recent studies have suggested that the lack of suitable biosecurity increases the risk of IAV transmission from wild birds to the backyard poultry and swine (Hamilton-West *et al*. 2012; Hill *et al*. 2019; Chauhan and Gordon 2021). Furthermore, 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) and Cameroon in 2014 (Larison *et al*. 2014) are the evidence of reverse-zoonotic transmission of IAV from human to swine. These observations suggest a requirement of active IAV surveillance in backyard swine populations to safeguard backyard swine farming and public health.

The climate and geographical location of South Africa are unique for the avian migration for over-wintering and breeding (Szép *et al*. 2006). The interaction of backyard swine with wild and migratory birds poses a severe risk of IAV transmission. Since there was no information on IAV prevalence in backyard swine in the country, the present study, for the first time performed molecular surveillance to identify IAV prevalence in backyard swine populations in the uMgungundlovu District of the KwaZulu-Natal province of South Africa.

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MATERIALS AND METHODS

Ethics statement

This study had the full approval of the research protocol from the Animal Research Ethics Committee, University of KwaZulu-Natal, Durban, vide Reference# AREC/041/019D and the 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), South Africa, vide Reference# 12/11/1/5/4 (1425).

Sample collection

A total of 102 swine oral secretion (saliva) samples were collected during March 2021, from three backyard swine farms located within the uMgungundlovu District of the KwaZulu-Natal province in South Africa (Fig 1, Table 1). Samples were collected using a three-strand twisted 100% cotton rope following a standard non-invasive protocol detailed and reviewed previously (Prickett *et al*. 2008; Henao-Diaz *et al*. 2020). The data related to backyard swine farming such as biosecurity, awareness of virus diseases in swine, vaccination status and routine swine handling were collected on a questionnaire to assess the risk of IAV transmission within the backyard. In addition, data regarding the presence of poultry, cattle and wild birds and previous disease outbreaks in swine were collected to assess the risk of IAV transmission within the backyard. An Animal Health Technician (AHT) from the State Veterinary Department assisted in identifying any apparent disease symptoms in the backyard swine under study. Saliva samples were collected from both healthy as well as symptomatic swine from the backyard farms under investigation (Table 2).

Each saliva sample that was collected from 1- 5-month-old swine (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 1-3 years old swine (n = 54) represented an individual sample. At least one millilitre (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 the sterile 15 ml Falcon tubes and transported to the laboratory on dry ice. Each sample was aliquoted into three replicates in the laboratory using 2 ml sterile cryovials to avoid freeze-thawing under aseptic conditions inside a class II biological safety cabinet, properly labelled and stored in a -80°C freezer for downstream processing in a BSL-2 laboratory located in the Doris Duke Medical Research Institute at the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

Table 1: Backyard swine samples included in the present study.

Swine age	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 the swine of that age.

RNA extraction and quantification

Total RNA was extracted from swine saliva 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°C to eliminate the cells and other contaminants present in the swine saliva. Total 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 concentration and integrity of a subset of the randomly selected RNA samples were verified using Qubit Fluorometer and PerkinElmer's 'LabChip' equipment, respectively. The RNA samples were stored in a -80°C freezer for downstream processing.

Real-time RT-PCR

The total RNA was used to detect IAV using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen). A conserved sequence of IAV matrix gene was amplified using the protocol recommended by the Centers for Disease Control

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

and Prevention (CDC), Atlanta, USA. Briefly, the oligonucleotide primers including IAV-Forward: 5′- GAC CRA TCC TGT CAC CTC TGA C -3′and IAV-Reverse: 5′- AGG GCA TTY TGG ACA AAK CGT CTA- 3′ in the presence of a TaqMan probe 5′- TGC AGT CCT CGC TCA CTG GGC ACG- 3′ were used in the assay (CDC 2009). TaqMan probe was labeled at the 5′ -end with the reporter molecule 6-carboxyfluorescein (FAM) and with the quencher, Blackhole Quencher 1 (BHQ1) at the 3′ -end. While the RT-PCR assay is not approved by the DALRRD for the diagnosis of IAV in pigs in South Africa, it is widely used for the molecular detection of IAV in swine internationally as reviewed in previous studies (Chauhan and Gordon 2020; Chauhan and Gordon 2022c).

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

RESULTS AND DISCUSSION

The Nanodrop 2000c Spectrophotometer was used to assess the RNA samples under study (n=102). Most of the samples had RNA concentration in the range of 100-200 ng/µl, with only a few exceptions when RNA concentrations were either below or above this range. Further, the concentration and integrity of a subset of the randomly selected RNA samples (n = 10) was verified using Qubit Fluorometer and PerkinElmer's '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 (Fig 2). These observations confirmed the concentration and integrity of viral RNA in the backyard swine samples under investigation. To our surprise, none of the 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. It should be noted that the absence of IAV in a subset of backyard swine populations investigated in the present study does not necessarily rule out the possibility of IAV prevalence at other backyard swine holdings in South Africa.

It was noteworthy that during COVID-19 pandemic, a significantly decreased influenza activity was reported in the Southern Hemisphere, including South Africa. 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 in the flu season during 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, *viz*., Australia, Chile and South Africa. In contrast, 24,512 influenza-positive human specimens were reported out of 178,690 tested during April-July in 2017-2019 in these countries (Olsen *et al*. 2020). Substantial decrease in influenza circulation during COVID-19 pandemic may be influenced by increased alertness and behavioural changes in people for hygiene and using a face mask for day-to-day operations in public places to mitigate the transmission of the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV2). Most likely, the protective behaviour of people could significantly impact the

Table 2: Health status of backyard swine observed during sampling.

