UNIVERSIDADE FEDERAL DE PERNAMBUCO
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ADALÚCIA DA SILVA

VIGILÂNCIA EPIDEMIOLÓGICA DOS VÍRUS INFLUENZA A E ZIKA EM SUÍNOS DOMÉSTICOS NO ESTADO DE PERNAMBUCO

Recife
2020

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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

Área de concentração: Biologia Molecular

Orientador: Dr. Lindomar José Pena

Coorientador: Dr. Christian Robson de Souza Reis

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RESUMO

O vírus influenza A (IAV) é um importante causador de doença respiratória em suínos, mas a epidemiologia da influenza suína no Brasil ainda é pouco conhecida. Já o vírus Zika (ZIKV) causou uma epidemia em humanos no Brasil. Experimentalmente, suínos são susceptíveis à infecção pelo ZIKV, mas o papel desses animais na ecoepidemiologia do ZIKV sob condições naturais ainda não foi estabelecido. O objetivo principal desta proposta foi realizar a vigilância epidemiológica dos vírus influenza e Zika em suínos domésticos no Estado de Pernambuco. Pra isso, 500 amostras de *swab* nasal ou pulmão de suínos foram coletadas no Estado de Pernambuco. Das amostras avaliadas 9,4% (n=48) foram positivas para IAV. As análises filogenéticas dos genes HA e NA demonstraram estreita relação entre as cepas pernambucanas e as cepas H3N2 humanas circulantes na América Latina e na América do Norte entre 2012 e 2018 e os genes internos relacionados com cepas da China e Estados Unidos. A análise sorológica mostrou 92,6% de positividade para o subtipo H1N1 pdm09, 86,2% para o H3N2 e 6,18% eram negativas para ambos os subtipos. Os títulos contra H3N2 foram mais elevados quando comparados aos títulos contra H1N1. Em humanos de 2010 a agosto de 2019 6% dos pacientes foram positivos para IAV e 2% para influenza B (IBV) em Pernambuco. Dos diagnosticados com IAV, 50,76% eram H1N1 pdm09, seguido por 37,31% H3N2 e 11,93% não subtipado, sendo o H1N1pdm09 associado tanto a infecções mais brandas quanto infecções graves. Além disso, as taxas de positividade do IAV foram maiores nos meses de chuvosos e de temperatura mais amena. Os soros de suínos foram utilizados para ensaio de redução de neutralização de placa (PRNT), para detectar a presença de anticorpos neutralizantes contra ZIKV. Porém as amostras foram não reagentes para ZIKV, indicando que os animais não entraram em contato com o vírus e que suínos possivelmente não desempenham papel relevante na cadeia de transmissão do ZIKV. Juntos, esses estudos caracterizam de maneira inédita a epidemiologia de influenza A e Zika no estado de Pernambuco, fornecendo informações importantes para produtores, gestores de saúde e a comunidade científica.

Palavras Chaves: Influenza A. Suínos. ZIKV. Epidemiologia.

ABSTRACT

Influenza A virus (IAV) is a major cause of swine respiratory disease, but the epidemiology of swine influenza in Brazil is still poorly understood. Already the Zika virus (ZIKV) caused an epidemic in humans in Brazil. Experimentally, pigs are susceptible to ZIKV infection, but the role of these animals in the ecoepidemiology of ZIKV under natural conditions has not yet been established. The main objective of this proposal was to carry out epidemiological surveillance of influenza and Zika viruses in domestic swine in the state of Pernambuco. A total of 500 nasal swabs or lungs were collected from swine in Pernambuco State, Brazil. Of the samples evaluated 9.4% (n = 48) were positive for IAV. Phylogenetic analyzes of the HA and NA genes demonstrated a close relationship between Pernambuco strains and circulating human H3N2 strains in Latin America and North America between 2012 and 2018 and the internal genes related to strains from China and the United States. Serological analysis showed 92.6% positivity for H1N1 subtype pdm09, 86.2% for H3N2 and 6.18% negative for both subtypes. Titles against H3N2 were higher compared to titles against H1N1. In humans from 2010 to August 2019 6% of patients were positive for IAV and 2% for influenza B (IBV) in Pernambuco. Of those diagnosed with VAS, 50.76% were H1N1 pdm09, followed by 37.31% H3N2 and 11.93% not subtype, with H1N1pdm09 associated with both milder and severe infections. In addition, IAV positivity rates were higher in the rainy and milder months. Pig sera were used for plaque neutralization reduction assay (PRNT) to detect the presence of neutralizing antibodies against ZIKV. However, the samples were non-reactive to ZIKV, indicating that the animals did not come into contact with the virus and that pigs may not play a relevant role in the ZIKV chain of transmission. Together, these studies uniquely characterize the epidemiology of influenza A and Zika in the state of Pernambuco, providing important information to producers, health managers and the scientific community.

Key Words: Influenza A. Swine. ZIKV. Epidemiology.

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LISTA DE ABREVIATURAS E SIGLAS

Item	Definição
BA	**B**ahia
BHI	***B**rain **H**eart **I**nfusion* - Infusão de Cérebro e Coração
C	**C**apsídeo
CDC	***C**enter for **D**isease **C**ontrol and **P**revention* - Centro de Controle e Prevenção de Doenças dos Estados Unidos
cDNA	***C**omplementary **DNA*** - DNA complementar
CEUA	**C**omissão de **É**tica no **U**so de **A**nimais
CTNBio	**C**omissão **T**écnica **N**acional de **Bio**segurança
DENV	***D**engue **V**irus - Vírus da Dengue*
DMEM	***D**ulbecco's **M**odified **E**agle **M**edium*
FP	***F**unctional* **P**husion - Fusão funcional
HA	***H**emaglutinina*
HA	Teste de **H**emaglutinação
HEK 293	***H**uman **E**mbryonic **K**idneys cells* - Células de rim embrionário humano
HI	***H**emagglutination-**i**nhibition* - Inibição da Hemaglutinação
IAV	**I**nfluenza **A** **V**írus
IBV	**I**nfluenza **B** **V**írus
ICV	**I**nfluenza **C** **V**írus
IDV	**I**nfluenza **D** **V**írus
IFN-α	Interferon α
IFN-β	Interferon β
ILI	***I**nfluenza-**L**ike **I**llness* - Doença semelhante a gripe
JEV	***J**apanese **E**ncephalitis **V**irus* - Vírus da Encefalite Japonesa
MAPA	**M**inistério da **A**gricultura, **P**ecuária e **A**bastecimento

MDCK	***M**adin **D**arby **C**anine **K**idney cells* – Células de rim canino Madin Darby
NAS	***N**uclear **A**ccumulation **S**ignal* - Sinal de acumulação nuclear
NEP	***N**uclear **E**xport **P**rotein* - Proteína de exportação nuclear
NLS	***N**uclear **L**ocalization **S**ignal* - Sinal de localização nuclear
NPC	***N**uclear **P**ore **C**omplex- C*omplexo de poros nucleares
NS	***N**on **S**tructural protein*- Proteína não estrutural
OMS	**O**rganização **M**undial de **S**aúde
ORF	***O**pen **R**eading **F**rame* - Quadro de leitura aberta
PA	Polimerase ácida
PB1/2	Polimerase básica 1/2
PBS	***P**hosphate **B**uffered **S**aline* - Tampão fosfato salino
PE	**Pe**rnambuco
PKR	***P**rotein* **K**inase ***R*** - Proteína quinase R
prM	Proteína **Pr**ecursora da **M**embrana
PRNT	***P**laque **R**eduction **N**eutralization **T**est* - Teste de neutralização de redução de placa
qRT-PCR	***Q**uantitative **R**eal **T**ime **P**olymerase **C**hain **R**eaction* - Reação em cadeia da polimerase em tempo real quantitativo
RDE	***R**eceptor **D**estroying **E**nzyme* -Enzima destruidora de receptor
RN	**R**io Grande do **N**orte
RNP	**R**ibo**n**ucleo**p**roteína
RT-PCR	***R**everse **T**ranscription **P**olymerase **C**hain **R**eaction* - Reação em cadeia da polimerase por transcrição reversa
SA	***S**ialic **a**cid* - Ácido siálico
SARI	***S**evere **A**cute **R**espiratory **I**nfection*- Doença respiratória aguda grave
SIV	***S**wine **I**nfluenza **v**irus* - *V*írus da Influenza Suína
SRAG	**S**indrome **R**espiratória **A**guda **G**rave
STAT2	***S**ignal **T**ransducer and **A**ctivator of **T**ranscription **2*** -Transdutor de

	Sinais e Ativador da Transcrição 2
TBEV	***T**ick-**B**orne **E**ncephalitis **V**irus* - Vírus da encefalite transmitida por carrapatos
TRIG	***T**riple **R**eassortant **I**nternal **G**ene* - Genes internos triplo rearranjados
USDA	***U**nited **S**tates **D**epartment of **A**griculture* - Departamento de agricultura dos Estados Unidos
UTR	***Un**translated **R**egion* – Região não traduzida
vRNA	***V**iral **RNA-*** RNA viral
vRNP	***V**ira **RI**bonucleoprotein* - Ribonucleoproteína viral
YFV	***Y**ellow **F**ever **V**irus* - Vírus da Febre Amarela
WNV	***W**est **N**ile **V**irus*- Vírus do Oeste do Nilo
ZIKV	**Z**ika **v**írus- Vírus Zika

SUMÁRIO

1 INTRODUÇÃO

Os suínos desempenham um papel crítico na gênese e epidemiologia de diversos patógenos, sobretudo em lugares onde a produção não respeita as normas de higiene. Na região Nordeste do Brasil, as falha nas condições de higiene e manejo dos plantéis favorece a disseminação de patógenos. O conhecimento dos vírus circulantes pode fornecer alternativas de controle da disseminação de patógenos e favorecer o desenvolvimento econômico da região.

Os vírus influenza A pertencem à família *Orthomyxoviridae,* gênero *Alphainfluenzavirus* e como tal são partículas envelopadas com material genômico composto por oito segmentos de RNA de fita simples de sentido negativo. Esses vírus têm a capacidade de infectar humanos, equinos, aves e suínos. Quando ocorre intercâmbio viral entre humanos e animais, são geradas mutações decorrentes de rearranjos gênicos, favorecendo o surgimento de novas cepas virais.

O vírus Zika é um arbovírus pertencente à família *Flaviviridae*, isolado pela primeira vez em macacos na década de 40. Em 2015, o vírus Zika reemergiu causando doenças neurológicas congênitas em humanos, sobretudo na região nordeste do Brasil. A transmissão é principalmente urbana e silvestre, com humanos servindo como hospedeiros primários de amplificação em áreas onde não há primatas não humanos. Porém pouco se sabe a respeito da participação de outros animais nesse processo. Por isso investigar outros animais como possíveis reservatórios do vírus Zika se faz primordial para o controle da doença.

No Brasil o conhecimento dos principais vírus circulantes é escasso e ainda não existe nenhum sistema de monitoramento oficial desses números em animais. Assim, o presente projeto tem como objetivo a caracterização das cepas de influenza suína circulantes e também a busca sorológica do vírus Zika nos suínos no Estado de Pernambuco.

1.1. OBJETIVOS

1.1.1. Objetivo geral

Realizar a vigilância epidemiológica dos vírus influenza e Zika em suínos domésticos no Estado de Pernambuco.

1.1.2. Objetivos específicos

1. Identificar à presença de vírus influenza A em suínos domésticos residentes no Estado de Pernambuco;
2. Caracterizar geneticamente amostras do vírus da influenza de suínos no estado de Pernambuco;
3. Determinar a soroprevalência dos vírus H3N2 de origem humana e do vírus pandêmico H1N1 pdm09 em suínos de Pernambuco;
4. Mapear geneticamente amostras do vírus da influenza isolado de humanos em Pernambuco;
5. Realizar o levantamento epidemiológico da influenza entre seres humanos que apresentam Síndrome gripal (*Influenza Like Illness – ILI*) e Síndrome respiratória aguda grave (*Severe acute respiratory infection* – SARI) em Pernambuco, entre os anos de 2010 a 2019;
6. Obter evidências de uma possível circulação zoonótica do vírus Zika em suínos domésticos.

2 REFERENCIAL TEÓRICO

2.1 OS VÍRUS INFLUENZA

Os vírus Influenza são partículas envelopadas pertencentes a família *Orthomyxoviridae* e seu material genômico é constituído por 7-8 segmentos de RNA fita simples de sentido negativo (BOUVIER; PALESE, 2008). Eles são classificados em quatro tipos: A, B, C e D. Os vírus influenza A (IAV) infectam uma grande variedade de espécies, desde mamíferos até aves, os principais hospedeiros dos vírus influenza B (IBV) e C (ICV) são os humanos e os vírus influenza D (IDV), até o momento, foram encontrados infectando bovinos, cabras e suínos (ZHAI; ZHANG; CHEN; ZHOU *et al.*, 2017).

2.1.1 Propriedades gerais dos vírus Influenza A

O vírion pode ser dividido em três componentes: o envelope, a camada intermediária e o core (FUJIYOSHI; KUME; SAKATA; SATO, 1994; STEVAERT; NAESENS, 2016).

O envelope é formado por uma bicamada lipídica onde estão inseridas as proteínas transmembrana hemaglutinina (HA), neuraminidase (NA) e o canal de íon M2. A camada intermediária é formada pela proteína matriz (M1) e o core viral é composto pela ribonucleoproteína viral (vRNP) composto pela nucleoproteína (NP), 08 segmentos do RNA viral (vRNA), a proteína de exportação nuclear (NEP) (proteína não estrutural NS2) e o complexo de polimerases viral (PB2, PB1, PA) **(Figura 1)** (NAYAK; BALOGUN; YAMADA; ZHOU *et al.*, 2009; ZHANG; PEKOSZ; LAMB, 2000).

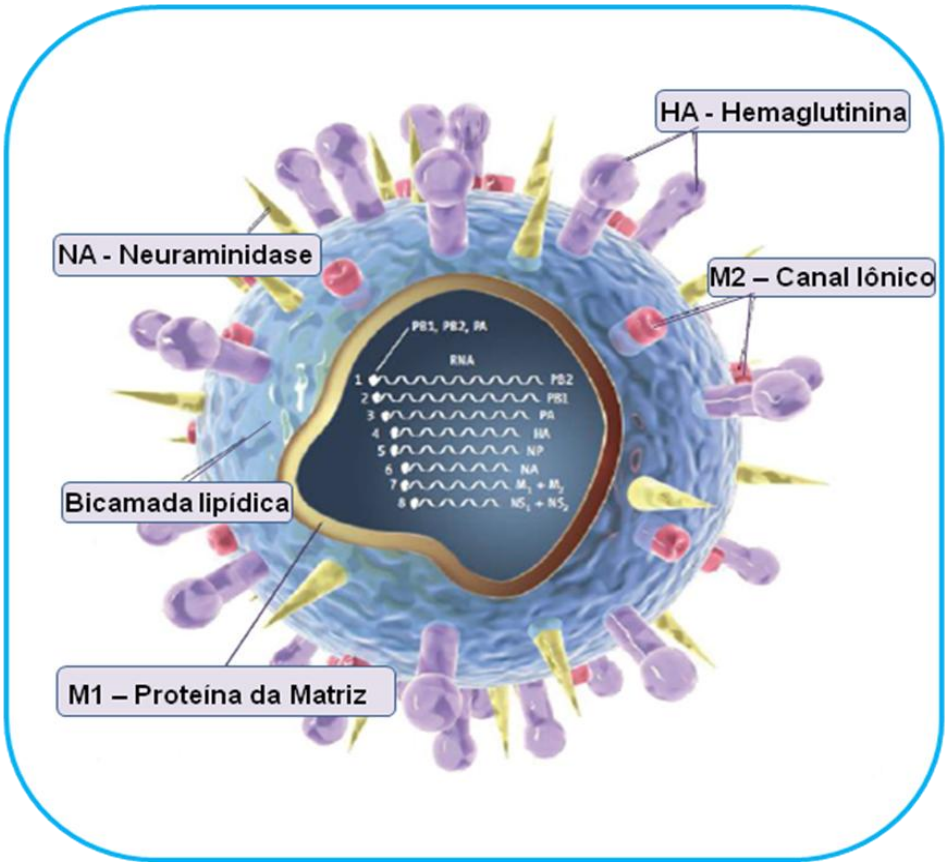

Figura 1. Figura esquemática do vírion do vírus Influenza A. Existem três proteínas incorporadas ao envelope dos vírus Influenza A: hemaglutinina, neuraminidase e proteína da matriz 2. Abaixo do envelope está a matriz, composta pela proteína da matriz 1, que envolve o genoma de RNA de cadeia negativa segmentado. O genoma consiste em oito segmentos que são agrupados em complexos de ribonucleoproteínas, com as três subunidades de polimerase (turquesa, verde e laranja) montadas nos terminais de RNA genômico. A proteína não estrutural 2 também é incorporada ao Vírion. **Fonte:** Adaptado de (SCHOTSAERT; DE FILETTE; FIERS; SAELENS, 2009).

No IAV, os segmentos de vRNA 1, 2 e 3 codificam as proteínas que compõem o complexo de polimerases viral PB2, PB1 e PA, respectivamente (STEVAERT; NAESENS, 2016).

O segmento 4 codifica a hemaglutitina (HA), proteína responsável pela ligação do vírus a receptores de ácido siálico na célula hospedeira. Esta proteína é conhecida por ser imuno-dominante e por essa razão é bastante estudada como alvo vacinal (BLIJLEVEN; BOONSTRA; ONCK; VAN DER GIESSEN *et al.*, 2016). O segmento 5 codifica a Nucleoproteína (NP), que se liga ao vRNA, estabilizando-o e protegendo-o da ação de RNAses (GALLAGHER; TORIAN; MCCRAW; HARRIS, 2017).

A neuroaminidase é codificada pelo segmento 6, além de ser uma estrutura antigênica, participa na liberação de novos vírions da superfície da célula hospedeira, permitindo assim a disseminação viral devido a sua atividade sialidásica (SHTYRYA; MOCHALOVA; BOVIN, 2009).

As proteínas M1 e o canal iônico M2, ambas codificadas pelo segmento 7, M1 estabiliza a estrutura do envelope viral atuando como “esqueleto” para ancoragem de outras proteínas virais e M2 atua como viroportina mediando a acidificação do envelope viral e a fusão do envelope viral a na membrana da célula hospedeira citosol (LEIDING; WANG; MARTINSSON; DEGRADO *et al.*, 2010; STAUFFER; FENG; NEBIOGLU; HEILIG *et al.*, 2014).

O segmento 8 codifica as proteínas NS1 e NS2. A NS1 está envolvida na fuga dos mecanismos antivirais das células hospedeiras, bem como a regulação da expressão gênica do hospedeiro e do vírus (GARCÍA-SASTRE, 2011) e NS2, também conhecida como proteína de exportação nuclear (NEP), participa da exportação do complexo ribonucleoproteico viral (vRNP) do núcleo para o citoplasma e interage com as nucleoporinas, provavelmente atuando como um adaptador entre o vRNP e o complexo de poros nucleares (PATERSON; FODOR, 2012).

Na **Tabela 1** são apresentadas o conjunto de todas proteínas do vírus Influenza A e suas principais funções de forma resumida.

Tabela 1: Proteínas do vírus Influenza A e suas respectivas funções

Segmento gênico	Proteína	Função da proteína	Referência
1	**PB2**	Componente do complexo da polimerase viral; participa do processo de transcrição, com o reconhecimento e captura de *Cap* e também no processo de replicação. Interage diretamente com PA	(LI; RAO; KRUG, 2001; LONG; FODOR, 2016; SHI; SUMMERS; PENG; GALARZ, 1995)
1	**PB2-S1**	Liga-se a PB1, localiza-se nas mitocôndrias e inibe a via de sinalização	(YAMAYOSHI; WATANABE; GOTO;

		dependente de RIG-I	KAWAOKA, 2016)
2	**PB1**	Componente do complexo da polimerase viral. Subunidade catalítica da RNA polimerase dependente de RNA viral, responsável pelo alongamento da cadeia de RNA e requerida tanto para replicação quanto para transcrição, interage com PB2 e PA.	(STEVAERT; NAESENS, 2016)
2	**PB1-F2**	Derivada de uma ORF alternativa na posição +1. PB1-F2 é o fator de virulência, induz a apoptose associada à mitocôndria.	(KAMAL; ALYMOVA; YORK, 2017; ZAMARIN; ORTIGOZA; PALESE, 2006)
2	**PB1-40**	PB1-N40 é a forma truncada N-terminal de PB1, atua inibindo a resposta ao Interferon.	(VASIN; TEMKINA; EGOROV; KLOTCHENKO *et al.*, 2014; WISE; FOEGLEIN; SUN; DALTON *et al.*, 2009)
3	**PA**	Componente do complexo da polimerase viral e participa no processo de transcrição e replicação, por fornecer uma atividade de endonuclease de RNA (*cap snatching*). PA interage com PB1.	(DATTA; WOLKERSTORFER; SZOLAR; CUSACK *et al.*, 2013; HARA; SCHMIDT; CROW; BROWNLEE, 2006)
3	**PA-X**	PA-X modula a resposta do hospedeiro e a virulência viral.	(BAVAGNOLI; CUCUZZA; CAMPANINI; ROVIDA *et al.*, 2015)
3	**PA-N155** **PA-N182**	Provável função durante o ciclo de replicação viral	(MURAMOTO; NODA; KAWAKAMI; AKKINA *et al.*, 2013)
4	**HA**	Importante fator de tropismo viral; receptor de ligação; atividade de fusão; e maior antígeno viral.	(BLIJLEVEN; BOONSTRA; ONCK; VAN DER GIESSEN *et al.*, 2016; BYRD-LEOTIS; GALLOWAY; AGBOGU; STEINHAUER, 2015)
5	**NP**	Principal componente do complexo RNP viral,controla o transporte nuclear- citoplasmático do vRNA.	(CHUTIWITOONCHAI; KAKISAKA; YAMADA; AIDA, 2014; NAYAK; BALOGUN; YAMADA; ZHOU *et al.*, 2009; YU; LIU; CAO; ZHAO *et al.*, 2012)
6	**NA**	Glicoproteína de superfície; cliva o ácido siálico dos receptores celulares do HA para permitir a fuga da progênie das células infectadas.	(PIZZORNO; ABED; BOUHY; BEAULIEU *et al.*, 2012; ZANIN; DUAN; WONG; KUMAR *et al.*, 2017)
7	**M1**	M1 é o principal componente da	(ARZT; PETIT;

		membrana viral; é subjacente ao envelope viral e desempenha vários papéis na montagem e infecção do virion.	BURMEISTER; RUIGROK *et al.*, 2004; ZHIRNOV; MANYKIN; ROSSMAN; KLENK, 2016)
7	**M2**	Proteína de membrana que forma um canal de prótons que é ativado sob condições de baixo pH; importante para descompactar o genoma durante a entrada do vírus	(LEIDING; WANG; MARTINSSON; DEGRADO *et al.*, 2010)
7	**M42**	Funcionalmente semelhante a M2, porém com diferença na localização celular.	(WISE; HUTCHINSON; JAGGER; STUART *et al.*, 2012)
8	**NS1**	A NS1 é uma proteína multifuncional que está envolvida em inúmeras interações vírus - hospedeiro, incluindo a fuga dos mecanismos antivirais das células hospedeiras, bem como a regulação da expressão gênica do hospedeiro e do vírus.	(ABDELWHAB; VEITS; BREITHAUPT; GOHRBANDT *et al.*, 2016; BERGMANN; GARCIA-SASTRE; CARNERO; PEHAMBERGER *et al.*, 2000)
8	**NEP/NS2**	Proteína não estrurural de exportação nuclear dos RNPs virais	(PATERSON; FODOR, 2012)
8	**NS3**	O seu papel pode estar associado à adaptação viral a outros hospedeiros.	(SELMAN; DANKAR; FORBES; JIA *et al.*, 2012; VASIN; TEMKINA; EGOROV; KLOTCHENKO *et al.*, 2014)

Fonte: A autora

2.1.2 Replicação

Cada segmento de vRNA constitui uma unidade de replicação individual que juntamente com o complexo polimerase heterotrimérico ligado nos terminais 5' e 3' pareados do vRNA e múltiplas cópias de NP, forma a ribonucleoproteínas virais (vRNPs) (EISFELD; NEUMANN; KAWAOKA, 2015; GALLAGHER; TORIAN; MCCRAW; HARRIS, 2017).

Os IAVs entram nas células hospedeiras através de endocitose após a proteína HA viral se ligar às moléculas receptoras de ácido siálico na membrana plasmática do hospedeiro (SRIWILAIJAROEN; SUZUKI, 2012; THOENNES; LI; LEE; LANGLEY *et al.*, 2008). Após a internalização, ocorre a acidificação do endossomo e alterações conformacionais do HA, o que leva à fusão entre o vírion

e as membranas endossomais, proporcionando ao genoma do vírus uma porta de acesso ao citoplasma (SHEN; ZHANG; LIU, 2013).

Ao mesmo tempo, o canal iônico M2 viral promove a acidificação do interior do vírion, fazendo com que a proteína matriz M1 se dissocie do genoma viral (STAUFFER; FENG; NEBIOGLU; HEILIG *et al.*, 2014). As vRNPs liberadas são transportadas para o núcleo, e a transcrição primária resulta na produção de mRNAs virais, que são exportados para o citoplasma e traduzidos para proteínas pelos ribossomos celulares. As proteínas virais recém traduzidas são então transportadas para o núcleo (PB2, PB1, PA, NP, M1 e NEP) ou para a membrana plasmática (HA, NA e M2) (PATERSON; FODOR, 2012).

Após a entrada nuclear de PB2, PB1, PA e NP, é iniciada a replicação do genoma e novas vRNPs são produzidas. As vRNPs recém sintetizadas são exportados para o citoplasma com a assistência das proteínas M1 e NEP e posteriormente levadas para a membrana plasmática em vesículas Rab11 incorporadas em partículas de vírus da progênie contendo HA, NA, M2 e M1(NAYAK; HUI; BARMAN, 2004; PATERSON; FODOR, 2012).

A liberação de vírus da membrana plasmática é mediada pelas atividades de pelo menos duas proteínas da superfície do virion, M1 e NA: M1 promove a agregação dos segmentos de vRNA, enquanto o NA previne a agregação do vírus na superfície celular **(Figura 2).** Todos os segmentos de vRNA possuem regiões altamente conservadas de 12 ou 13 nucleotídeos em cada uma de suas extremidades, que servem como sinalizadores para o processo de “empacotamento”. Mesmo assim, muitas partículas virais não terão todos os oito segmentos de vRNA ou terão vRNAs em excesso, essas partículas são chamadas de defectivas, pois não possuem a capacidade de se replicar nas células hospedeiras de maneira eficiente (NAYAK; HUI; BARMAN, 2004; SAMJI, 2009; ZANIN; DUAN; WONG; KUMAR *et al.*, 2017).

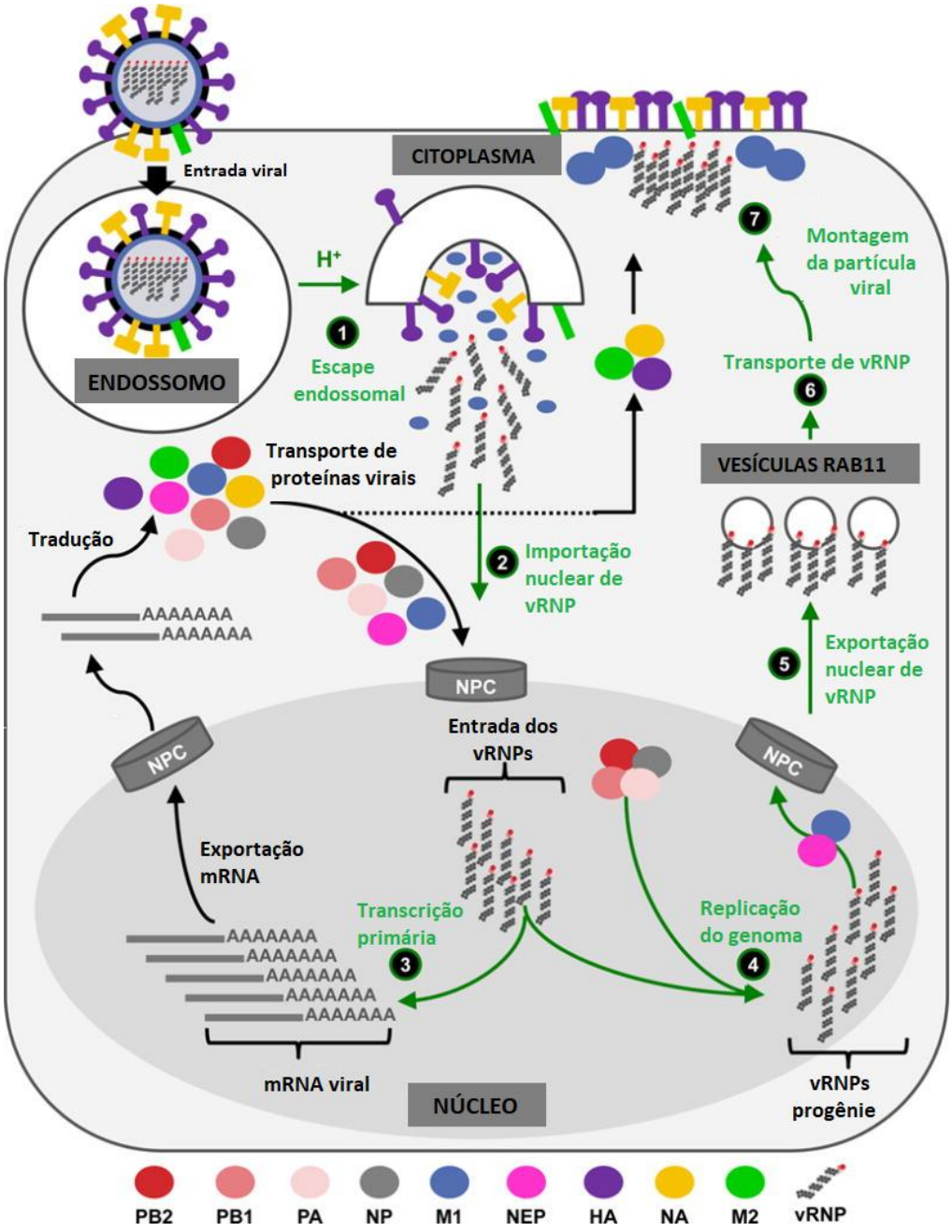

Figura 2: Ciclo Replicativo dos IAV. IAVs são endocitados pelas células após a ligação de HA às moléculas receptoras na membrana plasmática. Na **etapa 1** o escape das vRNPs do endossomo após a acidificação e transporte através do complexo de poros nucleares (NPC) **(etapa 2)**, a transcrição primária de mRNAs virais, que são serão traduzidos por ribossomos celulares **(etapa 3).** Na etapa 4 replicação do genoma e produção de vRNPs da progênie que são então exportadas para o citoplasma **(etapa 5)** e levadas a membrana plasmática em vesículas RAB11 **(etapa 6)** e incorporadas aos vírions e então liberadas **(etapa 7). Fonte:** adaptado de **(EISFELD; NEUMANN; KAWAOKA, 2015).**

2.1.3 Mecanismos evolutivos dos IAV

Os IAV possuem um grande e antigenicamente diverso reservatório viral em aves aquáticas e aves migratórias selvagens, nas quais a infecção é geralmente assintomática. Através de sua migração, os vírus são transmitidos globalmente,

podendo cruzar a barreira das espécies (OLSEN; MUNSTER; WALLENSTEN; WALDENSTRÖM *et al.*, 2006) **(Figura 3)**.

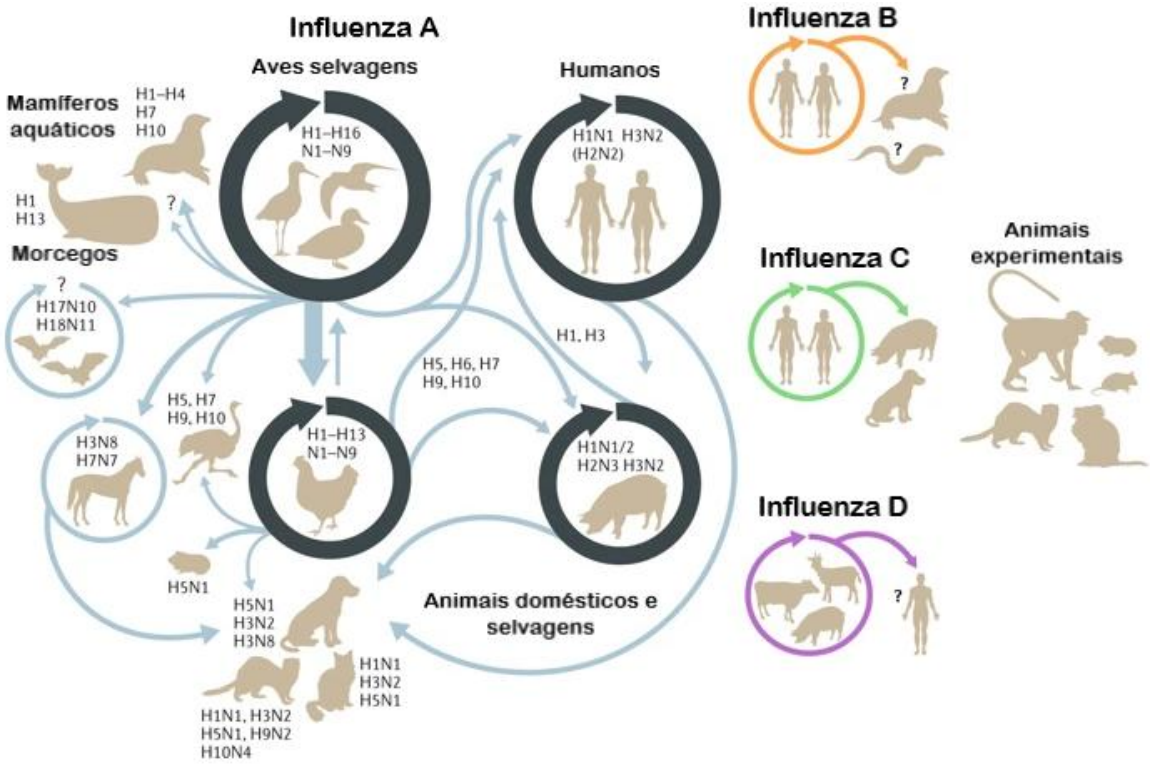

Figura 3. Ecologia dos vírus influenza. Os IAV (subtipos de hemaglutinina (HA) 1 a 16) circulam no reservatório de aves selvagens. Os subtipos deste reservatório são capazes de infectar muitas espécies diferentes, às vezes por meio de hospedeiros intermediários e às vezes exigindo mutações adaptativas (setas azuis claras). Subtipos específicos predominam em certas espécies (círculos azuis escuros). Os IAVs dos subtipos H1, H2 e H3 circulam em humanos; os mesmos três subtipos também circularam em suínos. Os subtipos H5, H6, H7, H9 e H10 infectam os seres humanos após a exposição a aves infectadas e geralmente não são transmitidos entre humanos. Os subtipos de HA H17 e H18 circulam apenas em morcegos. Os vírus da influenza B circulam em humanos, embora infecções em focas tenham sido descritas. Os vírus da influenza C circulam em humanos e suínos. Os vírus Influenza D circulam em bovinos, cabras e suínos. Muitas espécies foram infectadas experimentalmente por vírus influenza, incluindo furões, camundongos, porquinhos da índia, macacos e saguis. **Fonte:** Adaptado de (LONG; MISTRY; HASLAM; BARCLAY, 2019)

Ocasionalmente, os vírus influenza de animais infectam seres humanos. Muitas dessas zoonoses não conseguem se dispersar, isto é, não há transmissão subsequente e ocorrem em apenas um pequeno número de indivíduos. Os vírus recém-adquiridos tornam-se enzoóticos em uma nova espécie somente após a acumulação de alterações genéticas que adaptam o vírus ao seu novo hospedeiro e suportam replicação e transmissão eficientes (LONG; MISTRY; HASLAM; BARCLAY, 2019; TAUBENBERGER; KASH, 2010). Essa evolução ocorre através do acúmulo de mutações pela polimerase viral propensa a erros e a seleção de vírus

que são mais capazes de se replicar e transmitir. Esse fenômeno, conhecido como deriva antigênica *(antigenic drift)*, também explica à ocorrência de epidemias sazonais de influenza A nas populações humana e animal (BEDFORD; SUCHARD; LEMEY; DUDAS *et al.*, 2014) **(Figura 4 A).**

O genoma do vírus influenza é segmentado, e isso permite a recombinação por rearranjo *(antigenic shift)*, no qual genes inteiros, que permitem rápida adaptação a uma nova espécie, podem ser rapidamente adquiridos. Em uma nova espécie, a incompatibilidade restringirá a replicação de um vírus não adaptado, no entanto, se uma única célula é co-infectada por um AIV aviário restrito e um vírus adaptado aos mamíferos, o rearranjo de genes pode dar origem a vírus com uma nova combinação de segmentos gênicos que superam a restrição (TAUBENBERGER; KASH, 2010) **(Figura 4 B).**

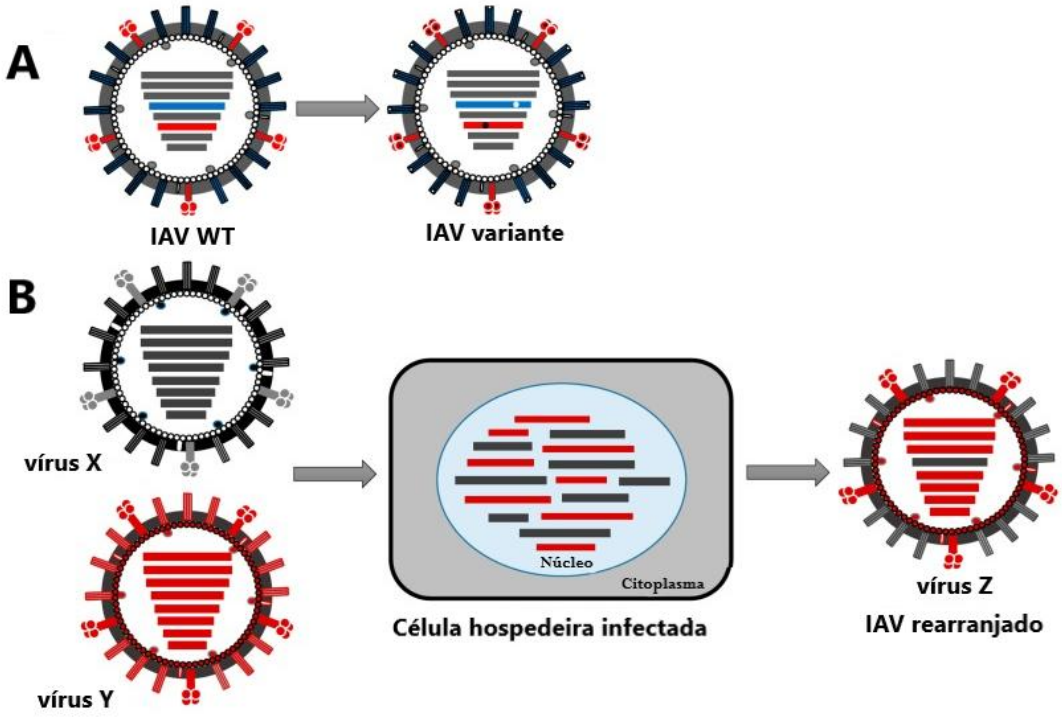

Figura 4. Mecanismos de evolução do IAV. A) Deriva antigênica *(antigenic drift):* O acúmulo gradual de mutações no genoma dos IAVs leva ao surgimento de novas variantes de vírus. Mutações no HA (azul) e NA (vermelho) podem afetar os epítopos antigênicos, levando a novas variantes antigênicas. **B)** Rearrajo *(antigenic shift)*: A troca/reagrupamento de segmentos gênicos entre dois ou mais IAVs invasores em uma célula hospedeira pode levar ao surgimento de novos subtipos distintos (antigenicamente). Adaptado de (MOSTAFA; ABDELWHAB; METTENLEITER; PLESCHKA, 2018)

Nos últimos 100 anos, houve pandemias em 1918, 1957, 1968, 1977 e 2009 (NAYAK; BALOGUN; YAMADA; ZHOU *et al.*, 2009).

Em 1918, a Gripe Espanhola, considerada a pior pandemia da história registrada, causou aproximadamente 20-40 milhões de mortes em todo o mundo. A

Gripe Espanhola foi causada pelo vírus H1N1 com genes de origem aviária e apesar de não se saber a sua origem exata, ele se espalhou pelo mundo entre os anos de 1918-1919 (REID; TAUBENBERGER; FANNING, 2001).

A Gripe asiática, em 1957, causada pelo vírus H2N2 que se originou de um vírus aviário, incluindo os genes da hemaglutinina H2 e da neuraminidase N2 e genes da influenza sazonal humana, foi responsável por cerca de 1,1 milhão mortes em todo o mundo ((CDC); JACKSON, 2009; LOURIA; BLUMENFELD; ELLIS; KILBOURNE *et al.*, 1959; SCHOLTISSEK; ROHDE; VON HOYNINGEN; ROTT, 1978).

Em 1968, a Gripe de Hong Kong, causada pelo vírus H3N2, composto por genes de origem aviária (H3N2) e humana (H2N2) que resultaram em 34.000 mortes apenas nos Estados Unidos (SCHOLTISSEK; ROHDE; VON HOYNINGEN; ROTT, 1978; TAUBENBERGER; MORENS, 2009).

Após as pandemias, os IAVs recém-emergidos continuam a circular como gripe sazonal e acumulam mutações selecionadas para evitar o acúmulo de respostas imunes na população exposta (LONG; MISTRY; HASLAM; BARCLAY, 2019).

Em abril de 2009, um novo IAV (H1N1) de origem suína, designado como A (H1N1) pdm09, surgiu no México e nos Estados Unidos e se espalhou rapidamente pelo mundo ((CDC), 2009). Análises genéticas e evolutivas revelaram que esse vírus pandêmico contém uma combinação de segmentos gênicos que não haviam sido relatados anteriormente em vírus da gripe suína ou humana em nenhuma parte do mundo **(Figura 5)**. No final dos anos 90, a reorganização entre os vírus aviário norte-americano (subtipo desconhecido), humano A (H3N2) e suíno clássico A (H1N1) resultou em três vírus suínos recombinantes A (H3N2) e A (H1N2). Um vírus triplo recombinante dos suínos A (H1N2) foi então rearranjado com um vírus aviário do tipo A (H1N1) da Eurásia, resultando no vírus A (H1N1) pdm09 (GARTEN; DAVIS; RUSSELL; SHU *et al.*, 2009; SMITH; VIJAYKRISHNA; BAHL; LYCETT *et al.*, 2009). Os segmentos PB2 e PA foram derivados da linhagem do vírus aviário, enquanto o segmento gênico PB1 era do vírus humano A (H3N2). Os segmentos gênicos HA, NP e NS eram do vírus da espécie suína clássica A (H1N1). Os segmentos gênicos NA e M eram do vírus da espécie suína A (H1N1) do tipo aviário da Eurásia (SMITH; VIJAYKRISHNA; BAHL; LYCETT *et al.*, 2009).

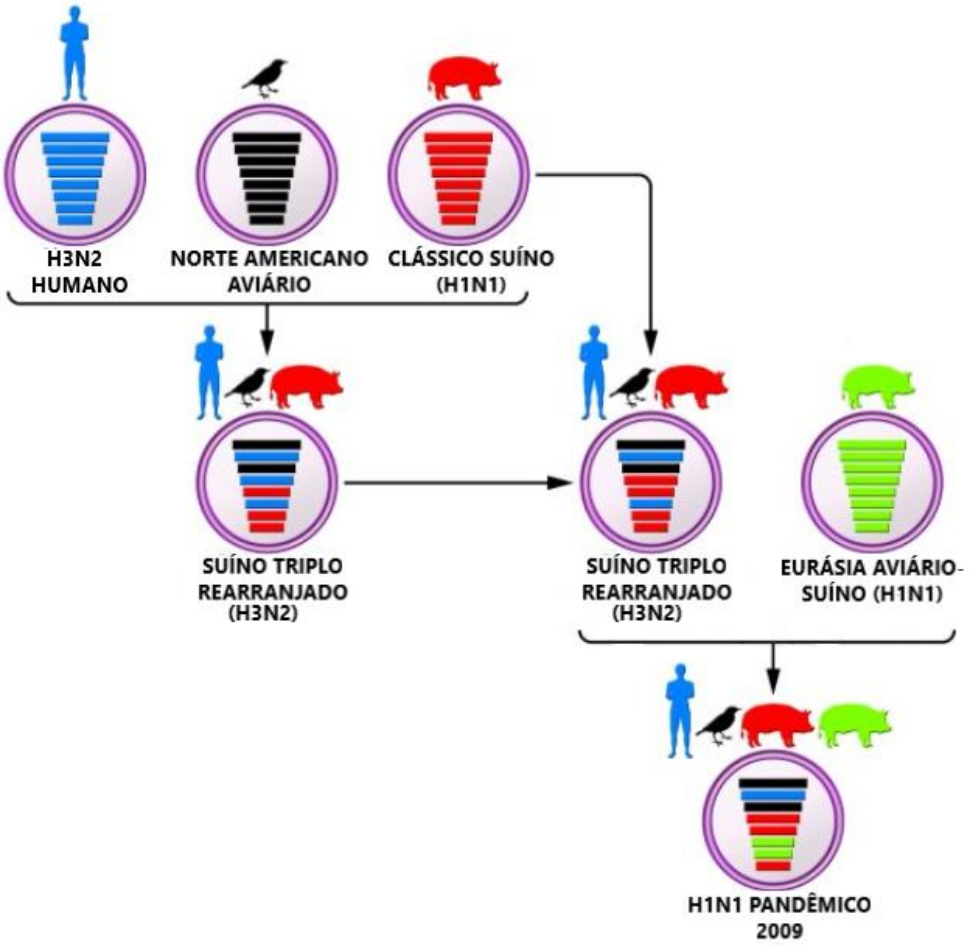

Figura 5. Origem do vírus H1N1 pdm09. O rearranjo dos vírus da América do Norte H3N2 e H1N2 triplos rearranjos (de origem norte-americana aviária, humana [H3N2] e suína clássica [H1N1]) com vírus suínos eurasiáticos do tipo aviário (H1N1) resultou no vírus pandêmico de 2009. Cada segmento gênico de origem aviária, humana ou suína corresponde a uma característica na superfície da partícula viral esquemática **Fonte:** Adaptado de (TSCHERNE; GARCÍA-SASTRE, 2011).

2.1.4 Papel dos suínos

O HA se liga às porções de ácido siálico (SA), que são os carboidratos terminais ligados a glicanos maiores de glicoproteínas e glicolipídios (glicoconjugados) na superfície das células dos vertebrados. As diferenças estruturais desses receptores entre as espécies determinam a suscetibilidade específica da espécie à infecção pelo vírus influenza (WU; MENG; SEITZ; VALENTIN-WEIGAND *et al.*, 2015).

Os vírus aviários ligam-se preferencialmente a receptores de ácido siálico alpha2,3-galactose (α2,3-AS), enquanto que as estirpes humanas se ligam a receptores alfa-2,6-galactose- (α2,6-AS). Os suínos são suscetíveis a cepas de vírus humana e aviária isso se deve ao fato de o epitélio respiratório de suínos

expressarem moléculas de ácido siálico humano e aviário (WAHLGREN, 2011). Por muitos anos, pensou-se que a barreira interespécies só poderia ser cruzada após a adaptação de um vírus da gripe aviária em suínos, uma vez que os suínos expressam ambos os tipos de receptores (α-2,3-AS e α-2,6-AS), porém, posteriormente, observou-se que os subtipos de vírus da gripe aviária H5 e H7 poderiam ser diretamente transmitidos de aves para humanos, apesar de terem especificidade de receptor α-2,3-AS (ABDELWHAB; VEITS; BREITHAUPT; GOHRBANDT *et al.*, 2016; CLAAS; OSTERHAUS; VAN BEEK; DE JONG *et al.*, 1998; COSTA; CHAVES; VALLE; DARJI *et al.*, 2012).

São descritos dois mecanismos básicos que permitiriam aos suínos servir como hospedeiros intermediários para a geração de vírus de influenza pandêmicos**.** Em um deles, os vírus aviários e humanos se rearranjariam, dando origem a uma cepa híbrida com potencial pandêmico. No outro, um vírus aviário adquire a capacidade de se ligar de forma eficiente para receptores da superfície de células humanas de modo que pudesse ser prontamente transmitidos a um hospedeiro humano sem um requisito para a recombinação genética. Esses modelos podem não ser mutuamente exclusivos. Muito possivelmente, um vírus aviário poderia se combinar com um vírus humano antes ou depois de se adaptar à ligação NeuAcα2,6Gal, resultando em um rearranjo com capacidade proliferativa aumentada (ITO; COUCEIRO; KELM; BAUM *et al.*, 1998; TSCHERNE; GARCÍA-SASTRE, 2011).

A necessidade de melhorar a vigilância epidemiológica do IAV nos suínos foi destacada pelo surgimento do vírus da pandemia H1N1 em 2009. Três principais subtipos do vírus da Influenza Suína (SIV) têm circulado em populações suínas em todo o mundo: H1N1, H1N2 e H3N2. Esses subtipos evoluem constantemente, produzindo diferentes linhagens contendo componentes genéticos derivados de cepas de influenza aviária e humana. Até o momento, a persistência endêmica do SIV em rebanhos suínos inclui diversos vírus recombinantes (CADOR; ANDRAUD; WILLEM; ROSE, 2017; VINCENT; AWADA; BROWN; CHEN *et al.*, 2014). O aumento da vigilância global da gripe em suínos fornece a primeira oportunidade de estimar a extensão da transmissão humano-suíno do IAVs.

2.2 VÍRUS ZIKA

O Zika vírus (ZIKV), arbovírus pertencente à família *Flaviviridade,* foi isolado pela primeira vez em 1947 de um macaco *rhesus* febril na Floresta Zika de Uganda e, posteriormente, identificado em mosquitos *Aedes africanus* da mesma floresta (DICK; KITCHEN; HADDOW, 1952).

Poucos estudos foram desenvolvidos desde o aparecimento do Zika até o ano de 2007, quando o vírus reemergiu causando epidemias de uma doença semelhante a dengue nas ilhas do Pacífico e na América Latina, com casos relatados também em outros continentes (DUFFY; CHEN; HANCOCK; POWERS *et al.*, 2009). No início de 2015 diversos estados do Nordeste do Brasil relataram surtos de infecção por ZIKV (ZANLUCA; MELO; MOSIMANN; SANTOS *et al.*, 2015). A associação entre infecção por ZIKV e sinais neurológicos em adultos foi confirmada em outubro de 2015, a associação entre a infecção ZIKV e os casos de microcefalia neonatal foi estabelecida, através da investigação clínica e epidemiológica (TEIXEIRA; COSTA; DE OLIVEIRA; NUNES *et al.*, 2016).

Embora a maioria dos pacientes infectados pelo ZIKV tenha uma doença febril aguda, alguns pacientes podem desenvolver complicações neurológicas graves, como a síndrome de *Guillain-Barré*, ou infecções fatais disseminadas (CHAN; YIP; TSANG; TEE *et al.*, 2016).

2.2.1 Organização genômica

O ZIKV pertence à família *Flaviviridae*, gênero *Flavivirus*, que compreende os vírus do Oeste do Nilo (WNV), Febre Amarela (YFV) e Dengue (DENV) (BASARAB; BOWMAN; AARONS; CROPLEY, 2016). O ZIKV é um vírus RNA fita simples de sentido positivo não segmentado, com aproximadamente 11Kb (CUNHA; ESPOSITO; ROCCO; MAEDA *et al.*, 2016). A partícula viral é esférica, com um diâmetro de 50 nm, e é envolvida por uma proteína de superfície disposta de maneira semelhante ao icosaédro (ATIF; AZEEM; SARWAR; BASHIR, 2016).

O genoma do ZIKV é traduzido em uma poliproteína longa no citoplasma das células infectadas. A poliproteína é adicionalmente clivada e processada por proteases do hospedeiro ou proteases virais em três proteínas estruturais (proteína precursora da membrana (prM), proteína envelope (E) e proteína capsídeo (C) e

sete proteínas não estruturais (NS1, NS2A, NS2B, NS3 , NS4A, NS4B e NS5) em um estágio posterior **(Figura 6)** (CHAMBERS; HAHN; GALLER; RICE, 1990).

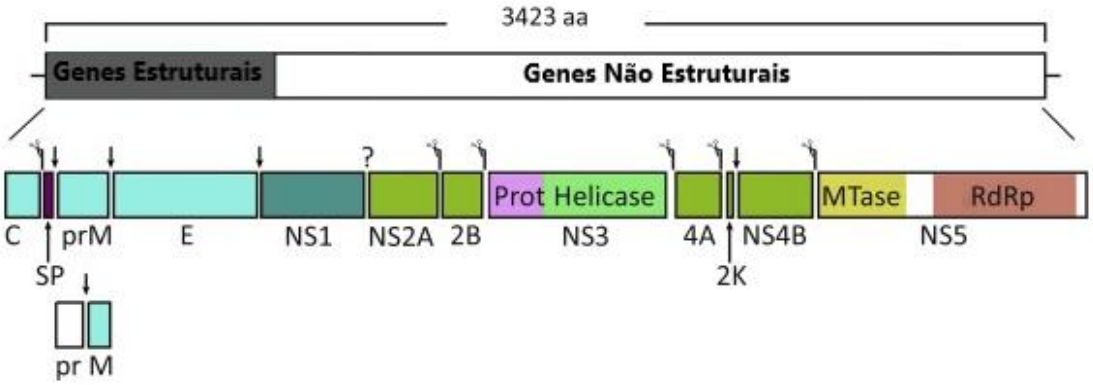

Figura 6. Organização genômica do ZIKV. Após processamento, a poliproteína dá origem a três proteínas estruturais e sete não estruturais. Adaptado de (SHI; GAO, 2017)

2.2.2 Proteínas virais

As proteínas estruturais formam a partícula viral, enquanto as proteínas não estruturais desempenham funções essenciais na replicação do genoma, processamento de poliproteínas e manipulação de respostas do hospedeiro para vantagem viral.

As proteínas estruturais são o capsídeo (C) que codifica a proteína-C, a proteína precursora da membrana prM e o envelope que codifica a proteína E (SHI; GAO, 2017).

A proteína do capsídeo dos flavivírus é considerada um importante alvo para o desenvolvimento de fármacos anti-flavivírus devido ao seu importante papel na biologia viral. Múltiplas subunidades da proteína C formam o nucleocapsídeo do vírus e envolve o material genético viral (MISHRA; UVERSKY; GIRI, 2018). O capsídeo viral é feito de proteína C, tem forma icosaédrica e é circundada por uma membrana lipídica derivada do hospedeiro e, além do papel de apoio à estrutura, a proteína capsidial também está envolvida nas funções associadas à replicação viral (SHI; GAO, 2017).

A proteína E é o principal componente envolvido na ligação ao receptor, fusão na membrana e reconhecimento imunológico do hospedeiro, e a proteína M está escondida sob a camada de proteína E. Semelhante a outros flavivírus, a proteína E do ZIKV compreende quatro domínios: o domínio transmembrana, que é responsável pela âncora de membrana; e os domínios I, II e III, que constituem o

restante predominantemente da porção superficial da cadeia proteica(SHI; GAO, 2017).