Backyard farm ID	Swine age	Health status of swine sampled*
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. swine had red patches on the skin, one swine had red eyes, one swine appeared lethargic and inactive
	1-year	Apparently healthy
	2-years	Apparently healthy
	3-years	Apparently healthy

*An Animal Health Technician (AHT) from State Veterinary Department visually identified the disease symptoms in backyard swine under investigation.

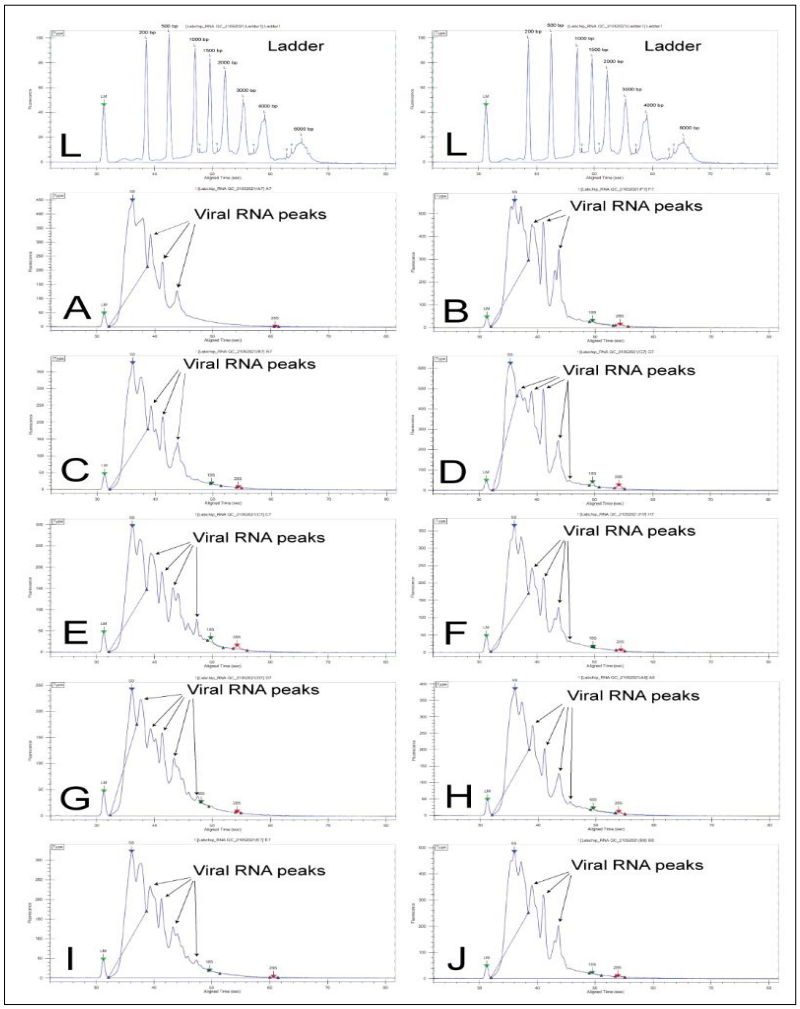

Fig 2: 'L' represents 'Ladder' ranging from 200 to 6000 nucleotides. 'A' to 'J' represent peaks of the viral RNA samples under investigation, ranged between approximately 300 to 1500 nucleotides. The negligible quantities of 18s and 28s ribosomal RNAs verified the elimination of cells and contaminants during RNA extraction by centrifugation of the swine saliva samples at 1,500 g.

transmission of IAV, provided that the SARS-CoV2 and IAV share the same transmission route (Cowling *et al*. 2020). We noticed that the backyard swine farmers and the household members except one were wearing a face mask during our visits for backyard swine sampling. Such practices would limit the transmission of IAV between humans and swine.

In this study, all the backyard swine farmers mentioned the interactions of wild birds with their swine. While two of the backyard farms kept their swine confined within the pens, the third backyard farm had their swine free-roaming and contained within the pens. It was noticed that some of the piglets at the second backyard farm could exit the pens because the gates were not kept in good condition and also it was observed that the free-roaming swine at the third backyard farm were in contact with sheep. Interestingly, few piglets and growers at this backyard farm had redness in the eyes and red patches on the skin. These observations indicated a considerable risk that exists in terms of disease transmission between animal species that were in contact.

Interestingly, it was noticed that a few piglets at the second and third backyard farms were coughing and sneezing during sampling, while a few others appeared to be lethargic and inactive. In addition, the farmers observed diarrhoea and pneumonia in some of the swine in previous months. Interestingly, Padmanabhan and Hause (2016) reported that Porcine astrovirus-4 infection in piglets in the USA was associated with acute respiratory symptoms, mimicking influenza-like illness. The observance of acute respiratory symptoms in some of the piglets in the present study and their association with Porcine astroviruses requires further investigation.

CONCLUSION

The inadequate biosecurity standards at the South African backyard swine farms put the backyard swine farming at high risk of disease transmission. Therefore, periodic active surveillance of IAV in South African backyard swine populations to safeguard the backyard swine farming practices and public health is essential. The extracted viral

RNA that was used in present study can be further used for the detection of other RNA viruses of swine.

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Conflict of interest: None.

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