A proteina M compreende uma volta no terminal N (anel M) e região transmembranares, que ancoram a proteína M à membrana lipídica. A prM possui atividade semelhante à chaperona para a proteína Envelope e desempenha um papel fundamental na maturação da partícula viral de um estado imaturo para uma forma madura. A forma madura do vírus é virulenta, fusogênica e infecciosa, e também tem tendência de aderência com a membrana da célula hospedeira (WANG; THURMOND; ISLAS; HUI *et al.*, 2017).

A proteína NS1 está associada á patogênese viral. Esta proteína é translocada para o lúmen do retículo endoplasmático e neste compartimento assume uma forma dimérica em associação com a membrana das células infectadas. O complexo de replicação é formado por proteínas virais transmembranares, enzimas virais e proteínas NS1 (BROWN; AKEY; KONWERSKI; TARRASCH *et al.*, 2016).

A NS1 é secretada como partícula de lipoproteína hexamérica das células infectada. Os mecanismos moleculares da patogênese da NS1 estão relativamente bem estabelecidos para os flavivírus DENV e WNV, porém ainda não estão bem estabelecidos para o ZIKV (EDELING; DIAMOND; FREMONT, 2014).

O NS2A está funcionalmente envolvido na montagem do vírus, e juntamente com os componentes do complexo de replicação, participa da replicação viral, se ligando à 3′ UTR (região não traduzida) do RNA viral (PHOO; LI; ZHANG; LEE *et al.*, 2016). A proteína NS2A tem papel funcional na secreção e montagem de partículas virais, assim como está envolvida na síntese de RNA viral e participa na formação de membrana induzida por vírus e na inibição da resposta do interferon α / β (MISHRA; UVERSKY; GIRI, 2018).

O ZIKV NS2B é uma proteína de membrana de 130 resíduos de comprimento acoplada à interação com o NS3 para formar um complexo protease NS2B-NS3 ativo (WANG; THURMOND; ISLAS; HUI *et al.*, 2017).

A proteína NS3 do ZIKV é composta de 617 resíduos de aminoácidos. É uma enzima que contem um domínio N-terminal com função de protease e um domínio C-terminal com função de helicase. O domínio de protease é formado pelo trecho de resíduos de aminoácidos 1-167, enquanto o domínio helicase compreende a região entre os resíduos 168-617. Tanto a atividade enzimática de protease e helicase de NS3 são essenciais para o processamento de poliproteínas e replicação viral,

respectivamente. O complexo protease NS2B-NS3 é responsável por todas as clivagens citoplasmáticas, incluindo nas junções entre as proteínas NS2A / NS2B, NS2B / NS3, NS3 / NS4A e NS4B / NS5 e dentro das proteínas da cápside, NS2A e NS4A (LUO; VASUDEVAN; LESCAR, 2015).

A replicação de flavivírus é facilitada pelo complexo associado à membrana viral, onde as proteínas NS2A, NS2B, NS4A e NS4B agem como um suporte para a formação do complexo. Outro papel da NS4A é controlar a atividade ATPase da helicase NS3, e a proteína NS4B participa na inibição da resposta imune via sinalização do interferon. A NS4A e a proteína NS4B envolvidas na inibição da via de sinalização de Akt-mTOR levam à neurogênese defeituosa que resulta em autofagia em células-tronco neurais de fetos humanos (LIANG; LUO; ZENG; CHEN *et al.*, 2016).

Esta proteína não estrutural é necessária para a replicação do genoma de RNA dos flavivírus. NS5 é uma proteína complexa com atividade catalítica dupla, onde o domínio N-terminal de ZIKV NS5 (resíduos 1 a 255) é caracterizado pela atividade metiltransferase, enquanto o domínio C-terminal (resíduos 274-892) tem atividade de RNA polimerase dependente da RNA (RdRp). Outro papel importante da NS5 é suprimir a sinalização de IFN, que é mediada pela degradação dependente de proteassomas do STAT2 humano (UPADHYAY; CYR; LONGENECKER; TRIPATHI *et al.*, 2017).

2.2.3 Suínos como Modelo Animal da infecção por ZIKV

O ZIKV é transmitido para os seres humanos principalmente pela picada de mosquitos infectados. Porém, até o momento, a pesquisa do ZIKV tem sido focada principalmente na patogênese das complicações neurológicas associadas ao ZIKV. Já seus potenciais reservatórios animais ainda é controverso. Recentemente nosso grupo publicou um artigo de revisão sobre os modelos *in vitro* e *in vivo* para o estudo do ZIKV (PENA; MIRANDA GUARINES; DUARTE SILVA; SALES LEAL *et al.*, 2018).

Os suínos são usados como modelo para a pesquisa biomédica porque compartilham semelhanças em vários aspectos da anatomia, fisiologia, genética e resposta imunológica. Em relação à sua imunidade, os suínos assemelham-se muito aos humanos em mais de 80% dos parâmetros imunológicos, em oposição a menos de 10% dos camundongos (MEURENS; SUMMERFIELD; NAUWYNCK; SAIF *et al.*, 2012). Além disso, os suínos são suscetíveis a vários flavivírus, incluindo DENV, WNV e JEV, devido a essa suscetibilidade, é importante avaliar o comportamento

desses animais frente a infecção pelo ZIKV (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017).

Com base nisso, leitões neonatos infectados experimentalmente com uma cepa porto-riquenha de ZIKV de 2015 usando três diferentes rotas de inoculação: intracerebral, intradérmica e intraperitoneal, demonstraram que dois dos onze leitões inoculados intracerebralmente, uma rota não natural de infecção, exibiam fraqueza nas pernas, ataxia e tremor. A infecção pelo ZIKV em leitões resulta em soroconversão e viremia de baixo título, viriúria e replicação do vírus em órgãos internos, demonstrando que os suínos recém-nascidos podem ser usados como modelo para estudar alguns aspectos da biologia do ZIKV. Um estudo independente usando leitões de três meses de idade não detectou viremia ou sinais clínicos evidentes, embora os animais soroconverteram após a inoculação (RAGAN; BLIZZARD; GORDY; BOWEN, 2017).

Darbellay et al.,2017 inoculou o ZIKV diretamente nos fetos porcinos *in utero* no líquido amniótico ou intracerebral. Na prole infectada, alguns leitões apresentaram retardo do crescimento, tinham cérebros subdimensionados, convulsões, perna de trás espalhada, taxa de crescimento reduzida, comportamento agressivo (DARBELLAY; COX; LAI; DELGADO-ORTEGA *et al.*, 2017).

Em outro estudo, leitões recém-nascidos foram inoculados com ZIKV por via intracerebral e por via intraperitoneal, ambos os grupos de animais soroconverteram e apresentaram baixa viremia, virúria e replicação viral em órgãos internos, demonstrando que, suínos inoculados com ZIKV através de diferentes vias são capazes de produzir IgM, IgG e anticorpos neutralizantes contra o ZIKV (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017).

No trabalho de Wichgers Schreu *et al.,*2018, observou-se microcefalia em dois dos 15 suínos inoculados *in utero* e neuropatologia leve a grave, caracterizada por depleção neuronal nos córtices cerebrais de vários lobos, em todos os fetos (WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018).

A partir desses estudos iniciais, parece que os suínos poderiam servir como reservatórios para o ZIKV, mas não como hospedeiros amplificadores, dada a baixa viremia após a infecção. No entanto, estudos adicionais usando um número maior de animais são necessários para esclarecer o papel dos suínos na epidemiologia do ZIKV.

Até o momento nenhum caso de infecção natural pelo vírus Zika em suínos foi descrita. Contudo com base na ampla distribuição de *A. aegypti*, *A. albopictus* e outros gêneros de mosquitos, como *Culex*, capazes de transmitir o vírus, e a proximidade desses mosquitos com diversas espécies de mamíferos nas áreas urbanas e rurais em todo o país, o transbordamento do ZIKV para outros animais é um cenário potencialmente real. Um ciclo de vida selvagem lançaria uma nova dinâmica de transmissão com impactos desconhecidos em outras espécies animais, incluindo seres humanos.

O ensaio PRNT faz parte de um dos 3 métodos para diagnosticar a infecção pelo ZIKV. Ainda é considerado o padrão-ouro para a sorologia de arbovírus, pois em infecções primárias, é altamente específico. Os outros dois métodos consistem na detecção de RNA viral por qRT-PCR e detecção de anticorpos IgM reativos ao ZIKV por ELISA. Os testes moleculares são os mais indicados para a detecção do ZIKV na fase aguda e os ensaios sorológicos são indicados a partir do quarto dia de infecção. Amostras positivas nos ensaios de ELISA devem ser confirmadas pelo PRNT devido à possibilidade de reações cruzadas com outros flavivírus (SHAN; XIE; REN; LOEFFELHOLZ *et al.*, 2017) (MUSSO; GUBLER, 2016).

2.2.4 Situação epidemiológica da infecção por ZIKV

Em 2007, o primeiro surto de ZIKV foi relatado na ilha de Yap, Micronésia, seguido por epidemias em várias ilhas do Pacífico, incluindo a Polinésia Francesa, entre 2013 e 2014 (DUFFY; CHEN; HANCOCK; POWERS *et al.*, 2009). Os primeiros relatos de um surto desconhecido de doença exantemática no Brasil, posteriormente identificado como infecção pelo ZIKV, foram emitidos em dezembro de 2014. Em maio de 2015, a disseminação do ZIKV entre a população local foi confirmada laboratorialmente, primeiro nos estados de Pernambuco (PE). Rio Grande do Norte (RN) e Bahia (BA) na região Nordeste, depois em outros estados das regiões Centro-Oeste e Sudeste. Até setembro de 2019 foram registrados 9.813 casos prováveis de Zika em todo país, com dois óbitos confirmados no Estado da Paraíba **(Figura 7)**.

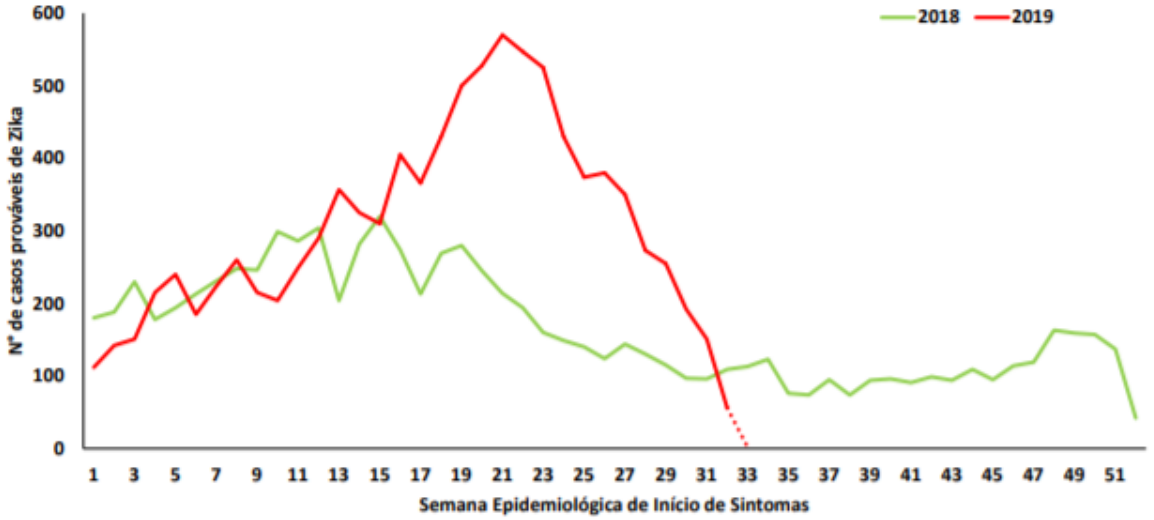

Figura 7. Casos prováveis de infecção aguda pelo vírus Zika por semana epedemiológica até a semana 33. No gráfico são mostrados os casos nos anos de 2018 e 2019. **Fonte:** Sinan NET

2.3 Sanidade nos Planteis Suinícolas no Estado de Pernambuco

Segundo dados mais recentes do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), em 2016, o Brasil ocupava o quinto lugar no *ranking* mundial de produção de suínos, com uma população de 39 milhões de cabeças, sendo 6,32 milhões apenas na região Nordeste do país. Entre 15-18% da produção é vendida para o mercado externo, tornando o Brasil o 4º maior exportador de carne suína mundial (SUÍNOS, 2017).

A produção de suínos tem crescido nos últimos 15 anos devido a melhorias realizadas na cadeia produtiva, como a tecnificação das granjas, melhoramentos genéticos para produção de matrizes de alto desempenho e o desenvolvimento de vacinas e fármacos para combate das principais doenças que prejudicam a produção. Porém, uma das principais barreiras para o comércio internacional da carne suína brasileira para mercados exigentes é a ocorrência de enfermidades de notificação obrigatória e de importância econômica no país, entre elas, citam-se peste suína clássica, doença de Aujeszky e Influenza Suína (SUÍNOS, 2017).

A gripe suína é uma infecção viral altamente contagiosa em suínos, sendo uma doença de grande importância econômica para a indústria suinícola, devido ao seu envolvimento como um dos principais cofatores para o desenvolvimento de infecções secundárias tanto por vírus como por bactérias e também por gerar casos

graves de doença pulmonar especialmente em leitões, levando a retardo de crescimento e diminuição de peso, o que leva a perdas econômicas (DEBLANC; ROBERT; PINARD; GORIN *et al.*, 2013; FABLET; MAROIS-CRÉHAN; SIMON; GRASLAND *et al.*, 2012; JIMÉNEZ; RAMÍREZ NIETO; ALFONSO; CORREA, 2014).

A escassez de informação sobre os vírus circulantes em suínos na Região Nordeste e no Estado de Pernambuco dificultam a tomada de medidas preventivas. Portanto, este estudo é um passo importante para determinar a situação epidemiológica em que se encontram os rebanhos do Estado de Pernambuco, para prevenir a gênese e disseminação de novos vírus para humanos.

3 RESULTADOS

3.1 MOLECULAR PREVALENCE OF SWINE INFLUENZA VIRUS ON FARMS IN NORTHEAST BRAZIL

Abstract

Swine production in Brazil has grown over the last 15 years due to improvements in the production chain, such as farm modernization, genetic improvement for the production of high-performance matrices and breeding herds and control of the main diseases that hinder production. Influenza A virus (IAV) is a major cause of respiratory disease in pigs, but the epidemiology of swine influenza in Brazil is still poorly understood. A molecular survey was carried out in 500 animals from 15 farms and 2 municipal slaughterhouses in the state of Pernambuco, northeastern Brazil. We found 9.6% (48/500) of the samples positive for influenza type A by qRT-PCR, Some of the viruses were sequenced by NGS and phylogenetic analyzes revealed that they grouped with human H3N2 subtypes that circulated between 2015 and 2017. In conclusion, our results demonstrated the circulation of influenza human-like strains between farms and slaughterhouses in northeastern Brazil.

1. Introduction

Swine influenza is an acute respiratory disease caused by the influenza A virus (IAV) (VINCENT; AWADA; BROWN; CHEN et al., 2014). IAV belongs to the *Orthomyxoviridae* family whose genetic material consists of eigth negative sense single stranded RNA segments (BOUVIER; PALESE, 2008). Hemagglutinin (HA) and neuraminidase (NA) glycoproteins expressed on the surface of viral particles play a role in adherence, spread and are vital for antigenicity (SRIWILAIJAROEN; SUZUKI, 2012; ZANIN; DUAN; WONG; KUMAR et al., 2017).

Pigs infected with swine influenza virus (SIV) show clinical signs of acute respiratory disease and the severity of which depends on several factors, such as the animal's immune status, viral strain and the presence of secondary infections, and may present clinical signs ranging from cough and sneezing to prostration (HEALTH, 2009; VINCENT; AWADA; BROWN; CHEN *et al.*, 2014).In addition to economic

losses, SIV also poses a zoonotic risk and can be transmitted to people who have close contact with these animals (MOSTAFA; ABDELWHAB; METTENLEITER; PLESCHKA, 2018; SCHRAUWEN; FOUCHIER, 2014).

In April 2009, a new swine IAV (H1N1), designated as A (H1N1) pdm09, emerged in Mexico and the United States and spread rapidly throughout the world ((CDC), 2009). This new virus was generated by the rearrangement between circulating strains in swine (SMITH; VIJAYKRISHNA; BAHL; LYCETT *et al.*, 2009). The zoonotic potential of SIV represents a threat to public health, since the swine to human and human to swine transmission has been documented (NELSON; GRAMER; VINCENT; HOLMES, 2012; RITH; NETRABUKKANA; SORN; MUMFORD *et al.*, 2013; SONG; LEE; PASCUA; BAEK *et al.*, 2010). Therefore, the control and monitoring of SIV is important to minimize the economic impact of this pathogen to the pork industry and the consequences for public health.

In Brazil, because it is not a disease of compulsory notification, the data about circulating strains in the country is scarce, especially in the Northeast of the country where the production is basically made for subsistence, and the production is little modernized (Associação Brasileira de Criadores de Suínos, 2017). The aim of the present study was to provide a molecular and serological view of SIV in swine from northeastern Brazil.

2. Materials and methods

2.1. Sample collection

Collection of swine nasal swab or lung specimens was carried out on farms and municipal slaughterhouses during 2017-2018. Swine samples of varying ages were collected. Nasal or lung swabs (in the case of slaughterhouses) were also collected in cryogenic vials containing transport medium (brain heart infusion medium 3.4%, supplemented with 10,000 U / mL penicillin G, 2 mg / mL streptomycin, 1 mg / ml gentamicin and 0.02 mg / ml amphotericin B). These were immediately transferred to an ice pack for transport to the laboratory for testing and storage at -80 ° C.

2.2. RNA extraction

Viral RNA was extracted from swine respiratory specimens using QIAamp viral RNA mini kit (Qiagen Inc., Valencia, CA, EUA) according to the manufacturer's instructions. Nanodrop reader (NanoDrop 2000/2000c Spectrophotometer, Thermo

Scientific, Wilmington, DE, USA) was used to test the concentration and purity of the extracted RNAs.

2.3. Real time RT-PCR (RT-qPCR)

RT-qPCR assay targeting the M gene (118nt) of influenza type A were performed with 5 µL of RNA as described previously (HEALTH, 2009). Detection was performed using the GoTaq® probe-1-step RT-qPCR system kit (Promega) according to the manufacturer's protocol. For each reaction one negative control *no-template* containing water and a positive RNA control of a reference virus (A/PR/8/1934(H1N1)) were included in each run.

2.4. RT-PCR and Next Generation Sequencing

The multisegment reverse transcription of influenza A-positive samples was performed using the ImProm-II™ Reverse Transcription System (Promega, USA) and the cDNA was amplified using Hot Start Taq DNA Polymerase®(New England Biolabs) according to manufacturer's instructions using the Opti1 primer set with influenza-specific universal primers complementary to the conserved 12–3 nucleotides at the end of all eight genomic segments: Opti1-F1 5′-GTTACGCGCCAGCAAAAGCAGG, Opti1-F2 5′ GTTACGCGCCAGCGAAAGCAGG and Opti1-R1 5′ GTTACGCGCCAGTAGAAACAAGG. Influenza genome was sequencing using the Illumina MiSeq platform. Paired-end library was prepared using the Nextera™ XT DNA library kit (Illumina, San Diego, CA, USA) and sequencing was performed using a 150-cycle (2 × 75-bp paired-end) MiSeq v3 reagent kit (Illumina) via MiSeq platform according to the manufacturer's protocols. Low-quality reads in demultiplexed data were removed and 3′ terminal nucleotides were trimmed with Trimmomatic 0.36 software and de novo assemble was performed using Velvet 1.2.10.

To determine the evolutionary relationships of the virus, phylogenetic analyses were performed using the neighbor-joining method with 1000 bootstrap using the MEGA v.4 (TAMURA; DUDLEY; NEI; KUMAR, 2007)

2.5. Ethical considerations

This project was approved by the Animal Use Ethics Committee (CEUA) of Fiocruz / PE (protocol number 90/2015) and also by the National Technical Biosafety

Commission (CTNBio) (technical opinion No. 5.643 / 2017) meets all the requirements of the “Arouca Law” (Law 11,794, of 10.8.2008).

3. Results

3.2. Molecular detection of SIV infection

Molecular diagnosis by qRT-PCR showed that 9.6% (48/500) of the samples were positive for influenza type A (detection of genes in the matrix). Positive samples were from São Bento do Una (70.83%), Pedra (10.41%), Alagoinha (6.25%), Paulista (8.3%), Recife (2.08%) and Lajedo (2.08%) (Fig 1).

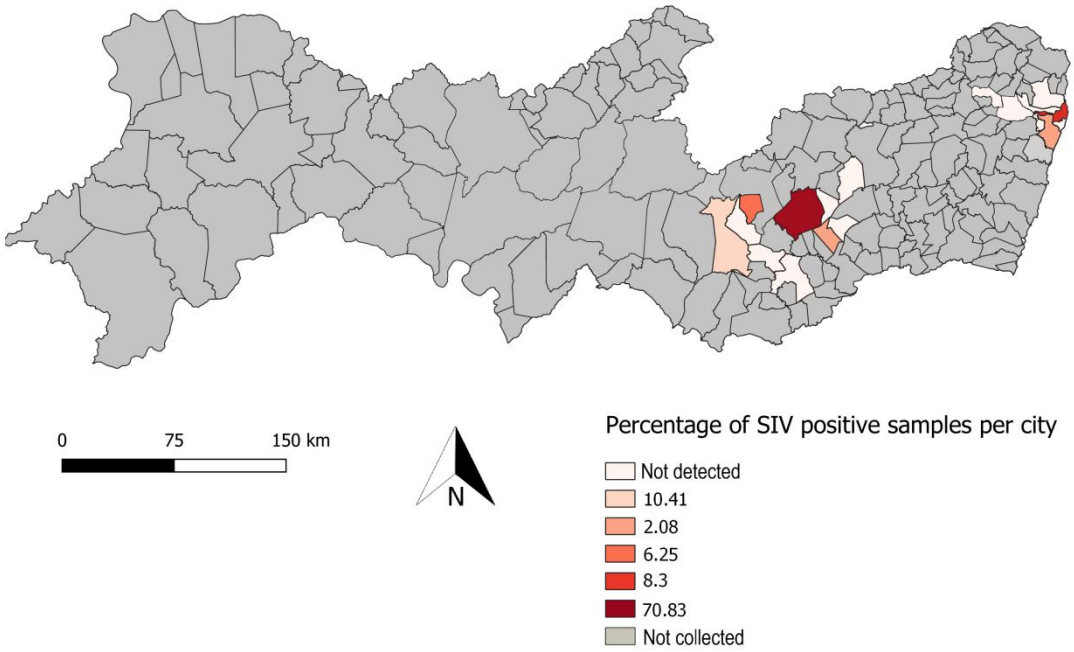

Fig.1. Spatial pattern of influenza A infection among pigs in Pernambuco, Brazil, 2017-2018. Map shows the collected cities and their respective percentages of positivity in the metropolitan region; Cities represented in white were collected, but there were no IAV positive animals in qRT-PCR; the cities represented in gray were not collected.

Of the 17 sites used for the study, the IAV was confirmed in nine sites. The proportion of cases per positive location is shown in Table 1.

Table 1. Distribution of cases of influenza A per location during the study

Local government area	Location	Sample size (n)	Type of sample	Number of positive cases	Detection rate per location (%)
Metropolitan	Paulista	20	Lung	04	20
	Recife	14	Swab	01	7.14

Region (MR)	Igarassu	07	Swab	-	-
	Abreu e Lima	10	Lung	-	-
Total per region		**51**		**05**	**9.8**
Zona da Mata (ZM)	Paudalho	15	Lung	-	-
	Carpina (Farm 1)	11	Swab	-	-
	Carpina (Farm 2)	08	Swab	-	-
Total per region		**34**		**0**	**0**
Agreste (AG)	Alagoinha	69	Swab	03	4.35
	Cachoeirinha	62	Swab	-	-
	Caetés	09	Swab	-	-
	Garanhuns	16	Swab	-	-
	Lajedo	18	Lung	01	5.55
	Pedra	81	Swab	05	6.17
	São Bento do Una (Farm 1)	21	Swab	08	38.09
	São Bento do Una (Farm 2)	30	Swab	11	36.66
	São Bento do Una (Farm 3)	39	Swab	15	38.46
	Venturosa	70	Swab	-	-
Total per region		**415**		**43**	**10.36**
TOTAL		**500**		**48**	

3.3. Next-generation sequencing

In the analysis readings similar to the swine genomes and some known swine bacteria were filtered and the remaining readings were tested for similarity to influenza virus from the GenBank and Fludb databases. Among the ~2M readings generated, 15% were found as swine genomic material, and ~ 58% mapped to bacterial genomes were filtered, leaving ~ 540,000 readings to be further analyzed. Bowtie 2.0 was used to assemble the remaining readings were gathered into 2,000 larger segments (contigs). The vast majority of these contigs have been mapped as a bacterial and fungal source. Virus mapped contigs were mapped to Hepatitis B virus (HBV) and two contigs mapped Influenza B virus (IBV).

Only a small number of mapped contigs were mapped to IAV (~21 contigs between 400- 1300bp with average coverage~22,631,887). For each segment: PB2 (1 contig; 400bp), PB1 (1 contig; 430bp), PA (3 contigs; 481-1008bp), HA (4 contigs;

400-1300bp), NP (1contig; 300bp), NA (3 contigs ; 300-400bp), M (2 contigs; 400-500bp) and NS (4 contgs; 420-440bp).

Phylogenetic analysis revealed 4 HA fragments obtained from Pernambuco pigs during this study grouped with H3 subtype strains that circulated in humans between 2012 and 2014. These fragments had similarities with the human strains circulating in the state of Pernambuco in 2014 (Fig. 2A). The 3 fragments from NA gene clusttered with N2 subtype from swine circulate in Chile (Curico and Maule) in 2015 (Fig.2B).

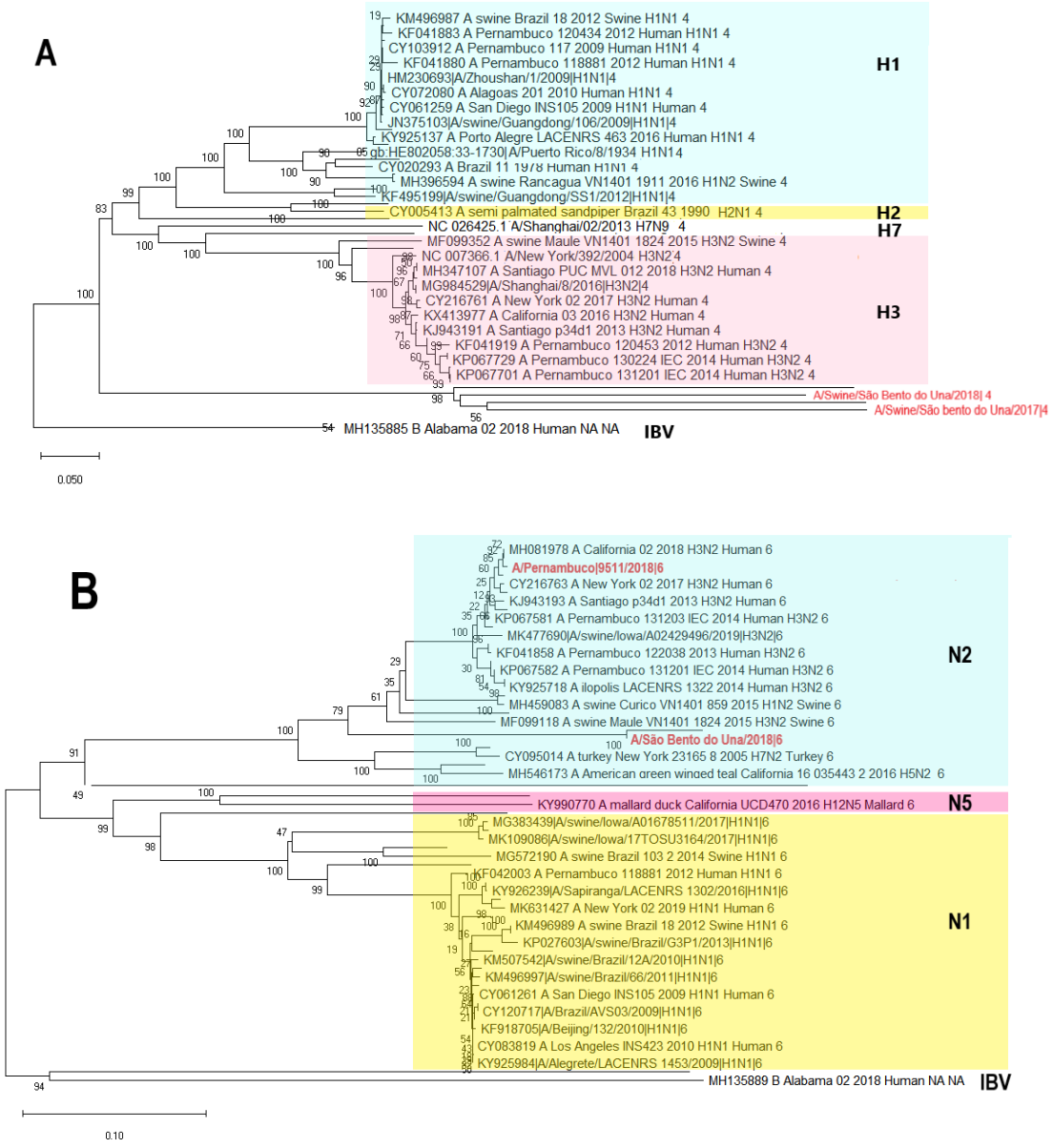

Fig.2. Evolutionary relationships of partial nucleotide sequences (200-6509 bp) of swine samples. (a) HA from the H3 subtype and **(b)** NA from de N2 subtype. Phylogenetic analyses were performed and the tree was constructed via the neighbor-joining method with 1000 bootstrap replicates in MEGA4.

Phylogenetic analysis of the polymerases fragment-genes showed that the Pernambuco virus sequences were distributed in the South American lineage group, which has human's strains from Santiago- Chile and South Brazil (Rio grande do Sul and Santa Catarina)(Fig.3).

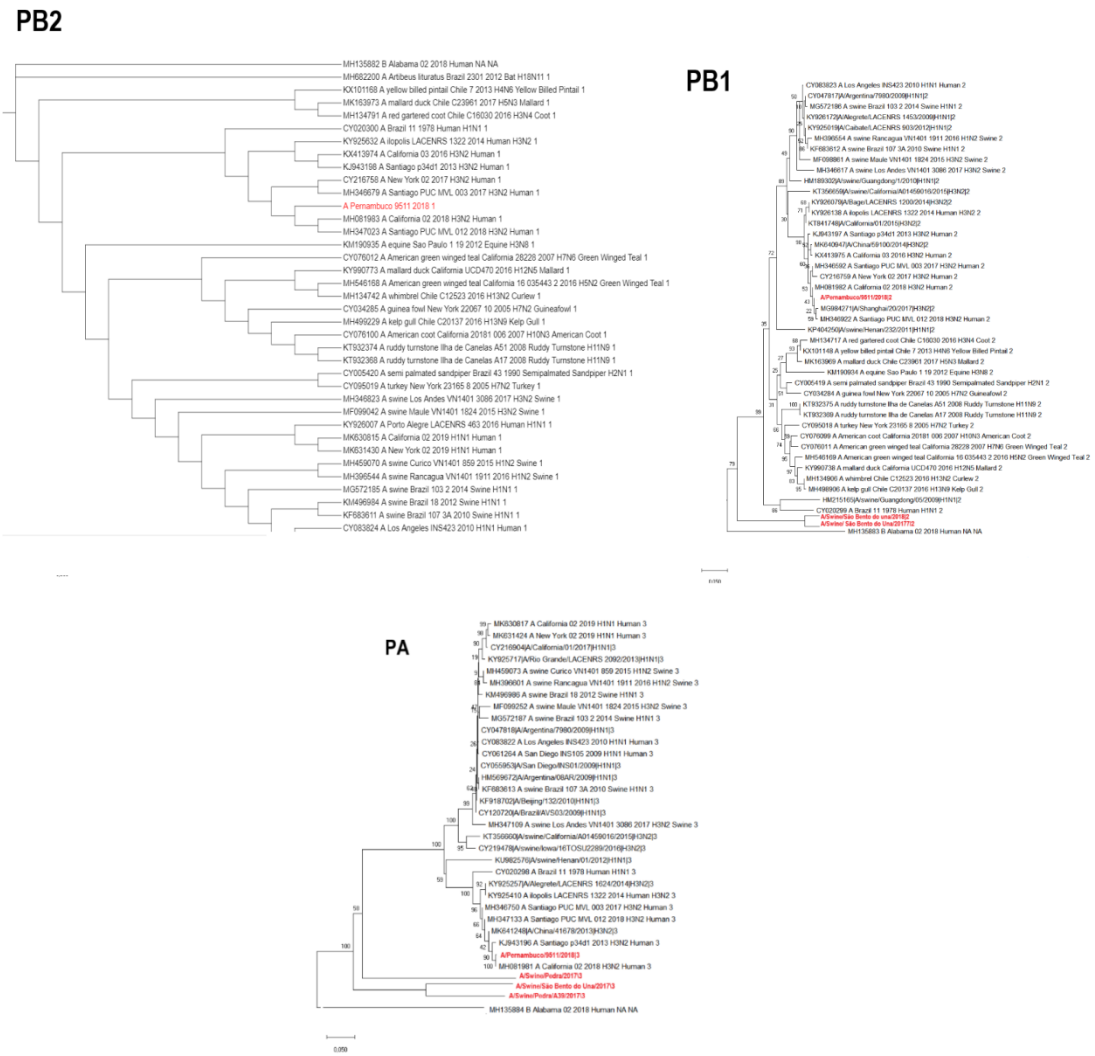

Fig.3. Phylogenetic trees of the PB2, PB1 and PA genes of Pernambuco swine influenza viruses.

Notably, the NP, M and NS genes of the sequenced strains were closely related to those of the H3N2 virus emerging in China and the United States (Fig. 4).

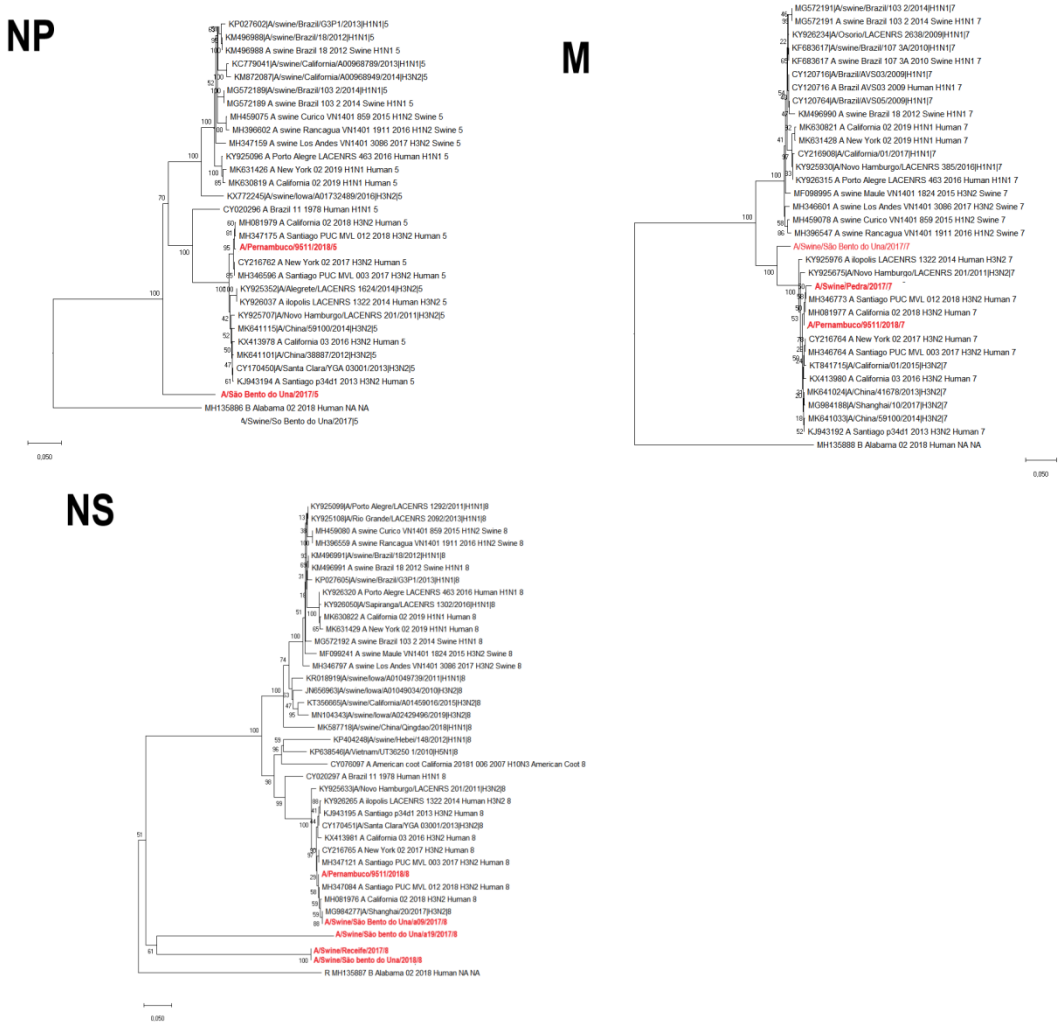

Fig.4. Phylogenetic trees of the NP, M and NS genes of Pernambuco swine influenza viruses.

4. Discussion

Respiratory diseases in pigs are a major health problem in swine production, with Influenza A virus being a major agent (ROSE; HERVÉ; EVENO; BARBIER *et al.*, 2013). Besides economic losses, the importance of swine in influenza virus generation and dispersion is well described and numerous environmental and host factors are involved in this process (LONG; MISTRY; HASLAM; BARCLAY, 2019).

During this study, the overall prevalence of SIV infection among Pernambuco pigs, northeastern Brazil (9.6%) similar rates were reported in other regions of the world where the prevalence of positivity in the diagnosis for SIV ranged from 7,8% to 12,3% (BROOKES; IRVINE; NUNEZ; CLIFFORD *et al.*, 2009; ROSE; HERVÉ; EVENO; BARBIER *et al.*, 2013). This findings also corroborate with clinical status observed in the herds studied, in which many animals presented mild to intense respiratory symptoms at the time of collection.

Most of the positive samples (70,86%) were found in the Agreste region where there was a greater number of visited farms and collected animals. In addition, swine rearing is extremely rudimentary and many farmers feed the animals with unprocessed poultry carcasses. This facilitates viral exchange and favors the emergence of potentially infectious recombinant viruses for humans and animals and represent a risk factor for animals to acquire viruses and other pathogens (CARNERO; KITAYAMA; DIAZ; GARVICH *et al.*, 2018).

Of the 18 IAV positive samples that were sequenced using the NGS it was not possible to sequence the complete genome of the IAVs present in the samples, generating many related readings of contaminant genomes present in the sample. However, it was possible to identify the presence of other bacterial and viral pathogens, which may be of interest to identify pathogens associated with SIV infections in swine.

In this study, we used the amplicon sequencing protocol of clinical samples to analyze the full genome of the influenza virus, unfortunately it was not possible to retrieve the full genome of the samples due to the large number of bacterial and genomic contaminant sequences. Previously, Rosseel *et al.*, 2012 demonstrated that analysis of metagenomic sequencing could detect unknown viruses in clinical specimens (ROSSEEL; SCHEUCH; HÖPER; DE REGGE *et al.*, 2012). However, this clinical sample sequencing scheme has lower levels of sensitivity and specificity compared to the isolated sample amplicon sequencing protocol due to contamination by large amounts of host genome fragments and also other microorganisms present in the sample (IMAI; TAMURA; TANIGAKI; TAKIZAWA *et al.*, 2018). Depletion of host/microbiota components (ribosomal RNA and poly-A RNA) and total DNA / cDNA amplification are routine methods for improving recovery results (MATRANGA; ANDERSEN; WINNICKI; BUSBY *et al.*, 2014), but Li *et al.*,2016 demonstrated these methods may introduce biased distributions of reading coverage over the genome of the IAV, making it difficult to assemble the genome. Also demonstrated that viral serotyping was also affected by pre-treatments (LI; LI; ZHOU; QU *et al.*, 2016).

Phylogenetic analysis of the HA and NA fragments indicated that the sequenced strains belonged to H3N2 strains with circulating human strains in Latin and North America between 2012 and 2018. Internal genes were closely related to H3 viruses in China and the United States. These results follow an intense viral exchange between humans and swine, however, due to the small amount of human

and swine sequences from Brazil, we could not analyze in more detail the sequences found in this study with the most recent viral sequences.

So far few studies have performed the diagnosis and molecular characterization of SIV here in Brazil, but the presence of human influenza H1N1 virus (pandemic 2009) has been confirmed in swine respiratory disease outbreaks on farms in southern regions (Paraná and Rio Grande do Sul), Midwest (Mato Grosso) and Southeast (Minas Gerais and São Paulo) of Brazil, during the years 2009-2010, which proves the spread of human viruses in Brazilian swine herds (RAJÃO; COSTA; BRASIL; DEL PUERTO *et al.*, 2013), however to date there is no data in the herds in Northeast Brazil.

In conclusion, we confirm the circulation of IAV among swine in northeastern Brazil, and we also report the similarity to IAV strains similar to human strains circulating between 2015 and 2017. This study highlights the importance of monitoring circulating strains among pigs to prevent the occurrence of zoonoses.

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3.2 SEROPREVALENCE OF INFLUENZA A H1N1PDM AND H3N2 VIRUS IN DOMESTIC SWINE IN PERNAMBUCO, BRAZIL

Abstract

The aim of this study was to provide an overview of the serological status of the H1N1 pdm09 and H3N2 influenza viruses in pigs residing in northeastern Pernambuco in 2017-2018. A serological survey was carried out on 343 pigs from 14 farms and 2 slaughterhouses located in Pernambuco, Brazil. Serological data showed that 92.60% of the sera were positive for the H1N1pdm09 subtype, 86.2% for the H3N2 subtype and 84.54% for both subtypes. All farms collected showed positive animals for both viral subtypes the level of seropositivity suggests high exposure to virus. About 42.60% of the collected farms used to feed pigs with carcasses of raw birds that resulting in indirect but frequent interspecies contacts. In summary, our results showed high seroprevalence of IAV, H1N1pdm09 and H3N2, in pig populations in the state of Pernambuco, northeastern Brazil during 2017 and 2018 this high prevalence may be related to factors related to animal management and low biosafety.

1. INTRODUCTION

Brazilian pig production has grown over the past 15 years due to improvements in the production chain, such as the farm technification, genetic improvement for the production of high-performance breeding stock and the development of vaccines and drugs to combat the main diseases that hinder production. However, one of the main barriers to the international trade in Brazilian pork for demanding markets is the occurrence of economically important diseases in the country, including classical swine fever, Aujeszky's disease and swine influenza (SUÍNOS, 2017).

Swine influenza is a highly contagious viral infection in pigs, being a disease of great economic importance for the pig industry, due to its involvement as one of the main cofactors for the development of secondary infections by both viruses and bacteria and also by generating cases severe lung disease, especially in piglets, leading to growth retardation and weight loss, which leads to economic losses (DEBLANC; ROBERT; PINARD; GORIN *et al.*, 2013; FABLET; MAROIS-CRÉHAN;

SIMON; GRASLAND *et al.*, 2012; JIMÉNEZ; RAMÍREZ NIETO; ALFONSO; CORREA, 2014).

Influenza A (IAV) viruses H1N1, H1N2 and H3N2 are endemic in swine populations worldwide. After the 2009 human pandemic (H1N1pdm09), several cases of transmission of this virus between humans and pigs have been reported, as evidence of reverse zoonosis, in addition to the appearance of several recombinant viruses (GARTEN; DAVIS; RUSSELL; SHU *et al.*, 2009; NELSON; GRAMER; VINCENT; HOLMES, 2012; NELSON; VINCENT, 2015).

The transmission of influenza viruses between pigs and humans is linked to the evolution of the virus and to the emergence of new transmissible strains capable of infecting human beings and spreading from person to person that can lead to pandemics (MOSTAFA; ABDELWHAB; METTENLEITER; PLESCHKA, 2018).

Studies in Latin America have shown seroprevalence of IAV and evidence of transmission from humans to pigs (DIBÁRBORA; CAPPUCCIO; OLIVERA; QUIROGA *et al.*, 2013; GONZALEZ-REICHE; RAMÍREZ; MÜLLER; ORELLANA *et al.*, 2017; TINOCO; MONTGOMERY; KASPER; NELSON *et al.*, 2016). However, there is still little information about the seroprevalence of IAV in the swine population in Brazil, especially in the Northeast region.

Our study aimed to estimate the seroprevalence of IAV in populations of pigs living in the state of Pernambuco and to evaluate factors associated with the seroprevalence of IAV in these populations.

2. MATERIALS AND METHODS

2.1 Study area

The study was carried out in the state of Pernambuco, Brazil (Fig. 1), the state is subdivided into five regions, with the Agreste region having the highest proportion of small-scale pig farmers in the state. Within the State, we selected three areas with the largest number of pig farms and breeding pigs to collect.

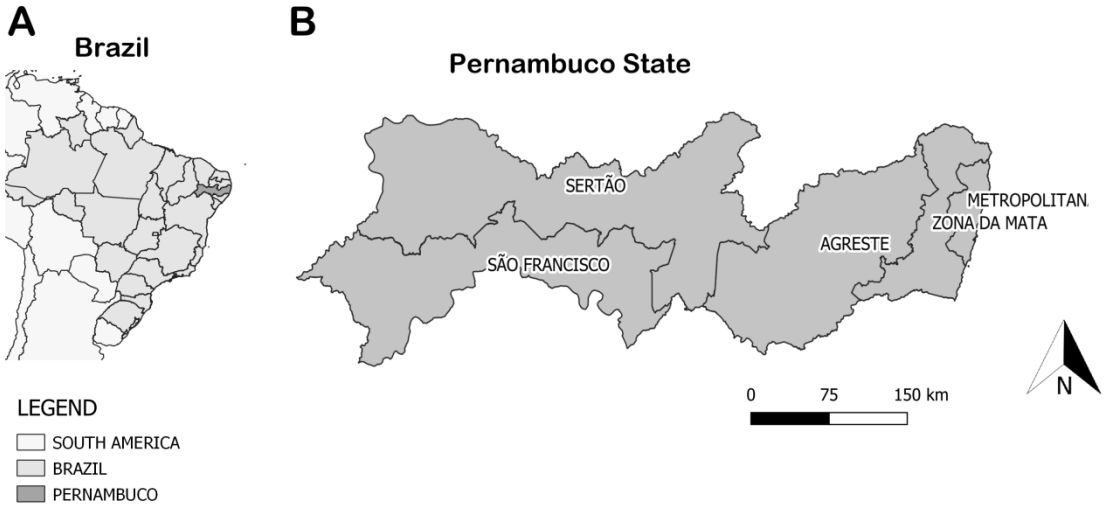

Figure 1. Spatial distribution of State of Pernambuco. (a) The location of Pernambuco State in Brazil; **(b)** Pernambuco is divided into 5 regions: Metropolitana, Mata, Agreste, Sertão and São Francisco. Sampling was performed on farms and slaughterhouses within the first 3 regions.

2.2. Study design and sampling

The collections were carried out between February 2017 and August 2018 in 14 properties and 2 municipal slaughterhouses. At the time of sample collection, a form with the sanitary conditions of the place was filled out (cleaning of facility, food, exposure to birds and clinical signs such as fever, cough, nasal discharge).

Swine blood were collected from all age groups, including piglets, weaners, producers, finishers and sows. The number of pigs sampled was proportional to the size of the herd, with all animals sampled in small herds (<10 pigs) and up to 30 animals sampled in herds with > 10 pigs.

2.2. Viruses Isolation and Reverse Genetic rescue

The A/Pernambuco/9511/2018 (H3N2) influenza virus was isolated in MDCK cells from a human patient with an influenza-like illness in January 2019. The A/Califórnia/04/2009 (H1N1) pandemic H1N1/09 virus was recovered through eight plasmids expressing viral genes kindly provided Dr. Daniel Perez (University of Georgia, USA). The H1N1/09 virus was generated by cell transfection in a co-culture of MDCK and HEK 293T cells following the protocol described previously by Hoffmann *et al.,* 2002 (HOFFMANN; KRAUSS; PEREZ; WEBBY *et al.*, 2002). These

viruses were propagated in MDCK cells, and used as the test viruses for hemagglutination inhibition (HI) assays.

2.3. Hemagglutination-inhibition (HI) assay

Serum samples were treated with receptor-destroying enzyme (RDE) (Sigma-Aldrich) (1 part serum, 3 parts RDE) for 18 hours at 37 ° C, followed by heat inactivation at 56° C for 30 minutes. The viral strains tested were A/California/04/2009 (H1N1) and A/Pernambuco/9511/2018 (H3N3). A BALB/c mouse serum was used as negative control and a vaccinated human serum was used as positive control. Briefly, 25 µL of serial dilutions (1: 2) of the treated serum samples then mixed with 4 hemagglutinating units of the virus in a 96-well plate and incubated in room temperature for 30 minutes. Then 50 µl of 0.5% turkey red blood cells were added to each well and incubated at room temperature for 30 minutes. HI antibody titer is defined as the reciprocal of the highest serum dilution that completely inhibits the haemagglutination reaction. We defined HI antibody titers ≥20 were considered positive.

2.4. Data analysis

To estimate statistically significant differences, Fisher's exact test was carried out to compare the determined SIV antibody titers for each virus and based on location. Analyses were performed with a confidence level of 95% in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

2.5. Ethical considerations

This project was approved by the Animal Use Ethics Committee (CEUA) of Fiocruz / PE (protocol number 90/2015) and also by the National Technical Biosafety Commission (CTNBio) (technical opinion No. 5.643 / 2017) meets all the requirements of the “Arouca Law” (Law 11,794, of 10.8.2008).

3. RESULTS AND DISCUSSION

3.1. Serological survey of human H3N2 and pandemic H1N1/09

The sample collection sites included 14 farms and 2 municipal slaughterhouses in the Metropolitan, Mata and Agreste of Pernambuco. The sampled

farms were between 07-100 animals. In all farms, positive samples were found for both viral subtypes tested. Serological analysis revealed that 86.2% of sera samples were positive for H3N2 subtype, 92.6% for H1N1 subtype and 6.18% were negative for both subtypes. Antibody titers were higher for H3N2 (media between 115 and 866) than for H1N1 subtype (media between 204 and 229; p <0.05; Fig. 2A). H3N2 titers were also higher when farms were analyzed individually (Fig. 2B).

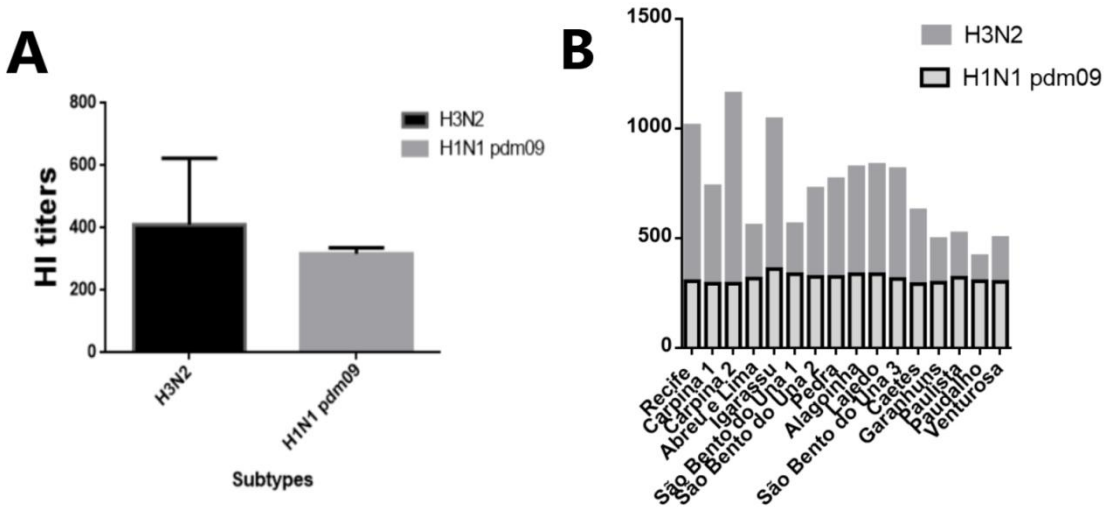

Fig. 2. Serology for IAV H3N2 and H1N1 subtypes in pig sera (n = 343). Antibody detection was performed by HI. Animals with titers less than or equal to 10 were considered negative. A) Comparison between serological titers for H3N2 and H1N1 subtypes; B) distribution of seroprevalence by farm and by subtype.

3.2 Seroprevalence and associated factors

The frequency of IAV positive herds and the estimated seroprevalence for each year are shown in Table 1. Regarding the IAV subtypes analyzed by the HI assay, 97.63% were positive for H1N1 pdm09 and 99.05 for H3N2 in 2017. One sera was negative for both IAV strains in the HI assay. In 2018, 67.44% were positive for H1N1pdm09, 64.34% were H3N2 and 29.355 (34/129) sera were negative in the HI test. HI titers ranged from 40 to ≥1280.

Table 1. Seroprevalence of influenza A virus in pigs in 2017 and 2018

Year	H1N1 pdm09 Prevalence %	H3N2 Prevalence %	Frequency positive herds
2017	97.63 (206/211)	99.05 (209/211)	100 (8/8)
2018	67.44 (87/129)	64.34 (83/129)	100 (8/8)

In all the farms visited there were domestic animals, such as cattle, goats, dogs and cats. About 78.57% raised domestic birds (turkeys, ducks, chickens) in direct contact with pigs. Some characteristics of the facilities are shown in Table 2. None of the farms vaccinated the animals against influenza virus and 42.60% used raw poultry carcasses to feed the animals. Only one farm did not have animals with symptoms of respiratory disease at the time of collection.

Table 2. Characteristics of the farms collected

Farm	Type of production	Presence of Poultry	Animals vaccinated against SIV	Respiratory symptoms at the time of collection	Poultry offal feeding	Average herd size (n)
1	Farrow-to-finish	Yes	No	Yes	No	20
2	Farrow-to-finish	Yes	No	Yes	No	30
3	Farrow-to-nursery	No	No	No	No	100
4	Farrow-to-finish	Yes	No	Yes	Yes	80
5	Farrow-to-finish	Yes	No	Yes	Yes	50
6	Farrow-to-finish	Yes	No	Yes	Yes	100
7	Farrow-to-finish	Yes	No	Yes	Yes	60
8	Farrow-to-finish	Yes	No	Yes	Yes	20
9	Farrow-to-finish	No	No	Yes	No	80
10	Farrow-to-finish	Yes	No	Yes	Yes	60
11	Farrow-to-finish	Yes	No	Yes	Yes	60
12	Farrow-to-finish	Yes	No	Yes	Yes	20
13	Farrow-to-finish	No	No	Yes	No	120
14	Farrow-to-finish	No	No	Yes	No	200
Total		78.57 % (11/14)	100 % (14/14)	92.86% (13/14)	42.60% (6/14)	

In the municipal slaughterhouses, the animals came from several farms and small properties, but we did not have access to information from the breeders and the animals were in quarantine and without clinical symptoms at the time of collection.

4. DISCUSSION

Flu is an infectious disease caused by the IAV, the infection is characterized by fever, cough, muscle and joint pain, sore throat and runny nose, and can lead to hospitalization or death, especially in the elderly and children under 5 years. Annual influenza epidemics account for about three to five million cases of serious illness and 290,000 to 650,000 respiratory deaths worldwide (WHO, 2018). The importance of pigs in the generation and dispersion of the influenza virus is well described and several environmental and host factors are involved in this process (LONG; MISTRY; HASLAM; BARCLAY, 2019).

This study documents the high circulation of the influenza virus, demonstrated by 92.6% seropositivity for H1N1pdm09 and 86.2% for H3N2 virus among the studied swine populations. Serological studies in Europe and Asia found a higher prevalence of H1N1 subtype than H3N2 (KYRIAKIS; PAPATSIROS; ATHANASIOU; VALIAKOS *et al.*, 2016; LIU; WEI; TONG; TANG *et al.*, 2011). A study in southern Brazil revealed a higher prevalence of the H1N1 subtype (RAJÃO; ALVES; DEL PUERTO; BRAZ *et al.*, 2013). In northeastern Brazil, the H3N2 subtype presented the highest prevalence by the HI test.

Since swine influenza vaccination was not practiced by the farmers in our study, 100% of farms collected showed positive animals for both viral subtypes the level of seropositivity suggests high exposure to virus. A common practice is to raise pigs together with poultry (chickens, turkeys and birds), dogs and cats. This promiscuity in swine farming facilitates viral exchange and favors the emergence of potentially infectious recombinant viruses for humans and animals (CARNERO; KITAYAMA; DIAZ; GARVICH *et al.*, 2018).

About 42.60% of the collected farms used to feed pigs with carcasses of raw birds. This practice is common in the Agreste Region, where there is a large production of birds, which leads to the use of birds not slaughtered by small-scale swine farms, resulting in indirect but frequent interspecies contacts, which can lead to inter-species transmission of bacterial or viral pathogens such as the influenza A virus. (CARNERO; KITAYAMA; DIAZ; GARVICH *et al.*, 2018).

In summary, our results showed high seroprevalence of IAV, H1N1pdm09 and H3N2, in pig populations in the state of Pernambuco, northeastern Brazil during 2017

and 2018. The seroprevalence of IAV in the studied populations may be associated with factors related to animal management and low biosafety. This study emphasizes the relevance of continued influenza surveillance in these populations, including future studies of the genetic/antigenic characterization of IAVs to estimate the risk they may pose to public health.

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3.3 NEXT-GENERATION SEQUENCING OF AN HUMAN INFLUENZA A VIRUS (H3N2) FROM NORTHEAST BRAZIL

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ABSTRACT

Seasonal influenza is responsible for significant morbidity and mortality rates worldwide. Genomic characterization of circulating virus is essential to understand viral dynamics and emergence of new strains. Here, we sequenced the whole genome of a human influenza virus collected in the state of Pernambuco, northeastern Brazil, using next generation sequencing.

Infection by seasonal influenza A viruses (IAV) is characterized by fever, cough, muscle and joint pain, sore throat and runny nose, which can lead to hospitalization or death, especially in older people and children under 5 years of age. Annual IAV epidemics account for about 3 to 5 million cases of serious illness and 290,000 to 650,000 respiratory deaths worldwide (WHO, 2018). Based on their surface glycoproteins, IAV can be divided and classified into 18 HA subtypes and 11

NA subtypes, but IAV surveillance programs and advances in sequencing technologies may increase this number (TONG; ZHU; LI; SHI et al., 2013). Subtypes H1N1 and H3N2 are responsible for annual epidemics in humans worldwide (GROHSKOPF; SOKOLOW; BRODER; WALTER et al., 2018).

The genomes of IAV are highly variable and evolve very rapidly, which contributes to their ability to escape the human immune system, cross species barriers or potentially create highly virulent strains (ZELDOVICH; LIU; RENZETTE; FOLL et al., 2015). With the emergence of new viral strains, knowledge of the strains circulates in different regions of the world is essential for selecting the most appropriate vaccine strains and also for early monitoring IAV evolution (SHU; MCCAULEY, 2017; SIMON; PICHON; VALETTE; BURFIN et al., 2019). Few sequences for IAV are available in Northeast Brazil. Here, we report, for the first time, the full-length sequencing of an IAV isolated from a human patient in Pernambuco state, Northeast Brazil.

Viral RNA was extracted from MDCK culture supernatant infected with the strain A/Pernambuco/9511/2018(H3N2) using the QIAmp® Viral RNA Mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. A multisegment reverse transcription was performed using the ImProm-II™ Reverse Transcription System (Promega, USA) and the cDNA was amplified using Hot Start Taq DNA Polymerase®(New England Biolabs) according to manufacturer's instructions using the Opti1 primer set with influenza-specific universal primers complementary to the conserved 12–3 nucleotides at the end of all eight genomic segments: Opti1-F1 5′-GTTACGCGCCAGCAAAAGCAGG, Opti1-F2 5′ GTTACGCGCCAGCGAAAGCAGG, and Opti1-R1 5′ GTTACGCGCCAGTAGAAACAAGG. Virus was sequenced using the Illumina MiSeq platform(Illumina, San Diego, CA, USA).Paired-end library was prepared using the Nextera™ XT DNA library kit (Illumina) and sequencing was performed using a 150-cycle (2×75-bp paired-end) MiSeq v3 reagent kit (Illumina) according to the manufacturer's protocols. Low-quality reads in demultiplexed data were removed and 3′ terminal nucleotides were trimmed with Trimmomatic 0.36 software. Sequence assembly was performed using Bowtie2 and SAMTools using as a reference template the IAV strain A/New York/392/2004 (NCBI:txid335341).

Complete genome sequencing of A/Recife/9511/2018 (H3N2) revealed approximately 99% nucleotide sequence identities with human H3N2 IAV circulating

in United States and South America (Santiago-Chile and South of Brazil) between 2016 and 2018 (Table 1).

Table 1
Analysis of the PB2, PB1, PA, HA, NP, NA, M and NS genes for nucleotide similarity by BLAST with the most closely related strain for each respective segment.

Segment	Identity (%)	BLAST hit	Subtype	GenBank accession no.
PB2	99.83%	A/Connecticut/23/2017	H3N2	MH601793.1
	99.83%	A/Missouri/37/2018	H3N2	MH601793.1
PB1	99,96	A/New Hampshire/15/2018	H3N2	MK268442.1
	99,91	A/Rhode Island/06/2018	H3N2	MH358827.1
PA	99,78	A/Santiago/RMS_op010d0/2017	H3N2	MH347221.1
	99,73	A/USA/SC5633/2017	H3N2	MK168407.1
HA	99,65	A/Maine/10/2018	H3N2	MK077371.1
	99,15	A/Santiago/PUC-MVL_025/2018	H3N2	MK159859.1
NP	99,68	A/USA/SC5967/2018	H3N2	MK168437.1
	99,81	A/New Hampshire/29/2017	H3N2	MH082592.1
	99,68	A/California/52/2018	H3N2	MK240803.1
NA	99,73	A/Santiago/PUC-MVL_196/2017	H3N2	MH346578.1
	99.72	A/Delaware/34/2017	H3N2	CY258015.1
	99,66	A/Baltimore/0233/2017	H3N2	KY949690.1
M	99,71	A/Santiago/PUC-MVL_042/2018	H3N2	MK159828.1
	99.61	A/USA/SC4084/2017	H3N2	MK168383.1
NS	99,78	A/Santiago/PUC-MVL_025/2018	H3N2	MK159857.1
	99,55	A/Porto Alegre/LACENRS-1013/2016	H3N2	KY925441.1

We describe here the complete genome of a H3N2 IAV clinical strain circulating in the state of Pernambuco, Northeastern Brazil. This result supports the use of complete genomic sequencing as a tool for identifying genetic traits associated with severe influenza in the context of influenza surveillance.

Data availability. The GenBank accession numbers for the influenza virus genome sequence are MN787817-MN787824.

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3.4 SURVEILLANCE AND CHARACTERIZATION OF INFLUENZA VIRUSES AMONG PATIENTS WITH INFLUENZA-LIKE ILLNESS AND SEVERE ACUTE RESPIRATORY INFECTION IN PERNAMBUCO, NORTHEAST BRAZIL, 2010–2019

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Abstract

Background

Brazil is a country with a large territorial area, divided into five Regions with great cultural and climatic differences. Few studies address the difference between viral seasonality between different regions of Brazil. The State of Pernambuco, located in the northeast region, has several peculiarities as to geographical relief and climate, ranging from the coastal area, mountains and semiarid regions, which may imply viral transmission. This study aims to characterize and compare the epidemiological and clinical patterns of influenza viruses in humans in patients with severe influenza-like disease (ILI) and severe acute respiratory disease (SARI) in Pernambuco, Northeastern Brazil.

Methods

Diagnostic data from patients with ILI and SARI from all over the state of Pernambuco, submitted for diagnosis at the Central Laboratory of Pernambuco

(LACEN / PE) from January 2010 to August 2019, were analyzed. PCR-based assays were used for detection and subtyping of influenza viruses. Demographic, behavioral and clinical data were associated using chi-square tests and logistic regression.

Results

Out of the 15,412 ILI / SARI patients analyzed, 921 (6.10%) were positive for influenza A, 308 (2.04%) for influenza B and 6 (0.03%) for influenza A and B. Of those diagnosed with influenza A, the majority 50.76% was H1N1 pdm09, followed by 37.31% A/H3N2 and 11.93% unsubtyped. Clinical predictors of infection varied by virus type, ILI and SARI were most strongly associated with influenza A H1N1pdm09 (39.18% and 51.95% respectively). Influenza viruses were detected at various times throughout the study period, although IAV positivity rates were lower between September and November (spring), a time of year characterized by high temperatures and low rainfall in the Northeast region of the country.

Conclusion

Influenza circulates year-round among humans in Pernambuco with greater activity during the rainy months. Most cases of ILI and SARI are related to the H1N1 subtype pdm09, and the most affected group is young people aged 1-15 years. Continuous monitoring of influenza subtypes related to ILI and SARI cases can help local and regional public health authorities improve the vaccination calendar based on seasonality and circulating viral type in different regions of the country.

Key words

Influenza, Surveillance, virus, Northeast Brazil

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1. INTRODUCTION

Influenza viruses belong to the *Orthomyxoviridae* family and are enveloped negative-sense RNA viruses with a segmented genome and consist a group comprising four genera: A, B, C and D (BOUVIER; PALESE, 2008). Influenza A virus (IAV) infects a wide range of species, influenza B (IBV) and C (ICV) viruses have as their primary host humans, and influenza D virus (IDV) infects cattle (LONG; MISTRY; HASLAM; BARCLAY, 2019; WHITE; MA; MCDANIEL; GRAY *et al.*, 2016). IAV subtypes H3N2 and H1N1, together with IBV, are responsible for annual epidemics in human worldwide (GROHSKOPF; SOKOLOW; BRODER; WALTER *et al.*, 2018). Annual influenza epidemics account for about three to five million cases of serious respiratory illness and 290,000 to 650,000 deaths worldwide. Seasonal influenza infection is characterized by fever, cough, muscle and joint pain, sore throat and runny nose, which can lead to hospitalization or death, especially in older people and children under five years old (WHO, 2018).

They have a seasonal pattern that is influenced by social behavior, temperature and humidity, as well as aggravating factors with chronic respiratory diseases, age and immunodeficiency (KALIL; THOMAS, 2019; KUCHARSKI; KWOK; WEI; COWLING *et al.*, 2014; PARK; SON; RYU; CHOI *et al.*, 2019). In tropical and subtropical regions most cases of influenza occur in the rainy season (TAMERIUS; SHAMAN; ALONSO; BLOOM-FESHBACH *et al.*, 2013). In Brazil, due to the large territorial area, the climate and humidity differ among its various regions (ALMEIDA; CODEÇO; LUZ, 2018). The state of Pernambuco, located in northeastern Brazil, has a distinction for having a large coastal area with high temperatures and humidity and regions with lower temperatures and low humidity in the countryside of state. The regions near the Atlantic Ocean presented the highest precipitation values, while the regions 'Sertão' and 'Agreste', presented the lowest average precipitation depths throughout the year (ANTONIO G. PINHEIRO SOUZA, 2018). Few studies have shown the seasonality pattern of the influenza viruses in Northeast Brazil. Therefore, the knowing if there are differences in the patterns of seasonality and distribution of influenza subtypes in Pernambuco state is essential to improve understanding of these viruses, as well as to assist in the control and prevention.

Sentinel surveillance for influenza viruses was established at the public health service in Brazil in 2006 and encompasses the entire national territory. Cases of

influenza-like illness (ILI) as well as severe acute respiratory infection (SARI) are reported and referrals are sent to central laboratories for investigation of causes (Protocolo de Manejo Clínico de Síndrome Respiratória Aguda Grave(SRAG), 2018). Following WHO criteria, ILI is characterized as acute respiratory disease with a measured temperature ≥ 38 ° C and cough, beginning in the last 10 days. The case definition of SARI is an acute respiratory disease with a history of fever or fever measured ≥ 38 ° C and cough, starting in the last 10 days, requiring hospitalization. The same "start in the last 10 days" criterion was used in the case definitions of ILI and SARI to increase test specificity (FITZNER; QASMIEH; MOUNTS; ALEXANDER *et al.*, 2018).

The aim of our study was to perform influenza virus surveillance from January 2010 to August 2019 in patients with influenza-like diseases referred to the Central Laboratory of Pernambuco (LACEN/PE). This public health laboratory analyzes cases of viral respiratory infections seen in institutions, centers, clinics and hospitals in different cities of Pernambuco.

2. MATERIALS AND METHODS

2.1 Study design and Ethical statement

The present study is a transversal study that used documentary data. Confidentiality of the patients was assured, being respected the Resolution 466/2012 of the National Health Council (CNS) and under the approval of the University of Pernambuco (UPE) Research Ethics Committee (number/CAEE) 27607619.0.0000.5207.

2.2 Study population and data

Epidemiological data were obtained from epidemiological notification forms, and laboratory results refer to respiratory tract samples collected in accordance with the protocol for monitoring viral respiratory diseases of the Pernambuco State Department of Health. Samples were tested at the Pernambuco Central Laboratory (LACEN-PE) from January 2010 to August 2019 for respiratory viruses (adenovirus, human coronavirus, parainfluenza, influenza A and B). Epidemiology, clinical and laboratory data were included in the study. These data were collected through the

records deposited in the laboratory. Clinical characteristics were interpreted by physicians or reported by patients (or responsible for children).

2.3 Viral detection

Samples of patients with ILI (fever ≥38°C, cough, starting on the last 10 days, no need for hospitalization) and SARI (fever ≥38°C, cough, starting on the last 10 days, requiring hospitalization) sent to LACEN / PE for diagnosis. Samples of throat swabs / viscera or bronchial lavage were tested for influenza A and B viruses using real-time PCR method following the protocol described by the World Health Organization (WHO) and the CDC (WHO, 2017) Influenza A positive samples were subjected to subtyping of the haemaglutanin gene using single reactions following WHO recommended protocol. Some samples were tested for positivity for influenza viruses and other viruses (respiratory syncytial virus, metapneumovirus, human coronavirus, parainfluenza virus 1–3 and adenovirus) were tested by the indirect immunofluorescence method following the Brazil Ministry of Health protocol (Protocolo de Manejo Clínico de Síndrome Respiratória Aguda Grave (SRAG), 2018).

2.3 Statistical Analysis

Influenza positive patients were divided into three groups based on diagnostic results: A/H1N1 pdm09, A/H3N2, and influenza B. These groups were compared for age and clinical presentation. Epidemiological data were analyzed using a Pearson chi-square test to compare epidemiological factors (age groups, clinical presentation and ILI/SARI). The *p* values were considered significant if found <0.05. Statistical analysis was performed using the GraphPad Prism version 6 software (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1 Viral detection

From January 2010 to August 2019, 15,412 patients with symptoms of influenza-like illness (ILI) or severe acute respiratory infections (SARI) were referred

for diagnosis at LACEN/PE. The age of these patients ranged from <1 to 100 years old (mean 9.10 year and median 1 year). Among the 15,412 patients analyzed, 921 (6.10%) were positive for influenza A, 308 (2.04%) were positive for influenza B, and only six patients were tested positive for both influenza A and B (Fig. 1a). Among the 921 influenza A samples, 468 (50.81%) were subtyped as H1N1, 344 (37.35%) as H3N2 and 109 (11.83%) as unsubtyped IAV (Fig. 1b). Neither infections with more than one subtype of IAV were observed.

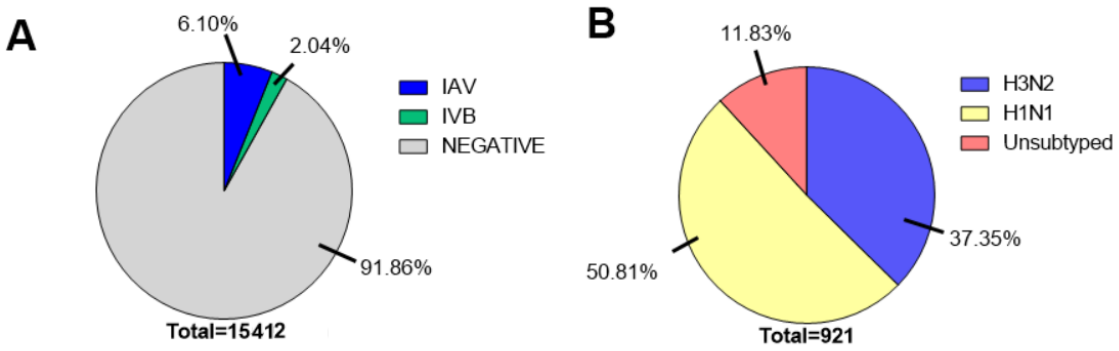

Fig.1. (a) Results of diagnostic tests of 15,412 patients with symptoms of respiratory infection in Pernambuco from 2010 to 2019 and **(b)** distribution of influenza A subtypes.

The pattern of distribution of positive cases for IAV and IBV were similar across geographic areas (Fig. 2).

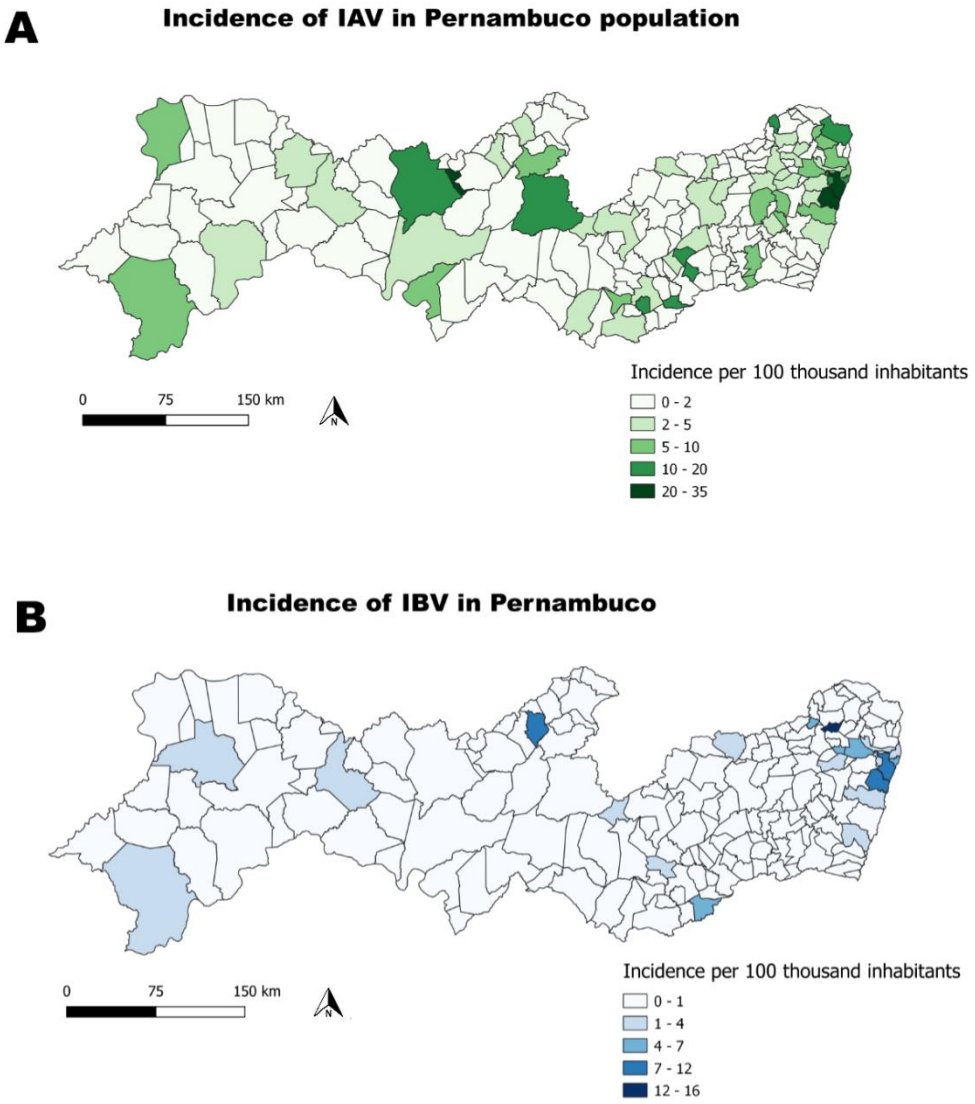

Fig.2. Pattern of distribution of positive cases for IAV and IBV in Pernambuco. a) Incidence of influenza A and **b)** Influenza B virus per 100 thousand inhabitant.

About 67.43% (621/921) of IAV cases and 75.65% (233/308) of IBV cases are concentrated in the Capital Recife and neighboring city Jaboatão dos Guararapes (Fig.3).

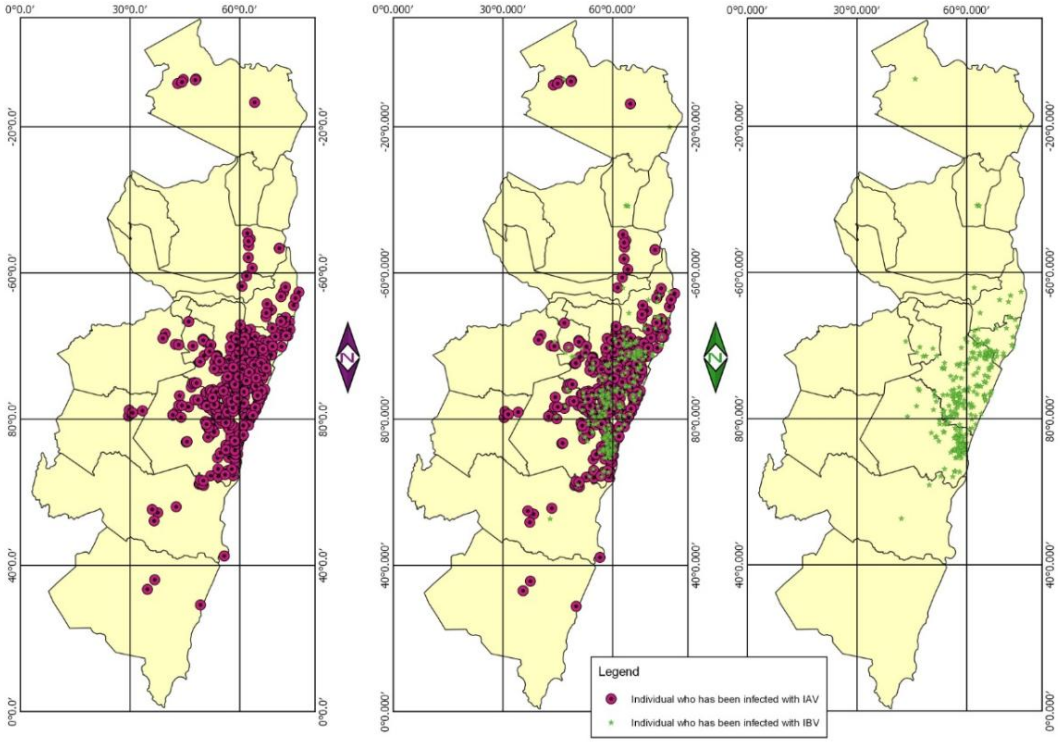

Fig.3. Distribution of positive cases for IAV and IBV in Metropolitan Region of Pernambuco. In the maps are distributed the cases of IAV (in red) and IBV (in green), where it can be observed the largest number of cases on the coast, where there is a higher rainfall rate and high humidity and population density.

3.2 Patients characterization

The ages of the patients ranged from <1 year to 100 years among the different subtypes. Analysis of the data included from January 2010 to August 2019 revealed that the age groups <1 year and 1-15 years had together the highest influenza A and B positivity rates (61.02% and 79.54%, respectively). From the age group of 1-15 years, 407 (44.19%) were positive for IAV and 182 (59.09%) were positive for IVB (Fig. 4). Male patients represented 49.51% (456/921) and 52.59% (162/308); while female patients represented 50.49% (465/921) and 47.39% (146/308) of the total number of IAV and IVB cases respectively.

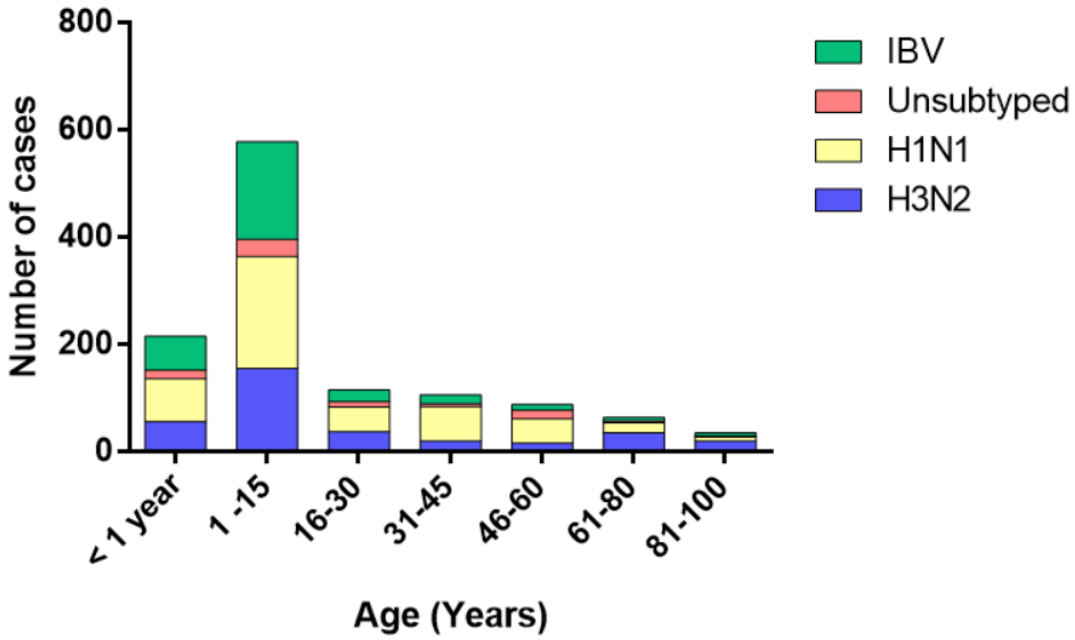

Fig. 4. Pattern of detection of influenza viruses by subtypes in different age groups. The groups of patients aged 1-15 and <1 year were the most affected by influenza viruses. Influenza B is shown in green, unsubtyped in red, A/H3N2 in blue and A/H1N1 pdm09 in yellow.

3.3 Annual distribution of viruses

A low number of cases of influenza A was observed in 2010 (1/209; 0.50%), 2011 (21/491; 4.30%), 2012 (10/537;1.90%), 2013 (31/1204; 2.60%), 2014 (42/1615; 2.60%) and 2015 (8/1494; 0.53%). We detected a constant increase between the years of 2016 (139/1894; 7.34%), 2017 (157/2167; 7.24%), 2018 (285/2752; 10.35%) and the first half of 2019 (227/3049; 7.45%) (Table 1).

Table 1. Distribution of Influenza A virus among different age groups and gender during 2010-19.

	Influenza A										
Age	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**Total**
>1 ano	-	1 (0.11%)	5 (0.54%)	7 (0.76%)	14 (1.52%)	-	14 (1.52%)	25 (2.74%)	45 (4.88%)	44 (4.78%)	155 (16.83%)
1 -15	1 (0.11%)	17 (1.84%)	5 (0.54%)	13 (1.41%)	16 (1.74%)	2 (0.22%)	54 (5.86%)	76 (8.25%)	135 (14.66%)	88 (9.55%)	407 (44.19%)
16-30	-	-	-	2 (0.22%)	5 (0.54%)	2 (0.22%)	24 (2.60%)	15 (1.63%)	32 (3.47%)	17 (1.84%)	97 (10.53%)
31-45	-	3 (0.32%)	-	3 (0.32%)	4 (0.43%)	1 (0.11%)	22 (2.39%)	10 (1.08%)	24 (2.60%)	29 (3.14%)	96 (10.42%)
46-60	-	-	-	5 (0.54%)	2 (0.22%)	2 (0.22%)	19 (2.06%)	6 (0.65%)	16 (1.74%)	27 (2.93%)	77 (8.36%)
61-80	-	-	-	1 (0.11%)	-	1 (0.11%)	6 (0.65%)	18 (1.95%)	19 (2.06%)	14 (1.52%)	59 (6.41%)
81-100	-	-	-	-	1 (0.11%)	-	-	7 (0.76%)	14 (1.52%)	8 (0.87%)	30 (3.26%)
Sex											
Male	1 (0.11%)	13 (1.41%)	10 (1.08%)	21 (2.28%)	18 (1.95%)	6 (0.65%)	65 (7.06%)	84 (9.12%)	137 (14.87%)	101 (10.97%)	456 (49.51%)
Female	-	8 (0.87%)	-	10 (1.08%)	24 (2.60%)	2 (0.22%)	74 (8.03%)	73 (7.93%)	148 (16.07%)	126 (13.68%)	465 (50.49%)

A basal number of IBV cases was observed, with a slight increase from 2016. We detected in each year: 2010 (2/209; 0.95%), 2011 (1/491; 0.20%), 2012 (1/537; 0.19%), 2013 (8/1204; 0.66%), 2014 (5/1615; 0.31%) and 2015 (15/1494; 1%), 2016 (41/1894; 2.16%), 2017 (92/2167; 4.24%), 2018 (26/2752; 0.94%) and the first half of 2019 (117/3049; 3.84%).

Table 2. Distribution of Influenza B virus among different age groups and gender during 2010-19.

	Influenza B										
Age	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**Total**
>1 ano	-	-	1 (0.32%)	-	1 (0.32%)	5 (1.63%)	11 (3.57%)	17 (5.52%)	4 (1.30%)	24 (7.79%)	63 (20.46%)
1 -15	1 (0.32%)	-	-	5 (1.63%)	1 (0.32%)	8 (2.60%)	20 (6.49%)	50 (16.24%)	16 (5.19%)	81 (26.30%)	182 (59.09%)
16-30	1 (0.32%)	1 (0.32%)	-	3 (0.98%)	1 (0.32%)	1 (0.32%)	5 (1.63%)	3 (0.98%)	2 (0.65%)	5 (1.63%)	22 (7.15%)
31-45	-	-	-	-	1 (0.32%)	1 (0.32%)	2 (0.65%)	8 (2.60%)	2 (0.65%)	3 (0.98%)	17 (5.52%)
46-60	-	-	-	-	-	-	1 (0.32%)	8 (2.60%)	1 (0.32%)	1 (0.32%)	11 (3.56%)
61-80	-	-	-	-	-	-	2 (0.65%)	4 (1.30%)	1 (0.32%)	-	7 (2.27%)
81-100	-	-	-	-	1 (0.32%)	-	-	2 (0.65%)	-	3 (0.98%)	6 (1.95%)
Sex											
Male	1 (0.32%)	1 (0.32%)	1 (0.32%)	4 (1.30%)	4 (1.30%)	11 (3.57%)	23 (7.47%)	38 (12.34%)	13 (4.22%)	66 (21.43%)	162 (52.59%)
Female	1 (0.32%)	-	-	4 (1.30%)	1 (0.32%)	4 (1.30%)	18 (5.84%)	54 (17.53%)	13 (4.22%)	51 (16.56%)	146 (47.39%)

3.4 Clinical Presentation

Of the 1,229 influenza virus-positive patients, 72.90% (896/1229) had ILI symptoms while about 27.10% (333/1229) had SARI. Of the patients diagnosed with ILI, 39.18% (351/896) were H1N1, 27.23% (244/896) H3N2, 5.25% (47/896) unsubtyped and 28.35% (254/896) IBV. In SARI group 51.95% (173/333) were H1N1, 24.63% (82/333) H3N2, 7.80%; (26/333) unsubtyped and 15.61% (52/333) IBV. (Fig.5)

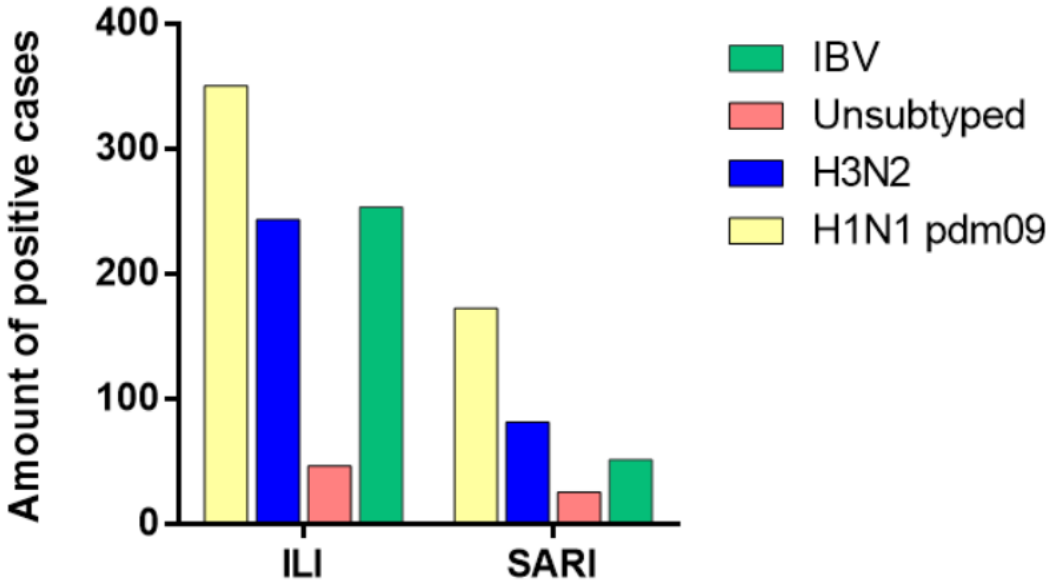

Fig.5. Relationship between influenza virus subtypes and ILI and SARI. In cases of ILI, A/H1N1 pdm09 and IBV present a higher number of cases, followed by A/H3N2 and unsubtyped; In SARI cases A/H1N1 pdm09 and A/H3N2 are the major. Influenza B is shown in green, unsubtyped in red, A/H3N2 in blue and A/H1N1 pdm09 in yellow.

3.5 Seasonal pattern

Influenza viruses were detected at various times throughout the study period (Fig. 6), although IAV positivity rates were lower between September and November (spring), a time of year characterized by high temperatures and low rainfall in the Northeast region of the country. There was an increase in positivity rates between ILI samples during the rainy season months and milder temperatures (between March and August), mainly related to H1N1 and IBV infections. In cases of SARI, there was also a higher incidence in the rainy period, with H1N1 being the major responsible.

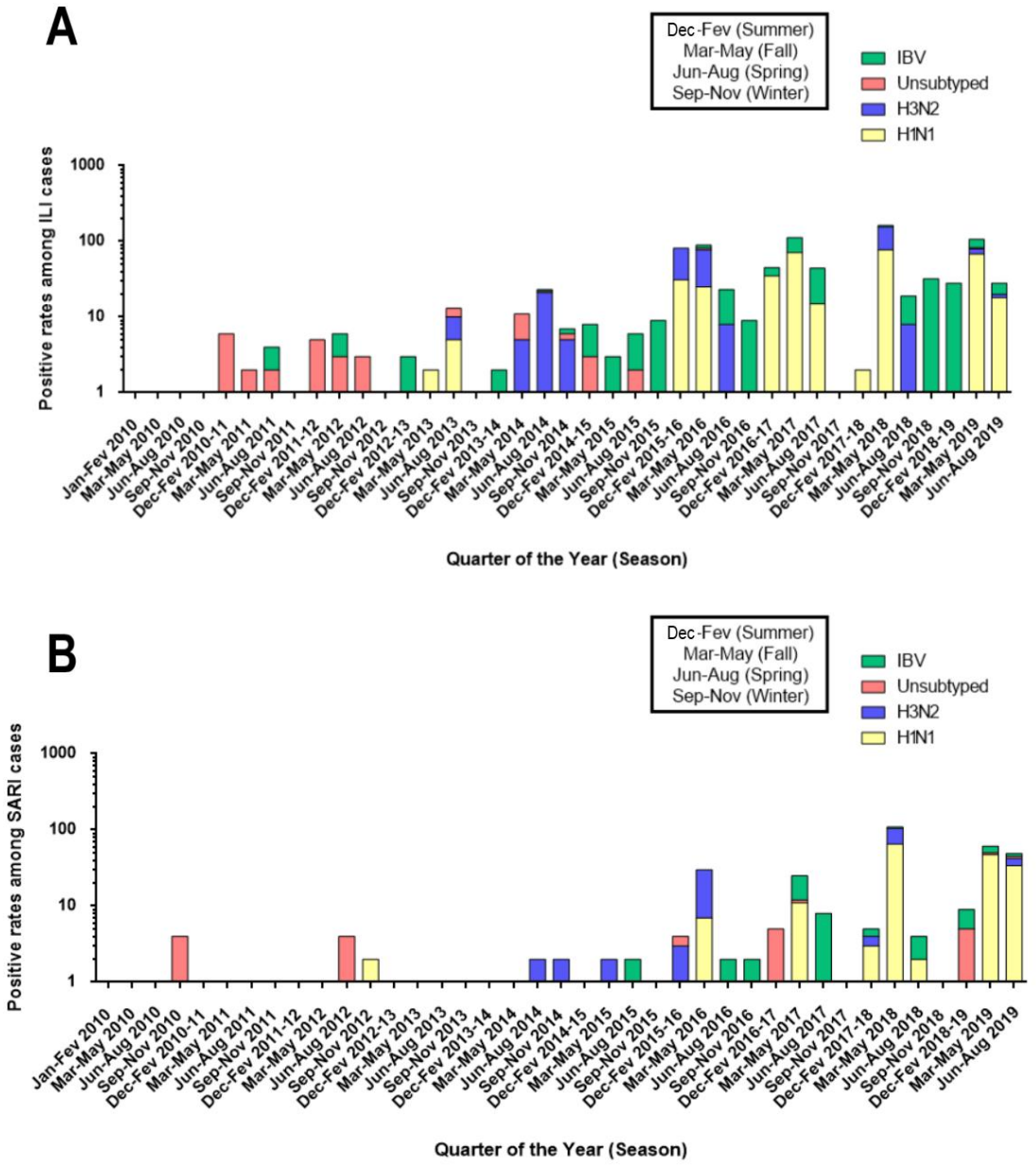

Fig. 6. Temporal analysis of influenza virus detection in patients with (a) ILI or (b) SARI in Pernambuco from January 2010 to August 2019. Each year quarter was divided according to year seasons: Dec-Feb (summer); Mar-May (Fall); April-July (Winter) and Sep-Nov (Spring).

4. Discussion

Influenza-like disease (ILI) and severe acute respiratory disease (SARI) is a clinical condition that is related to several respiratory pathogens, with influenza viruses being a major cause (RABONI; MOURA; CAETANO; AVANZI *et al.*,

2018). The establishment of influenza virus surveillance systems is of paramount importance for the preparation of new pandemics.

During 9 years of influenza surveillance among ILI/SARI patients in Pernambuco, Northeast Brazil between 2010 and 2019, it was found that approximately 10% of patients with symptoms of ILI or SARI were related to laboratory confirmed influenza. This result corroborate with the range (~ 6.5 – 22%) found in other Brazilian states and also with the data reported by the Brazilian Ministry of Health (BARROS; CINTRA; ROSSETTO; FREITAS *et al.*, 2016; PUIG-BARBERÀ; BURTSEVA; YU; COWLING *et al.*, 2016; RABONI; MOURA; CAETANO; AVANZI *et al.*, 2018).

There is a higher rate of positivity in the metropolitan region, where there is a higher concentration of people, besides being a warmer and wetter region due to the proximity to the sea(**ANTONIO G. PINHEIRO SOUZA**, 2018). However, this difference in positivity rates can be influenced by other factors, such as greater number / greater search for health units in this region.

Between the evaluated cases, 58.2% had symptoms of ILI, while about 41.8% had more complicated SARI. In the ILI and SARI cases, the main causative agent was IAV H1N1 followed by H3N2. The predominance of H1N1 and H3N2 in SARI cases has already been observed (BARROS; CINTRA; ROSSETTO; FREITAS *et al.*, 2016; OLIVEIRA; CARMO; PENNA; KUCHENBECKER *et al.*, 2009).

The data obtained in this study show the continuous circulation of influenza viruses in Pernambuco, which affect all age groups. This circulation was more frequent among the age groups <1 year and between 1-15 years old during the period from January 2010 to August 2019. Among the data obtained, IAV H3N2, H1N1pdm09 and Influenza B subtypes were detected. Studies suggest age as one of the risk factors for influenza infection, as well as behavioral patterns, showing higher infection rates among groups of 5 to 19 years (OLIVEIRA; CARMO; PENNA; KUCHENBECKER *et al.*, 2009; RABONI; MOURA; CAETANO; AVANZI *et al.*, 2018). Although some authors describe endocrine factors and pregnancy as one of the predisposing factors of female sex to influenza infections (GABRIEL; ARCK, 2014; KLEIN; HODGSON; ROBINSON, 2012), in the study we see no correlation between patient gender and increased susceptibility to influenza infection.

During the analyzed period, influenza viruses had a higher circulation during the rainy season (March-May and June-August), while in the dry season (September to November) only a few sporadic cases were detected. This pattern had been previously described, where analysis of the seasonality of influenza infections demonstrated the peak viral activity in May in the northeast region and spreads along the country's coast to the southern region (ALMEIDA; CODEÇO; LUZ, 2018; PARK; SON; RYU; CHOI *et al.*, 2019).

We conclude, therefore, that the circulation of influenza in Pernambuco showed a consistent seasonality with tropical regions during the study period. In addition, the group between 0 and 15 years was the most affected group, being IAV (H1N1 and H3N2) the main responsible for SARI and ILI. Continuous monitoring of influenza subtypes related to ILI and SARI cases can help local and regional public health authorities improve the vaccination calendar based on seasonality and circulating viral type in different regions of the country.

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3.5 LACK OF ZIKA VIRUS-SPECIFIC ANTIBODIES IN PIGS RAISED AT THE EPICENTER OF THE BRAZILIAN EPIDEMIC

ABSTRACT

The Zika virus (ZIKV) was first reported and isolated in primates in Uganda in 1947. Since then has been found natural and also experimental infection of a diverse range of wild and domestic animals. However, the spectrum of species involved in its transmission cycles remains poorly understood. Pigs have been used as animal models for several studies, including kinetic studies and pathogenicity of ZIKV. However until the date no case of ZIKV has been described in domestic pigs. The objective of this study was to detect the presence of neutralizing antibodies against ZIKV in pigs from farms and slaughterhouses in the state of Pernambuco, northwest of Brazil. Sampling was performed in slaughterhouses and farms in three regions of the state. A total of 343 sera were collected. Individual serum dilutions were submitted to serological examination for presence of antibodies against ZIKV using plaque reduction neutralization test (PRNT). However, neutralizing antibodies against ZIKV were not detected. But, it is still not possible to determine that ZIKV can cause endemics in herds.

Keywords: Zika virus; antibody; subclinical infection; domestic pigs

INTRODUCTION

The Zika virus (ZIKV) is mainly transmitted by arthropod vectors between humans by *Aedes* spp. mosquitoes (BARZON; TREVISAN; SINIGAGLIA; LAVEZZO *et al.*, 2016). The virus is a member of *Flavivirus* genus within the Flaviviridae family, the same family as the Dengue virus (DENV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) (BUENO; MARTINEZ; ABDALLA; DUARTE DOS SANTOS *et al.*, 2016).

Its first description occurred in the Zika forest of Uganda in 1947 in monkeys. However, it was later in 1952 that the first cases in humans were reported in the same region (DICK, 1952). The first outbreak of ZIKV infection was recorded in Micronesia, on the island of Yap in the year 2007, French Polynesia in 2013 and other countries and territories in the Pacific, where the ability of the virus to cause human outbreaks was clear (MUSSO; NILLES; CAO-LORMEAU, 2014). In Brazil, the first case of ZIKV infection was reported in 2015 that triggered a major outbreak of the disease. During this outbreak an association between virus and microcephaly was soon discovered in fetuses exposed to infection at the time. ZIKV infection was also associated with *Guillain-Barré* syndrome in adults (BRITO; CORDEIRO, 2016).

Soon after this association several studies were carried out to develop animal models for ZIKV infection (KUMAR; KRAUSE; AZOUZ; NAKANO *et al.*, 2017). Since not all primates infected in these works showed clinical signs of the disease, other small rodents such as mice, guinea pigs, and rabbits were required. In some cases, several serial passages are necessary in murine brains for manifestation of the disease, although death was sometimes been observed (ROSSI; TESH; AZAR; MURUATO *et al.*, 2016).

The infection caused by ZIKV has also been identified in other experimentally and naturally susceptible animal species (BUENO; MARTINEZ; ABDALLA; DUARTE DOS SANTOS *et al.*, 2016). The first viral isolate occurred from rhesus monkeys and these are presented the best model of non human primate for the study of the pathogenesis of ZIKV (NGUYEN; ANTONY; DUDLEY; KOHN *et al.*, 2017). However other animal reservoirs may be involved in the viral transmission cycle, the presence of anti-ZIKV antibodies were detected in wild mammals in Senegal in the 1960s. In Lombok, Indonesia, in 1978, anti-ZIKV antibodies were detected in ducks, goats, cows, horses, bats and carabahos, but not in chickens, rats or wild birds, indicating the wide circulation of the virus in domestic animals (VOROU, 2016).

Domestic pigs are used as a very useful model in preclinical studies, including human viral infections, such as caused by flaviviruses (CASSETTI; DURBIN; HARRIS; RICO-HESSE *et al.*, 2010; ILKAL; PRASANNA; JACOB; GEEVARGHESE *et al.*, 1994; LIND; MOUSTGAARD; JELSING; VAJTA *et al.*, 2007; RICKLIN; GARCÍA-NICOLÁS; BRECHBÜHL; PYTHON *et al.*, 2016; VODICKA; SMETANA; DVORÁNKOVÁ; EMERICK *et al.*, 2005). This due to the anatomical, physiological similarity and immune response with humans (PENA; MIRANDA GUARINES;

DUARTE SILVA; SALES LEAL *et al.*, 2018). Previous reports have been described that domestic pigs can be infected to other human flaviviruses, including DENV, WNV and JEV. In these infections viremia and viral replication in internal organs are observed (CASSETTI; DURBIN; HARRIS; RICO-HESSE *et al.*, 2010; ILKAL; PRASANNA; JACOB; GEEVARGHESE *et al.*, 1994; SCHERER; MOYER; IZUMI, 1959). A few studies show the occurrence in tropical and temperate climates of ZIKV infection in wild and domestic animals (VOROU, 2016).

The susceptibility of newborn piglets to ZIKV infection has been reported. A preliminary study has determined that neonatal pigs are predisposal to ZIKV infection (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017). In previous studies *in utero* inoculation of ZIKV in newborn piglets was able to reproduce clinical signs similar to those of humans (DARBELLAY; COX; LAI; DELGADO-ORTEGA *et al.*, 2017; WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018). In addition, it has been shown that ZIKV can infect and replicate in experimentally inoculated piglets.

Therefore, the present study aimed to evaluate the profile of neutralizing antibodies against ZIKV in pigs in slaughterhouses and farms living in the state of Pernambuco-Brazil.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Committee on Ethics and Animal Use of the Aggeu Magalhães Institute (CEUA-IAM) under protocol number 90/2015 and by the National Technical Committee on Biosafety (CTNBio) under protocol number 5.643/2017. According to the Principles of Animal Experimentation, the impact of procedures on animal welfare was minimized, ensuring that samples were collected by trained person.

Sample collection

The study was carried out in several municipalities of the State of Pernambuco, Brazil from February 2016 to August 2017. The collections were carried out in the Metropolitan Region (MR), Zona da Mata (ZM) and Agreste (AG) of the state. Samples were collected on farms and rural properties as well as in slaughterhouses in the region. In the farms the animals presented clinical sings such as diarrhea or bloody diarrhea, inappetence, walking stumbling, cough and weight

loss. In the slaughterhouses the animals were asymptomatic. 341 swine blood were collected and centrifuged at 2,500 × g for 5 minutes to obtain sera and stored at −80°C until use.

RNA extraction and qRT-PCR

Pools were made containing 10 sera each for RNA extraction. One pool with only negative samples were used as negative control. Viral RNA from sera samples was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN), according to manufacturer's instructions. ZIKV qRT-PCR was performed as previously described (LANCIOTTI; KOSOY; LAVEN; VELEZ *et al.*, 2008). We used RNA from a Zika PE243 culture serial diluted (1:10) as a positive control, the viral RNA concentrations in the standard curve were ≈270–0,027 ng. All qRT-PCR assays were performed on the Gotaq Probe 1-step RT-qPCR System (Promega) in 25 µL reactions with 4.5 µL RNA template. qRT-PCRs with cycle threshold (Ct) values higher than 30 cycles were considered negative.

Cells and Viruses

The African green monkey kidney cells (Vero) was cultivated in DMEM (Dulbecco's modified Eagle Medium – Gibco), supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml; Gibco), and 5% fetal bovine serum (Gibco). For the traditional PRNT assay, we used ZIKV Brazilian strain PE243 (GenBank accession no.KX197192.1).

PRNT methods

PRNT assays were performed by using monolayers of Vero cells in 24-well plates seeded with 0.5 mL of 1,5 × 10^5 Vero cell suspension per well in DMEM (Gibco) with 5% heat-inactivated fetal bovine serum and 100 units of penicillin and streptomycin (Gibco). Plates were incubated at 37°C with 5% CO_2 for 24 hours. After incubation, media was removed from the cell monolayer. Well-characterized human sera were used as positive controls. Test serum was heat inactivated at 56°C for 30 minutes. 4-fold dilutions of the test in DMEM beginning with a 1:20 dilution (1:20; 1:80; 1:320; and 1:1280) and the reference virus in DMEM were mixed (1/1 v/v) were incubated for 1 hour at 37ºC. After incubation, the media were aspirated of the 24-well plates and virus-serum mixture was inoculated (0.1 mL per well) and absorbed for 1 hour at 37ºC at which point the inoculums were removed. After incubation add 500 µl of semi-solid media (DMEM, 3% carboxymethylcellulose, 5% FBS and 1%

penicillin/streptomycin) to each well and incubate plates for 5 days at 37°C in a 5% CO_2 atmosphere. When virus plaques became visible, a second overlay containing crystal violet was added, and plaques were counted. The antibody titer was determined as the serum dilution that inhibited 50% of the tested virus inoculum (PRNT50).

RESULTS

In the present study, samples were collected from 341 swine in the state of Pernambuco, northeastern Brazil (Fig. 1). Many of the animals presented symptoms such as coughing, diarrhea, locomotor difficulties, weight loss and inappetence; these samples were collected in the period from February 2016 to August 2018.

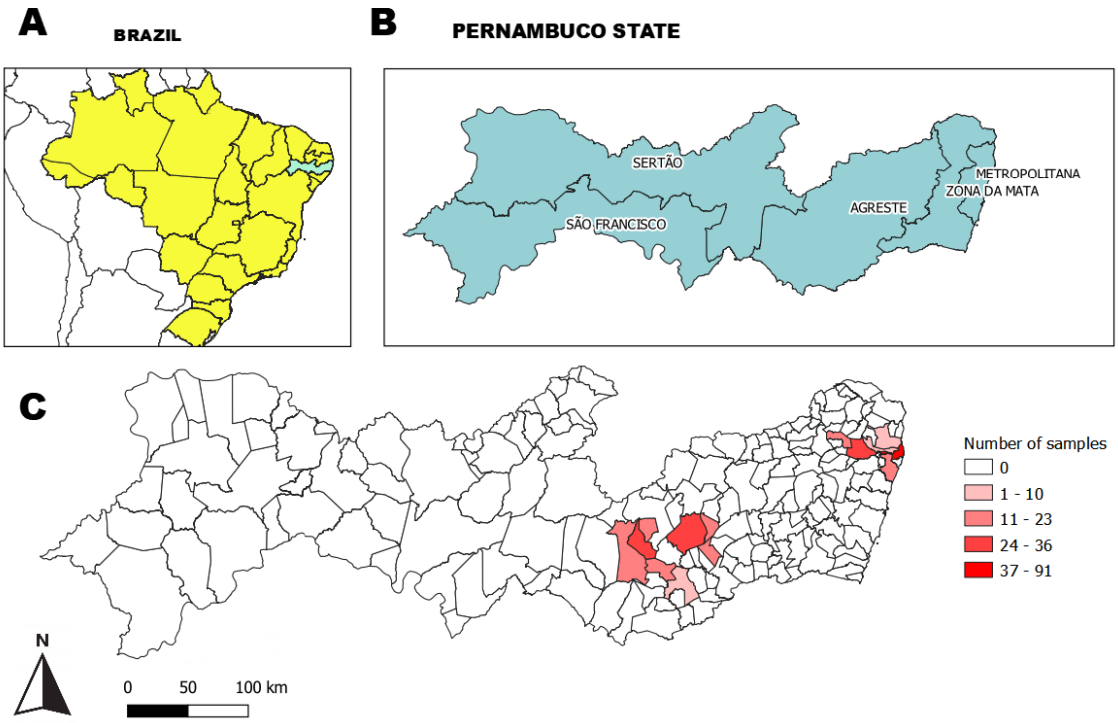

Figure 1. Spatial distribution of sampled collection in Pernambuco. A- the position of the state of Pernambuco (blue) in relation to Brazil; **B-** Mesoregions of the state of Pernambuco (Metropolitana, Mata and Agreste were the places collected); and **C-** Cities where collections were made, gradation indicates the number of samples collected (the larger the number of samples, the darker).

All 34 pools screened by real-time PCR for acute ZIKV infection had Ct values greater than 30, which were considered the cutoff point.

No positive animals for the presence of neutralizing antibodies against ZIKV were detected in any of the 341 samples analyzed (Table 1). There are reports of

ZIKV circulation and also other flaviviruses in wild and domestic animals in several countries (BUENO; MARTINEZ; ABDALLA; DUARTE DOS SANTOS *et al.*, 2016) and due to the presence of these viruses circulating in humans in the studied region, we cannot exclude the undetected circulation of ZIKV as well as other arboviruses in these animals.

Table 1. Results of the farm sero-survey.

Region	Location	Production type	Year	Specimen number	Plaque assay
	Abreu e Lima	Slaughterhouse	2017	08	<20(100%)
Metropolitan Region (MR)	Igarassu	Farrow-to-finish	2017	07	<20(100%)
	Paulista	Slaughterhouse	2017	91	<20(100%)
	Recife	Farrow-to-finish	2017	14	<20(100%)
Total per region				**120**	
Zona da Mata (ZM)	Carpina	Farrow-to-ursery	2017	19	<20(100%)
	Paudalho	Slaughterhouse	2016-2017	36	<20(100%)
Total per region				**55**	
	Alagoinha	Farrow-to-finish	2017	20	<20(100%)
	Cachoeirinha	Farrow-to-finish	2017	23	<20(100%)
	Caetés	Farrow-to-finish	2018	14	<20(100%)
Agreste (AG)	Garanhuns	Farrow-to-finish	2018	10	<20(100%)
	Lajedo	Slaughterhouse	2017	15	<20(100%)
	Pedra		2017	19	<20(100%)
	São Bento do Una	Farrow-to-ursery Farrow-to-finish	2017-2018	33	<20(100%)
	Venturosa	Farrow-to-finish	2017	32	<20(100%)
Total per region				**166**	
TOTAL				**341**	<20(100%)

DISCUSSION

Due to the recent ZIKV epidemic, the development of animal models for the study of viral pathogenesis has contributed to a better understanding of the

neuropathology induced by ZIKV. However, there is still limited information on the possible animal reservoirs of the ZIKV, including wild and domestic animals.

To date, no natural ZIKV infection has been reported in domestic pigs. However, the virus was isolated in several species of monkeys and the presence of antibodies against ZIKV was detected in domestic sheep, goats, horses, cows, ducks, rodents, bats, orangutans and carabaos (VOROU, 2016)

Flavivirus infection of pig has been demonstrated previously (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017). Scherer et al. (1952) showed that pigs were naturally infected or infected experimentally by the JEV using mosquitoes. Passively acquired antibodies were detected in animals with 2-7 weeks, showing the maternal transmission of them. Meanwhile, neutralizing antibodies were detected 45 days after infection (SCHERER; MOYER; IZUMI, 1959).

Pigs with serum samples positive for WNV were identified on farms in Mexico. The analysis was performed using ELISA and plaque neutralization assay. Positive samples were also found in the United States and Spain. The transmission of these viruses occurs through mosquitoes and evidence the possibility of the transmission cycle involving the vector (MERINO-RAMOS; LOZA-RUBIO; ROJAS-ANAYA; MARTÍN-ACEBES, 2018).

In the present study, we collected sera samples from 341 domestic pigs in farms and slaughterhouses in tree regions in the state of Pernambuco-Brazil. Sera samples were evaluated by qRT-PCR and PRNT tests. However, none of 34 pool analyzed were positive on qRT-PCR and neutralizing antibodies against ZIKV were not detected. PRNT assay is part of one of the 3 methods for diagnosing ZIKV infection. It is still considered the gold standard for arbovirus serology because, in primary infections, it is relatively specific. The other two methods consist of detection of viral RNA by qRT-PCR and detection of reactive IgM antibodies to ZIKV by ELISA. Molecular tests are the first indication for the detection of ZIKV. Positive samples in the ELISA assays should be confirmed by PRNT because of the possibility of cross-reactions with other flaviviruses (SHAN; XIE; REN; LOEFFELHOLZ *et al.*, 2017) (MUSSO; GUBLER, 2016).

Experimental infections of pigs have already been performed with the DENV(CASSETTI; DURBIN; HARRIS; RICO-HESSE *et al.*, 2010), WNV (ILKAL; PRASANNA; JACOB; GEEVARGHESE *et al.*, 1994), JEV (ILKAL; PRASANNA; JACOB; GEEVARGHESE *et al.*, 1994; PARK; HUANG; LYONS; AYERS *et al.*, 2018)

and ZIKV (WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018). Young piglets can be infected by ZIKV after experimental infection via intracerebral. Virus can replicate in the body of these animals causing neurological signs such as tremor and limb weakness (WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018). The presence of neutralizing antibodies is detected in animals inoculated by this route (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017). After in utero inoculation in pregnant females, viremia and neurological signs are also verified in the piglets (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017). The susceptibility of pig fetuses to ZIKV infection and the ability of ZIKV to persist in the porcine placenta to inoculation via this route were also seen (DARBELLAY; COX; LAI; DELGADO-ORTEGA *et al.*, 2017; WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018).

Swine are animals exposed to various natural and experimental infections and may be susceptible to natural ZIKV infection. Although the animals show clinical signs of infection during collection, our results show that swine collected had no ZIKV exposure, but we cannot exclude the role of these animals in the wild cycle of the virus, since these animals are subject to experimental infections by various (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017).

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4 DISCUSSÃO GERAL

A gripe é uma doença infecciosa causada pelo vírus Influenza, a infecção é caracterizada por febre, tosse, dores musculares e articulares, dor de garganta e coriza, e pode levar a hospitalização ou morte, principalmente em idosos e crianças menores de 5 anos. As epidemias anuais de gripe representam cerca de 3 a 5 milhões de casos de doenças graves e 290.000 a 650.000 mortes respiratórias em todo o mundo (WHO, 2018). A importância dos suínos na geração e dispersão do vírus influenza é bem descrita e vários fatores ambientais e do hospedeiro estão envolvidos nesse processo (LONG; MISTRY; HASLAM; BARCLAY, 2019).

No presente estudo, 500 amostras de *swab nasal* e pulmão foram coletadas de suínos residentes no Estado de Pernambuco, das quais 9,4% (n=48) foram positivas para o vírus Influenza A no qRT-PCR. Essa prevalência corrobora com dados encontrados por outros grupos ao redor do mundo onde a prevalência de positividade no diagnóstico para IAV nas amostras fica em torno de 7,8% e 12,3% (BROOKES; IRVINE; NUNEZ; CLIFFORD *et al.*, 2009; HOWDEN; BROCKHOFF; CAYA; MCLEOD *et al.*, 2009; ROSE; HERVÉ; EVENO; BARBIER *et al.*, 2013). Os achados também corroboram com o estado clínico observados nos rebanhos durante as coletas, em que muitos animais apresentaram sintomas respiratórios leves à intenso, o que aumenta a chance de identificação viral (HEALTH, 2009) .

Uma prática comum na criação de subsistência é a criação de suínos em conjunto com aves domésticas (galinhas, perus e pássaros), cães e gatos. Isso facilita o intercâmbio viral e favorece o surgimento de vírus recombinantes potencialmente infecciosos para humanos e animais (CARNERO; KITAYAMA; DIAZ; GARVICH *et al.*, 2018). Cerca de 70% (n=43) das amostras positivas, foram provenientes da região Agreste onde houve maior número de propriedades visitadas e animais coletados, além disso, a criação de suínos é extremamente rudimentar e muitos agricultores alimentam os animais com carcaças de aves não processadas, o que pode ser um fator de risco para animais adquirirem vírus e outros patógenos (CARNERO; KITAYAMA; DIAZ; GARVICH *et al.*, 2018).

A análise filogenética dos genes HA e NA das cepas pernambucanas indicou que as cepas sequenciadas se assemelham às cepas H3N2 humanas circulantes na

América Latina e na América do Norte entre 2012 e 2018. Os genes internos estão intimamente relacionados aos vírus H3 circulantes na China e nos Estados Unidos. Esses resultados mostram uma intensa troca viral entre humanos e suínos, no entanto, até o momento poucos estudos têm realizado o diagnostico e caracterização molecular de SIV no Brasil, mas a presença do vírus da influenza H1N1 humano (pandemia de 2009) foi confirmada em surtos de doença respiratórias em suínos em propriedades nas regiões sul (Paraná e Rio Grande do Sul), centro-oeste (Mato Grosso) e sudeste (Minas Gerais e São Paulo) do Brasil, durante os anos de 2009-2010, o que comprova a disseminação de vírus humanos nos rebanhos de suínos brasileiros (RAJÃO; COSTA; BRASIL; DEL PUERTO *et al.*, 2013), entretanto até o momento não há nenhum dado nos rebanhos no Nordeste do Brasil. Devido a isto, temos um número restrito de sequências humanas e suínas do Brasil disponíveis, o que dificulta a análise com mais detalhes das sequências encontradas neste estudo com as sequências virais brasileiras mais recentes.

Estudos sorológicos na Europa e na Ásia encontraram uma prevalência mais alta do subtipo H1N1 do que o H3N2 (KYRIAKIS; PAPATSIROS; ATHANASIOU; VALIAKOS *et al.*, 2016; LIU; WEI; TONG; TANG *et al.*, 2011). Um estudo realizado no sul do Brasil revelou uma maior prevalência do subtipo H1N1 (RAJÃO; ALVES; DEL PUERTO; BRAZ *et al.*, 2013). A análise sorológica revelou que 86,2% das amostras de soros eram positivas para o subtipo H3N2, 92,6% para o subtipo H1N1 pdm09 e 6,18% eram negativas para ambos os subtipos. Os títulos de anticorpos foram mais altos para H3N2 do que para o subtipo H1N1 pdm09. Todas as fazendas coletadas apresentaram animais positivos para ambos os subtipos virais. A maior prevalência de anticorpos contra o subtipo o subtipo H3N2 corrobora com os achados moleculares deste estudo, onde as cepas sequenciadas agrupadas com o subtipo H3N2 mais se correlacionaram com as cepas humanas.

Em humanos, 10% dos pacientes encaminhados para o diagnóstico de vírus respiratórios estavam relacionados à influenza confirmada laboratorialmente. Esse resultado corrobora com a faixa (~ 6,5 - 22%) encontrada em outros estados brasileiros e também com os dados relatados pelo Ministério da Saúde (BARROS; CINTRA; ROSSETTO; FREITAS *et al.*, 2016; PUIG-BARBERÀ; BURTSEVA; YU; COWLING *et al.*, 2016; RABONI; MOURA; CAETANO; AVANZI *et al.*, 2018).

Entre os casos avaliados, 58,2% apresentaram sintomas de ILI, enquanto cerca de 41% apresentaram quadro clínico mais complicado de SARI. Tanto nos

casos de ILI quanto SARI, o principal agente causador foi o IAV (H1N1 e H3N2), essa predominância já foi observada (BARROS; CINTRA; ROSSETTO; FREITAS *et al.*, 2016; OLIVEIRA; CARMO; PENNA; KUCHENBECKER *et al.*, 2009).

Os dados obtidos neste estudo mostram a circulação contínua dos vírus influenza em Pernambuco, que afetam todas as faixas etárias. Essa circulação foi mais frequente nas faixas etárias <1 ano e entre 1-15 anos, sendo a idade um dos fatores de risco para infecção por influenza (OLIVEIRA; CARMO; PENNA; KUCHENBECKER *et al.*, 2009; RABONI; MOURA; CAETANO; AVANZI *et al.*, 2018). Embora alguns autores descrevam os fatores endócrinos e a gravidez como um dos fatores predisponentes do sexo feminino às infecções por influenza(GABRIEL; ARCK, 2014; KLEIN; HODGSON; ROBINSON, 2012).

Durante o período analisado, os vírus influenza tiveram maior circulação durante a estação chuvosa (março-maio e junho-agosto), enquanto na estação seca (setembro a novembro) foram detectados apenas alguns casos esporádicos. Esse padrão já havia sido descrito anteriormente, onde a análise da sazonalidade das infecções por influenza demonstrou o pico de atividade viral em maio na região nordeste e se espalha ao longo da costa do país até a região sul (ALMEIDA; CODEÇO; LUZ, 2018; PARK; SON; RYU; CHOI *et al.*, 2019).

Portanto, confirmamos a circulação do IAV entre suínos no nordeste do Brasil e também relatamos a semelhança com cepas do SIV com cepas humanas que circulantes entre 2015 e 2017. E que a circulação da gripe humana em Pernambuco mostrou uma sazonalidade consistente com as regiões tropicais durante o período do estudo. Além disso, o grupo entre 0 e 15 anos foi o grupo mais afetado, sendo o H1N1 e o H3N2 os principais responsáveis por SARI e ILI, respectivamente.

A transmissão direta de patógenos animais para os seres humanos, mediada por vetores, é a fonte usual de infecção humana. Um exemplo dessa transmissão é a mais recente epidemia de ZIKV (VOROU, 2016). A compreensão do papel dos mamíferos selvagens e domésticos, na transmissão viral aos seres humanos, é de extrema importância para o controle da doença.

Até o momento, nenhuma infecção por ZIKV foi relatada em suínos domésticos sob condições naturais. No entanto, a infecção de suínos por flavivírus já foi demonstrada tanto experimentalmente como de forma natural (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017). Scherer *et al.* (1952) mostrou que suínos foram naturalmente infectados e infectados experimentalmente pelo vírus da encefalite

japonesa usando mosquitos. Anticorpos passivamente adquiridos foram detectados em animais com 2-7 semanas, mostrando a transmissão materna dos mesmos. Entretanto, anticorpos neutralizantes foram detectados 45 dias após a infecção (SCHERER; MOYER; IZUMI, 1959).

Os suínos já estão implicados na transmissão de outros arbovírus. No México, suínos com amostras de soro positivas para WNV foram identificados utilizando ELISA e PRNT. Amostras positivas também foram encontradas nos Estados Unidos e na Espanha. A transmissão desses vírus ocorre através de mosquitos e evidencia a possibilidade de o ciclo de transmissão envolver o vetor (MERINO-RAMOS; LOZA-RUBIO; ROJAS-ANAYA; MARTÍN-ACEBES, 2018).

No presente estudo, foram testadas 340 amostras de soro de suínos provenientes de fazendas e matadouros de três microrregiões do Estado de Pernambuco - Brasil. As amostras foram avaliadas para presença do vírus através de qRT-PCR e para presença de anticorpos neutralizantes contra ZIKV utilizando o ensaio de PRNT. Entretanto, nem a presença do vírus na corrente sanguínea nem a presença de anticorpos neutralizantes contra o ZIKV foram detectadas.

Infecções experimentais de suínos com DENV (CASSETTI; DURBIN; HARRIS; RICO-HESSE *et al.*, 2010), WNV (ILKAL; PRASANNA; JACOB; GEEVARGHESE *et al.*, 1994), JEV (ILKAL; PRASANNA; JACOB; GEEVARGHESE *et al.*, 1994; PARK; HUANG; LYONS; AYERS *et al.*, 2018) e ZIKV (WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018) já haviam sido testadas. Leitões jovens podem ser infectados pelo ZIKV após infecção por via intracerebral. O vírus pode se replicar no corpo desses animais causando sinais neurológicos, como tremores e fraqueza nos membros (WICHGERS SCHREUR; VAN KEULEN; ANJEMA; KANT *et al.*, 2018). A presença de anticorpos neutralizantes contra o ZIKV foi positiva em animais inoculados usando essa rota (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017). Após a inoculação *in utero* em fêmeas prenhes, viremia e sinais neurológicos também são verificados nos leitões (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017).

A falta de anticorpos neutralizantes não descarta o papel desses animais como possíveis reservatórios do ZIKV. Embora infecções experimentais por diferentes vias tenham mostrado viremia e sinais clínicos em suínos, ainda não é possível determinar que o ZIKV possa causar endemias em rebanhos (DARBELLAY; LAI; BABIUK; BERHANE *et al.*, 2017).

Este estudo destaca a importância de monitorar cepas circulantes entre porcos para evitar a ocorrência de zoonoses, podendo ajudar as autoridades locais e regionais de saúde pública a melhorar o calendário de vacinação com base na sazonalidade e no tipo viral circulante em diferentes regiões do país.

5 CONCLUSÕES

1. O vírus IAV foi identificado em 9,4% do rebanho de suínos domésticos residentes do Estado de Pernambuco, sendo a região Agreste com o maior índice de positividade, quando comparado às outras regiões estudadas;
2. As análises filogenéticas realizadas com genes HA e NA e dos genes internos demonstraram estreita relação entre as cepas pernambucanas e as cepas H3N2 humanas circulantes na América Latina, América do Norte e China entre 2012 e 2018.
3. A análise sorológica revelou que 86,2% das amostras de soros eram positivas para o subtipo H3N2, 92,6% para o subtipo H1N1 e 6,18% eram negativas para ambos os subtipos. Com títulos mais altos para H3N2 (média entre 115 e 866) do que para o subtipo H1N1 (média entre 204 e 229; $p < 0,05$;);
4. Nós descrevemos aqui o genoma completo de uma cepa IAV H3N2 circulante no estado de Pernambuco, Nordeste do Brasil.
5. Em humanos, observa-se maior prevalência do IAV em comparação com IBV. Sendo crianças e jovens entre 0-15 anos as faixas etárias mais acometidas;
6. A circulação do vírus influenza se concentra nas estações chuvosas (março-agosto) com casos esporádicos na estação mais seca (Setembro-novembro).
7. Dos animais testados para presença de anticorpos contra ZIKV 100% apresentaram títulos <20 no teste de PRNT. Porém a falta de anticorpos neutralizantes não descarta o papel desses animais como possíveis reservatórios do ZIKV.

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APÊNDICE A - ARTIGO PUBLICADO

In vitro and *in vivo* models for studying Zika virus biology

Artigo anexo na página seguinte.

A doutoranda e co-autora desse artigo de revisão publicado na *Journal of General Virology,* foi responsável pela produção do tópico *Reverse genetics systems for ZIKV*, onde resume os principais trabalhos relacionados a sistemas de genética reversa e clones infecciosos para o vírus Zika.

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REVIEW

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In vitro and *in vivo* models for studying Zika virus biology

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Abstract

The emergence and rapid spread of Zika virus (ZIKV) in the Americas has prompted the development of *in vitro* and *in vivo* models to understand several aspects of ZIKV biology and boost the development of vaccines and antivirals. *In vitro* model studies include reverse genetics systems, two-dimensional (2D) cell models, such as primary cells and cell lines, and *ex vivo* three-dimensional (3D) models derived from skin, brain and placenta. While these models are cost-effective and allow rigorous control of experimental variables, they do not always recapitulate *in vivo* scenarios. Thus, a number of *in vivo* models have been developed, including mosquitoes (*Aedes* sp. and *Culex* sp.), embryonated chicken eggs, immunocompetent and immunodeficient mice strains, hamsters, guinea pigs, conventional swine and non-human primates. In this review, we summarize the main research systems that have been developed in recent years and discuss their advantages, limitations and main applications.

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne arbovirus that has silently circulated in African and Asian countries for many decades and only caused outbreaks of a mild febrile illness. In 2007, however, a large ZIKV outbreak occurred in Yap Island. Later, the virus reemerged in French Polynesia in 2013 and rapidly spread throughout the Pacific [1, 2]. Nonetheless, the dramatic increase of birth defects reported in 2015 in Brazilian newborns changed the world's perspective on this hitherto overlooked pathogen [3]. Fifty-nine countries and territories have reported ZIKV cases from 2015 onwards. The virus continues to spread geographically to areas where competent vectors are present [4]. In the Americas continentalone, there have been 223477 confirmed autochthonous cases of Zika disease and 3720 cases of congenital Zika syndrome (CZS) since 2015 [5].

The virus belongs to the genus *Flavivirus* within the *Flaviviridae* family and as such it has a single-stranded positive-sense RNA genome that is approximately 11 Kb in length. The genome is translated as a single long open reading frame (ORF) that is flanked by 5′ and 3′ untranslated regions (UTRs) [6]. Translation of the ZIKV ORF generates three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The ZIKV genome produces two types of 3′UTR-derived, non-coding subgenomic flavivirus RNA (sfRNA), which play a role in antagonizing the host innate immune response [7, 8].

ZIKV is primarily transmitted to humans through the bites of infected mosquitoes from certain species. However, the virus can also be transmitted by blood transfusion, transplacentally, perinatally and sexually [9]. In most patients, infection by ZIKV causes a self-limiting exanthematous disease. However, foetuses infected with ZIKV may develop a number of serious teratogenic effects, including microcephaly, cerebral calcifications, ventriculomegaly, cerebellar hypoplasia, arthrogryposis, diaphragm paralysis, and visual and hearing impairments [10]. Moreover, in some patients the virus can cause a broad range of severe neurological manifestations, such as encephalomyelitis, myelitis, ophthalmologic disease, and Guillain–Barré syndrome (GBS) [11–13].

These severe clinical manifestations in humans have prompted the development of several *in vitro* and *in vivo* models aimed at uncovering the underlying mechanisms of ZIKV pathogenesis and transmission and boosting the development of countermeasures. In this review, we

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Keywords: ZIKV; animal models; *in vitro*; *ex vivo*; *in vivo*.
Abbreviations: DENV, dengue virus; JEV, Japanese encephalitis virus; TBEV, tick-borne encephalitis virus; WNV, West Nile virus; ZIKV, Zika virus.

summarize the main research systems that have been developed to study the biology of ZIKV in recent years and discuss their advantages, limitations and applications (Fig. 1).

IN VITRO MODELS

Two-dimensional cell culture models

Two-dimensional (2D) cell culture systems provide a simple and valuable system for studies in a highly controlled environment. Primary cells are isolated directly from animal or human tissue and usually have a limited lifespan. Conversely, cell lines can be continually passaged over a long period of time, since they have acquired mutations that allow them to proliferate readily. Primary cells better resemble the original tissue from which they were isolated, but they are more difficult to obtain, maintain and propagate compared to cell lines.

Primary cells

The infection of primary human epidermal keratinocytes obtained from neonatal foreskins and *in vitro*-generated immature dendritic cells (DCs) resulted in active ZIKV replication and the activation of the innate immune response [14]. Heparinized whole blood and human peripheral blood mononuclear cells (PBMCs) from healthy donors are also susceptible to ZIKV infection. $CD14^+$ blood monocytes are major targets for ZIKV and blood from pregnant women showed an enhanced susceptibility to infection by different ZIKV strains, suggesting differential immunomodulatory responses of blood monocytes during pregnancy [15, 16]. However, these data should be taken with caution, because they are based on studies carried out in countries were dengue is endemic and antibodies against dengue may modulate ZIKV infectivity by an antibody-dependent enhancement (ADE) mechanism [17]. Primary human DCs supported productive ZIKV replication following infection and exhibited donor-dependent variability in viral replication, but not viral binding. Different ZIKV strains antagonized type I interferon (IFN)-mediated phosphorylation of STAT1 and STAT2 [18].

Siemann and coworkers provided insights into the pathogenesis of ZIKV for male hosts by demonstrating that primary human Sertoli cells are susceptible to ZIKV infection.

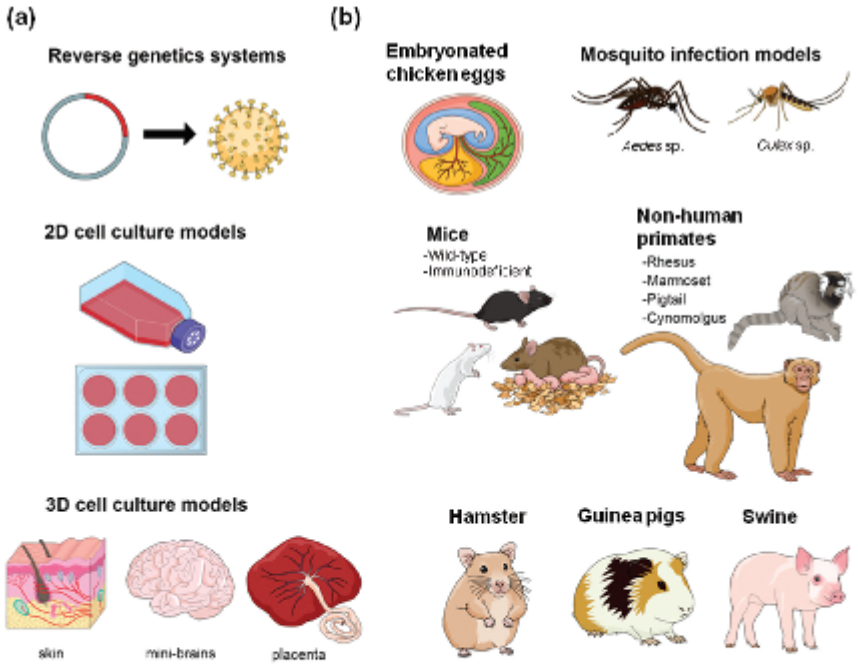

Fig. 1. *In vitro* and *in vivo* models for studying ZIKV biology. (a) *In vitro* models for ZIKV include reverse genetics systems, two-dimensional (2D) cell models (primary cells and cell lines) and *ex vivo* three-dimensional (3D) models (skin, brains and placenta). (b) A number of animal models have been developed to study ZIKV, including mosquitoes (*Aedes sp.* and *Culex sp.*), embryonated chicken eggs, several mice strains, hamster, guinea pigs, swine and non-human primates.

ZIKV infection of these cells leads to a strong antiviral response which compromises the integrity of the blood–testis barrier [19]. Amniotic epithelial cells (AmEpCs) isolated from human mid- and late-gestation placentas are productively infected by ZIKV. Trophoblast progenitor cells (TBPCs) from chorion, human placental fibroblasts (HPFs) and cytotrophoblasts (CTBs) from chorionic villi are also susceptible to ZIKV. Interestingly, cells from mid-gestation produced higher titres than cells from late gestation [20]. Jurado *et al.* demonstrated that ZIKV productively infects primary human placental macrophages, known as Hofbauer cells, and placental villous fibroblasts [21].

An independent study performed with human placental trophoblasts showed that ZIKV actively replicates in these cells without causing trophoblast dysfunction, senescence or death. This suggests that the placenta may serve as a silent portal for mother-to-foetus transmission [22]. Primary human endometrial stromal cells (HESC) supported the replication of different ZIKV strains. ZIKV replication and expression of the entry co-receptor AXL was enhanced by cyclic AMP and progesterone-induced decidualization of these cells, indicating that they may serve as virus sources for spreading to placental trophoblasts during pregnancy [23]. Primary human trophoblasts (PHTs), which are the barrier cells of the placenta, are refractory to ZIKV infection through the constitutive release of type III IFNA1, which functions in both a paracrine and an autocrine manner to protect trophoblast and non-trophoblast cells from infection [24].

Given the devastating neurological disorders linked to ZIKV affecting the peripheral and central nervous systems (PNS and CNS, respectively), Cumberworth *et al.* infected primary mouse PNS and CNS myelinating cells derived from wild-type and Ifnar1 knockout mice with ZIKV [25]. Through systematic quantification of ZIKV-infected cells in myelinating cultures, they found that CNS cells are considerably more susceptible to infection than PNS cells, especially CNS axons and myelinating oligodendrocytes. The infection of primary human astrocytes and microglia resulted in high viral replication and the induction of elevated levels of proinflammatory immune cytokines, which may be involved in neuropathogenesis [26, 27]. Primary human neural stem cells (hNSCs) derived from three different donors demonstrated donor-dependent ZIKA-mediated transcriptome alterations and reduction in neuronal differentiation, suggesting that a genetic component is involved in ZIKV neuropathology [28].

Cell lines

Viral growth kinetics in cell culture is useful to characterize different viral strains, and also to identify and test drug and vaccine candidates. In general, cell lines are considered to be genetically and phenotypically homogenous, but biological differences among the same cell line from different laboratories might occur and researchers should be aware of this possibility.

Like other flaviviruses, ZIKV replicates well in Vero and C6/36 cells, which are widely used for virus isolation from both clinical and mosquito samples [7, 29]. The susceptibility of different human and animal cell lines has been systematically characterized. Here we will summarize a few studies using different cell lines. Human cell lines derived from placenta (JEG-3), neurones (SF268), muscle (RD), retina (ARPE19), lung (Hep-2 and HFL), colon (Caco-2) and liver (Huh-7) allowed productive ZIKV replication and displayed cytopathic effects (CPE). In contrast, cell lines originating from prostate (LNCaP), testes (833KE), cervix (Hela), endometrium (HOSE6-3) and kidneys (HEK) supported viral replication but did not show CPE. Among animal cell lines, those of nonhuman primate (Vero and LLC-MK2), swine (PK-15), rabbit (RK-13), hamster (BHK21) and chicken (DF-1) origin permitted productive ZIKV replication [30]. Guinea pig lung fibroblast cells (JH4) are also susceptible to infection [31]. Recently, a human neuroblastoma cell line (SH-SY5Y) has been shown to be very useful for the evaluation of antiviral drugs against ZIKV [32].

ZIKV replicates in human monocytic leukaemia cells (THP-1) at a low rate, but triggers robust antiviral innate cytokine responses. The infection of first trimester human extravillous trophoblast cells (HTR8) demonstrated that ZIKV replicates efficiently in these cells and induces strong inflammatory cytokine and chemokine production [33]. ZIKV can also infect U87-MG (human glioblastoma) cells and produce NLRP3 inflammasome activation and IL-1β release after infection. Increased gene expression for superoxide dismutase 2 (*SOD2*) and heme oxygenase (*HemeOX*), two important antioxidant enzymes commonly used to assess oxidative stress, has also been observed. This suggests that ZIKV infection can cause oxidative stress and inflammasome activation, which can lead to cell death via pyroptosis and CNS damage [34].

Mosquito cell lines have been used to elucidate basic biological questions. For instance, Varjak *et al.* used the *Aedes aegypti*-derived Aag2, AF319 and AF5 cell lines to elucidate ZIKV–mosquito RNAi interactions [35]. C6/36 cells were also employed to investigate the cytoarchitecture of ZIKV during replication, and the authors suggested that these cells are good models for this kind of study [36–38]. To demonstrate ZIKV inhibition by *Wolbachia* strains in mosquito cell cultures, Schultz *et al.* used *Ae. albopictus* C710 and C/*w*Stri cells (derived from C710 cells). In this case, this study confirmed previous data obtained using *in vivo* models [39].

Three-dimensional (3D) cell culture models

Although 2D cell culture systems allow better control of experimental variables and are much easier to manipulate than 3D cell cultures, they do not exhibit the natural physiological conditions, cytoarchitecture and cellular complexity present *in vivo*. Therefore, 2D systems sometimes provide misleading or nonpredictive outcomes for *in vivo* settings [40]. Thus, researchers have chosen 3D systems to better characterize infection. Table 1 summarizes the main 3D cell

Table 1. Three-dimensional (3D) cell culture models

3D model	ZIKV strain	Major findings	Reference
Forebrain-specific human neural progenitor cells (hNPCs)	MR766 (original ZIKV strain)	ZIKV virus directly infects human cortical neural progenitor cells with high efficiency, resulting in stunted growth of this cell population and transcriptional deregulation	[50]
Developing human retina progenitors and cerebral organoid model	?	The candidate ZIKV receptor AXL is highly enriched in radial glia, the neural stem cells of the human foetal cerebral cortex, providing a hypothesis for why these cells are particularly vulnerable to ZIKV infection and a candidate mechanism for ZIKV-induced microcephaly	[48]
Induced pluripotent stem cells (iPSCs) growing as neurospheres and brain organoids	MR766	ZIKV induces cell death in human iPSC-derived neural stem cells (NSCs), disrupts the formation of neurospheres and reduces the growth of organoids	[46]
Forebrain-specific organoids from human iPSCs	MR766 and FSS13025 (Asian lineage)	Development of a forebrain organoid platform for chemical compound testing and modelling ZIKV infection. ZIKV, upon access to the foetal brain, targets NPSCs and causes microcephalic-like deficits in cortical development	[44]
Human cortical NPCs, neurons, neurospheres and cerebral organoids	ZIKV-BR (patient from Paraíba, 2015) and MR766	ZIKV-BR induces cell death in human NPCs, impairing the growth and morphogenesis of healthy neurospheres. Microcephaly is a distinctive feature of recent ZIKV Asian-lineage virus	[47]
Explants JEG-3 (trophoblast cell line) and BeWo (cytotrophoblast cell line)	H/PF/2013 (French Polynesia, 2013)	ZIKV infects pregnant dams and placenta, infecting the developing foetus, causing a foetal syndrome that resembles intrauterine growth restriction and spontaneous abortion	[72]
Chorionic villus explants	MR766, Nical-16 and Nica 2-16 (isolates from Nicaraguan patients)	ZIKV replicates in primary human placental cells from mid- and late gestation and villus explants from first-trimester human placentas, suggesting placental and paraplacental routes of transmission, and that infection of these cells is inhibited by the cyclic peptide duramycin	[20]
Primary human Hofbauer cells (HBCs) and cytotrophoblast cells from term placentas	ZIKV-UG/MR766 (Uganda 1947), ZIKV-CAM/FSS13025 (Cambodian/Asian isolate from 2010) and ZIKV-MEX/MEX 2-81 (Americas-derived virus isolated in 2016)	Primary placental-specific fibroblasts and HBCs are permissive for ZIKV replication in isolated cultures *in vitro*, while HBCs demonstrate susceptibility *ex vivo* in the context of placental tissues	[21]
iPSCs and cerebral organoids	ZIKV-UG (VR-1838/Uganda 1947) or ZIKV-PR (VR-1843/Puerto Rico outbreak, 2015)	Loss of AXL gene had no effect on infectivity or virus-mediated cell death in neural progenitors or cerebral organoids	[49]
First-trimester human maternal decidual tissues grown *ex vivo* as 3D organ cultures	PRVABC59 (patient from Puerto Rico outbreak, 2015) or MP1751 (mosquitoes from Zika Forest, Uganda, 1962)	Zika virus can replicate in the maternal decidua, identifying the maternal uterine aspect of the human placenta as a likely route of ZIKV transmission to the foetus	[51]

systems developed to date. Protocols for generating human brain region-specific organoids have been developed [41].

The skin is the site of ZIKV entry following the bite of an infected mosquito. Skin explants obtained from healthy donors following abdominoplastic surgery have proved valuable in understanding ZIKV pathogenesis in this site [14]. Because of their differentiation potential, pluripotent stem cells can generate virtually any cell type. Recently, structures resembling whole organs, termed organoids, have been generated from stem cells through the development of 3D culture systems [42]. Organoid cell culture systems are based on the properties of stem cells to differentiate and self-organize, creating multi-cellular tissues that resemble the structure of and function as an intact organ [43]. Several studies have used this system to study the connection between ZIKV infection and microcephaly [44–49]. Organoids also allow us to understand phenotypic and transcriptomic responses during neural development, for example [45]. ZIKV infects cells at different stages of brain maturation, leading to alterations in the cortical layer organization [47]. Qian *et al.* [44] engineered a miniaturized spinning bioreactor using 3D design and printing technology and developed protocols to generate forebrain-specific organoids from human iPSCs and for midbrain and hypothalamic organoids. They provided an accessible, cost-effective, simple-to-use and versatile platform for modelling human brain development and disease and for screening antiviral drug candidates. Garcez *et al.*

[46] used iPSCs cultured as neural stem cells (NSCs) neurospheres and brain organoids to demonstrate that ZIKV infection induces cell death in human iPSC-derived NSCs, disrupts the formation of neurospheres and reduces the growth of organoids. Tang *et al.* [50] also used a protocol to differentiate iPSCs into forebrain-specific human neural progenitor cells (hNPCs), which can be further differentiated into cortical neurons, as an *in vitro* model to investigate whether ZIKV directly infects human neural cells and the nature of its impact. They showed that ZIKV can directly infect hNPCs *in vitro* with high efficiency and that the infection of these cells led to attenuated population growth through virally induced caspase-3-mediated apoptosis and cell cycle deregulation.

Nowakowski *et al.* [48] hypothesized that protein expression may be promoting ZIKV entry and infectivity during neurogenesis. They analysed the expression of candidate genes mediating flavivirus entry across single cells from the developing human cerebral cortex and reported the importance of candidate ZIKV receptor AXL in vulnerability to ZIKV infection. In agreement with these findings, Wells *et al.* [49] studied the effects of AXL deletion and demonstrated that it was not able to protect against ZIKV infection.

ZIKV can replicate in first-trimester human maternal decidual tissues grown *ex vivo* as 3D organ cultures [51]. In explants of chorionic villi from first-trimester placentas, ZIKV infected proliferating villus cytotrophoblasts (CTBs), invasive CTBs and Hofbauer cells in the villus core, and expressed E and NS3 proteins, indicating viral replication [20, 21]. Tabata *et al.* suggested that ZIKV transmission occurs through placental and paraplacental routes and that the virus spreads from basal and parietal decidua to chorionic villi and amniochorionic membranes [52].

Reverse genetics systems for ZIKV

Reverse genetics is a powerful tool that allows important viral properties such as replication, virulence, cell penetration, transmission, host range and the function of coding or non-coding genomic regions to be studied. However, the construction of reverse genetics systems for flaviviruses is often difficult, as it involves multiple cloning of fragments of cDNA. The process is laborious and the difficulties encountered in replicating such clones in bacterial cells can cause viral sequences to be unstable and can even cause toxicity in bacterial hosts [53, 54]. Upon the emergence of ZIKV in the Americas, this technology was employed by several groups using the classical and epidemic strains [55–64]. The main ZIKV reverse genetics systems and their major applications are summarized in Table 2. The first system to be developed used the Cambodian ZIKV FSS13025 strain. Rescued viruses were shown to be highly infectious for *Ae. aegypti* mosquitoes and virulent to both A129 and AG129 mice, although it was more attenuated than the wild-type virus [55].

The Brazilian ZIKV Paraiba 01/2015 strain and the MR766 prototype of ZIKV have been used recently to generate infectious clones. Their genetic stability was further improved by inserting intron sequences into the NS1 and NS5 genes [56, 65]. ZIKV expressing reporter genes such as luciferase and GFP was proved to be a valuable tool for virus growth and replication analysis, as well as antiviral tests [55, 66]. The introduction of an NS1 K265E mutation significantly increased virus production on Vero cells, which has an impact on vaccine production [63]. Using reverse genetics, it was found that a single serine-to-asparagine substitution (S139N) in the prM protein of ZIKV contributes to foetal microcephaly, demonstrating the power of the system to identify genetic determinants of virulence [67].

A bacteria-free approach that does not require cloning, termed 'infectious subgenomic amplicons' (ISA), has been used to recover infectious viruses from PCR products in both mammalian and insect cells [59, 62]. The concept of ISA is based on the production by PCR of three to six overlapping DNA fragments that encompass the entire viral genome. The ZIKV genome is flanked by the CMV promoter and the hepatitis delta (HDV) ribozyme followed by the simian virus 40 (SV40) polyadenylation signal in the 5′ and 3′ ends, respectively. The amplicons are then mixed and transfected directly into susceptible cells to enable virus rescue through as yet unknown *in cellulo* recombination events. Unlike other bacterium-free approaches, the ISA method does not require any additional steps, such as cloning, propagation of cDNA into bacteria, or even RNA synthesis [59].

Setoh *et al.* used a modified circular polymerase extension reaction protocol to generate *de novo* a fully functional ZIKV directly from deep sequencing data. This technique has the advantage of generating infectious virus without the need for prior virus isolation and passaging in cell culture and/or suckling mice, which may result in the accumulation of adaptive mutations that may affect viral phenotypes [64].

More recently, infectious clones have been employed for *in vivo* and *in vitro* research on emerging mutations. A reverse genetics system was used to evaluate the effect of the V2634M mutation in NS5, a mutation associated with changes in viral replication efficiency as well as the incidence of microcephaly in Latin America. However, the mutant infectious clone showed no significant change in cell culture replication, and nor did it alter the pathogenesis characteristic and virulence of ZIKV in AG6 mice [68].

In addition to the infectious clones, the use of replicons has also been a useful tool to study viral replication, to investigate the role of specific mutations and to discover novel antiviral drugs. They have the advantage of not being infectious, which makes handling them in the laboratory safer [69, 70]. Taken together, these systems are valuable tools for the discovery of new antiviral compounds and for studying the physiopathology of ZIKV infection.

Table 2. ZIKV reverse genetics systems

ZIKV strain (country/year)	Nature of system	Molecular strategy	Major findings	Reference
Paraiba_01/2015 (Brazil/2015)	Full-length ZIKV cDNA inserted into the vector pACNR1811 between the CMV promoter and HDV ribozyme and poly (A) signal/RNA Pol-II terminator	Plasmid toxicity and stability in *Escherichia coli* (strain MC1061) was improved by inserting an intron sequence after nt position 2711 (NS1 gene) and after nt position 8882 (NS5 gene), respectively. A Vero adaption mutation (C-T) was introduced into nt position 5680 of the ZIKV genome (NS3 gene) to generate ZIKV-NS3m carrying a Ser356Phe substitution in the NS3 protein	The clone replicated efficiently in Vero, neuronal and placental cells. However, virus growth was lower than that for wild-type ZIKV	[56]
MR766 (Uganda, 1947)	Infectious subgenomic amplicon (ISA) fragments cloned into vector pUC57. ZIKV genome was cloned between the CMV promoter and HDV ribozyme and poly(A) signal/RNA Pol-II terminator	Four overlapping fragments covering the full-length viral genomic RNA was used. Two clones were generated and in 1 of them the eGFP gene and the protease 2A were fused in-frame to the first 33 amino acids of the ZIKV C protein	ZIKV expressing eGFP was suitable for viral replication studies in both mosquito and human cells and can be used for screening antiviral molecules and measuring neutralizing antibody titres	[66]
MR766 (Uganda, 1947)	The pWNII- GFP subgenomic West Nile virus (WNV) replicon plasmid was used as a scaffold. The ZIKV genome was cloned between the CMV promoter and HDV ribozyme and poly(A) signal/RNA Pol-II terminator	The ZIKV construct with the wild-type sequence had large deletions, which we deduced were probably a result of homologous recombination events. This was circumvented by inserting a synthetic intron into NS1, such that the coding sequence would be disrupted in bacteria but splicing in mammalian cells would restore the viral RNA	The infectious clone demonstrated similar growth to the parental virus. The addition of introns to the viral cDNA decreased the toxicity in bacteria, which improves the processes of cloning and rescue of the plasmids	[65]
FSS13025 (Cambodia/2010)	pCR2.1-TOPO with a T7 promoter and a HDV ribozyme sequence were engineered at the 5′ and 3′ ends of the complete viral cDNA for *in vitro* transcription and for generation of the authentic 3′ end of the RNA transcript, respectively	Five RT-PCR fragments (A–E) spanning the complete viral genome were individually cloned and assembled into the full-length cDNA of ZIKV. Regions spanning the viral prM-E-NS1 genes were into the low copy-number plasmid pACYC177 to reduce bacterial toxicity. A reporter gene was generated by inserting the fused template capsid luciferase gene and the FMD 2A peptide generated between the C-terminus of the luciferase gene and the ZIKV complete open reading frame	The recombinant virus had reduced replication in cell culture. However, it was virulent in mice and successfully infected *Ae. aegypti* mosquitoes experimentally. The Luciferase-expressing ZIKV was useful for antiviral screening	[55]
H/PF/2013 (French Polynesia/2013) taylori-tc/SEN/1984/41662-DAK (Senegal/1984) MRS_OPY_Martinique_PaRi_2015 (Martinique/2015)	The ISA method was used to recover infectious ZIKV. The viral fragments were flanked at the 5′ and 3′ untranslated regions by the pCMV and the HDR/SV40pA and transfected in three different mammalian cell lines (BHK-21, SW13 and HEK-293 cells)	Three overlapping genome fragments were generated for each virus by PCR. The amplicons were pooled and transfected into permissive cells to generate infectious ZIKV. Exchanging DNA fragments, inter- and intra-lineage chimeric ZIKV were produced. This is a bacteria-free approach, which overcomes the problems with plasmid stability in	Using the ISA method, it was possible to recover recombinant viruses derived from wild-type strains and also intra- and inter-lineage chimeras, and this could be a good tool to study the effect of genetic alterations and specific regions of the ZIKV genome. The replication kinetics of parental and recombinant viruses were essentially similar	[62]

Table 2. cont.

ZIKV strain (country/year)	Nature of system	Molecular strategy	Major findings	Reference
		bacteria		
H/PF/2013 (French Polynesia/2013) taylor-tc/SEN/1984/41662-DAK (Senegal/1984)	The TSA method was used to recover infectious ZIKV. Virus was rescued in both mosquitoes (C6/36) and mammalian (BHK-21) cells. The ZIKV genome is flanked in 5′and 3′ by the CMV promoter and the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal, respectively	Three fragments comprising the whole genome were transfected to generate an infectious clone. Three overlapping linear non-infectious subgenomic DNA fragments that encompass the entire viral genome were mixed and transfected directly into susceptible cells to enable the recovery of infectious viruses. This is a bacteria-free approach, which overcomes problems with plasmid stability in bacteria	The TSA method was used to produce recombinant viruses in mammalian cells and mosquitoes efficiently; in addition to ZIKV, other arboviruses (YFV, JEV, CHIKV and WNV) were also efficiently recovered by this methodology	[59]
SPH2015 (Brazil/2015) and SZ-WIV0 (China/2016)	pFK-JcIE2Flag vector. Artificial synthetic DNA sequences that contain ZIKV cDNA flanked by T7 promoter and HDV ribozyme were cloned into pFK plasmid, which is a low-copy vector	Several silent mutations were inserted into the viral genome sequence in order to reduce the activity of cryptic bacterial promoters from the infectious clone and thus facilitate rescue of the plasmid	The plasmid was stable in bacteria, and the recombinant ZIKV virus was able to infect a panel of cell lines, including Vero E6, C6/36, U-251MG, and cause lethal infection in AG6 mice. The V2634M substitution in NS5 caused negligible effects for the ZIKV life cycle in cell culture and pathogenesis in AG6 mice	[68]
GZ01 (China/2016)	Four ZIKV fragments were cloned into plasmids vectors. The SP6 promoter was placed upstream of the 5′ end of the ZIKV genome	The construction was engineered to contain a modified version of the group II self-splicing intron P.L.L.SU12 near the junction between the E and NS1 genes, which were removed from the RNA transcripts by an *in vitro* splicing reaction. Spliced viral RNA transcripts were used to generate infectious virus upon transfection of susceptible cells	Self-splicing ribozyme-based construction abolished the potential toxicity of ZIKV cDNA clones to the *E. coli* host. Two crucial cis-acting replication elements (5′-SLA and 5′-CS) of ZIKV were identified [60] and a S139N mutation in the prM protein of ZIKV was found to contribute to foetal microcephaly [67]	
Natal RGN (Brazil/2015)	Seven DNA fragments covering the entire genome were generated by circular polymerase extension reaction (CPER) and cloned into the pUC19 vector. An eighth pUC19 plasmid contained elements required for transcription [CMV, HDV ribozyme and a polyadenylation (pA) signal]	These plasmids were then used to generate cDNA fragments by PCR, with the resulting eight dsDNA fragments being mixed in equimolar amounts and subjected to cycles of CPER with a high-fidelity polymerase. The CPER products were then transfected into Vero cells for viral rescue	Fully functional Zika virus isolates were obtained directly from deep sequencing data from virus-infected tissues without the need for prior virus passaging and for the generation and propagation of full-length cDNA clones. The recombinant ZIKV was virulent for mice foetuses and transmitted efficiently in *Ae. aegypti* mosquitoes	[64]
PRVABC59 (Puerto Rico/2015)	The ZIKV genome was cloned in two separate pieces into the pACYC177 vector using Gibson assembly. The ZIKV genome was flanked by a T7 promoter and an HDV ribozyme sequence.	Plasmids were amplified, digested and ligated using Gibson assembly to create a plasmid containing the full-length virus sequence with a T7 promoter and hepatitis D virus ribozyme sequence, which was then transcribed into RNA using T7 polymerase. The resulting RNA was then electroporated into Vero cells to generate infectious virus. A single synonymous mutation was identified at nucleotide position C8489T,	Rescued ZIKV replicated similarly to wild-type virus in both human and mosquito cells. Infectious clone-derived virus initiated displayed similar rates of transmission in *Ae. aegypti* mosquitoes and similar pathogenesis in AG129 mice as compared to the wild-type virus	[57]

Table 2. cont.

ZIKV strain (country/year)	Nature of system	Molecular strategy	Major findings	Reference
		which allowed differentiation from wild-type virus		
MR766 (Uganda, 1947) H/PF/2013 (French Polynesia/2013) PRVABC59 (Puerto Rico/2015) BeH819015 (Brazil/2015) SPH2015 (Brazil/2015)	The genome of the virus is divided into four fragments using the diagrammed restriction endonucleases and cloned into pUC57 vector. ZIKV genome was flanked by a T7 promoter and the HDV ribozyme. Plasmids were amplified in *E. coli* strain MC1061	The four plasmids were digested, ligated, *in vitro*-transcribed and electroporated into C6/36 cells to rescue virus	A panel of six infectious clones was generated. This system allowed the identification of lethal errors in the published sequence of the SPH2015 virus. Recombinant viruses had satisfactory replication in cell culture, immunogenicity and virulence in mice	[58]
FSS13025 (Cambodia/2010)	The ZIKV replicon was constructed by replacing the viral structural genes with Rluc using a full-length cDNA infectious clone (pFLZIKV) obtained from a previous study [55]. A T7 promoter and an HDV ribozyme sequence were engineered at the 5′ and 3′ ends of the replicon cDNA for *in vitro* transcription and for generation of the authentic 3′ end of the RNA transcript, respectively	The C38-Rluc2A-E30 cassette encodes the N-terminal 38 amino acids of protein C, Rluc reporter, FMDV 2A protease and the 30 C-terminal amino acids of the E1 protein (E30) of ZIKV. The cassette was cloned into pFLZIKV at the NotI and SphI sites, replacing the structural genes. A second ZIKV replicon containing Rluc and Neo genes (Rep-Neo) was constructed by inserting an EMCV IRES into the 3′ UTR of the above Rluc replicon to generate a stable cell line expressing ZIKV proteins	The replicons were used to evaluate the effect of the NS5 mutation on viral RNA synthesis and the analysis of a known inhibitor of ZIKV. The replicon was useful for antiviral screening without the use of infectious virus, since the clones do not generate infectious particles, eliminating the risk of viral infection acquired in the laboratory	[70]

IN VIVO MODELS

Murine models

Murine models have contributed significantly to the acquisition of new insights into the biology of ZIKV infection [47, 71–73]. Mice have contributed to elucidate several aspects of ZIKV pathogenesis, including the link between ZIKV infection in pregnant women and congenital defects [46, 50, 74]. Mice have also been a valuable model for the evaluation of vaccine and antiviral candidates. Their small size, low cost and fast reproductive rate make these animals attractive models for ZIKV studies.

Immunocompetent mice strains

Immunocompetent adult mice show little susceptibility to ZIKV infection and do not emulate the spectrum of ZIKV clinical manifestations seen in humans (Table 3). One of the reasons for this phenotype is the ability of ZIKV to degrade STAT2, an IFN-regulated transcriptional activator in humans but not mice [75]. Since ZIKV is sensitive to the action of IFN types I, II and III, blockage of IFN receptors has been used to develop susceptible mouse models [14, 24, 76]. Lazear *et al.* used an IFNAR1-blocking monoclonal antibody (MAR1-5A3) in C57BL/6 mice to make them susceptible to ZIKV infection. These animals did not develop neurological manifestations and the disease was not as severe as that observed in *Ifnar1*$^{-/-}$ mice [76]. A similar approach was applied to develop a lethal C57BL/6 mice model. In this study, animals inoculated with the DAKAR D41525 strain via either the subcutaneous or the intraperitoneal route had 40 and 100 % mortality rates, respectively. ZIKV administration through either route caused viraemia, motor impairment and weight loss [77]. Immunocompetent mice treated with anti-IFNar1 antibodies have also been used for the discovery of antiviral drugs acting against ZIKV. Five-week-old CB57/6 mice previously treated with anti-ifnar1 antibodies and later infected with the mouse-adapted DAKAR 41519 strain showed a survival rate of almost 20 %, even when not treated with sofosbuvir, an inhibitor of the ZIKV NS5 RNA-dependent RNA polymerase (RdRp) [78]. The DAKAR D41519 strain is a lethal mouse-adapted ZIKV strain obtained following brain homogenate passage in Rag1$^{-/-}$ mice [79]. The antiviral action of sofosbuvir was also demonstrated in 3-day-old Swiss mice, without the need for IFN blockage [80].

Given that mouse models based on antibody blockage of IFN receptors are expensive for routine use and may not be able to completely deplete thr IFN response [78], alternative ways to develop immunocompromised models must be explored. One strategy is to administer immunosuppressive drugs. After ZIKV infection, dexamethasone-immunocompromised BALB/c mice sustained high viral replication in multiple organs, inflammation and mild weight loss after inoculation via the peritoneal route [81]. Interestingly, immunohistochemical analyses conducted in the tissues of

Table 3. Immunocompetent mice models for studying ZIKV biology

Mouse strain	Age	ZIKV strain (country/year)	Route of infection/dose	Major findings	Reference
C57BL/6J	5 weeks old	Dakar 41519 (Senegal/1984)	Subcutaneous/10^6 p.f.u.	Mice infected after treatment with antibody had a better rate of survival after the FDA-approved drug sofosbuvir was administered. However, almost 20 % of the untreated mice survived ZIKV infection	[78]
BALB/c	5–8 weeks old	PRVABC59 (Puerto Rico/2015)	Intraperitoneal/3.24×10^6 p.f.u.	High viral load was observed in dexamethasone immunosuppressed mice, as well as the development of several inflammations in organ tissues. Treatment with recombinant type I interferons was able to mitigate disease effects	[81]
129 Sv/Ev	5–6 weeks old	MP1751 (Uganda/1962)	Subcutaneous/10^6 p.f.u.	Low viraemia; no weight loss; no clinical disease; viral detection only in the brain, ovaries, spleen and liver	[87]
C57BL/6	5 weeks old	DAK AR D 41525 (Senegal/1984)	Subcutaneous or intraperitoneal/10^6 p.f.u.	Anti-Ifnar1-treated mice suffered severe lesions to CNS; high viraemia; weight loss; intraperitoneal injection led to a 100 % mortality rate	[77]
Swiss	1 day old	SPH 2015 (Brazil/2015)	Subcutaneous/intracranial/10 % suspension	Mild to severe CNS lesions observed; ataxia; paralysis; lethargy; subcutaneous infection caused spinal cord damage and myelopathy	[91]
FBV/NJ and C57BL/6J	5.5, 7.5,9.5 or 12.5 days *post-coitum*	HS-2015-BA-01 (Brazil/2015)	Intravenous (jugular vein)/10^6 p.f.u.	Earlier infections lead to severe malformations and intrauterine growth restriction; 10.5 days post-challenge (p.c.) the exposed mice developed both dysraphia and hydrocephalus; no significant effects noticed after 12.5 days p.c.	[92]
SJL	10–13 embryonic days	ZIKV^{BR} (Brazil/2015)	Intravenous/10^9, 4×10^{10}, 10^{12} p.f.u.	Neurological and optical malformations; intrauterine growth restriction; upregulation of apoptotic related genes	[47]

euthanized mice, especially from those that had been withdrawn from dexamethasone after day 9, indicated that the rapid systemic deterioration effects observed after viral challenge were partially due to the host immune response to ZIKV infection [81].

Previous studies have indicated the possible protective role of T-cell responses during ZIKV infection [82]. SJL mice are immunocompetent, but have elevated levels of circulating T-cells [83, 84]. Interestingly, pregnant SJL mice, when inoculated intravenously with a high dose of 10^{10}–10^{12} plaque-forming units (p.f.u.) of ZIKV, generated pups that presented neurological and opthalmological malformations similar to those observed in humans, in addition to intrauterine growth retardation [47]. Using C57BL/6 mice, the authors found that ZIKV was not able to cross the placental barrier in C57BL/6 females infected during gestation, as they had offspring that were free of infection [47]. In contrast, foetuses of C57BL/6 female mice that were inoculated intraperitoneally with an Asian lineage strain during gestation displayed signs of ZIKV infection in their brains and viral RNA was detected in mouse placentas at day 3 post-inoculation [85].

Similar to C57BL/6 mice, the outbred CD-1 mice have been demonstrated to be resistant to ZIKV infection. Infection of these animals at 3 weeks with the FSS13025 ZIKV Asian strain resulted in no clinical signs of infection and no detectable viraemia [73]. However, this apparent resistance might not be solely due to the route of infection chosen, but also to the viral strain used. When CD1 mice were challenged intracranially with the MR766 ZIKV strain, they exhibited an 80–100 % mortality rate that was age-independent. Intraperitoneal inoculation with the same strain generated morbidity signs, but no mortality in a dose-dependent manner [86]. Remarkably, 5–6-week-old female 129 Sv/Ev mice inoculated through the subcutaneous route with 10^6 pfu of ZIKV showed no clinical and histopathological signs of infection. Viraemia and low viral RNA levels were found in the brain, ovaries, liver and spleen after infection [87]. Remarkably, intravenous ZIKV infection of pregnant immunocompetent C57BL/6J pregnant mice led to profound placental pathology and a high frequency of foetal demise, in the absence of foetal infection [88].

Another immunocompetent mouse used in ZIKV research is the ICR strain, a highly prolific outbred mouse strain derived from Swiss mice. Direct intracerebral inoculation of mouse foetuses resulted in high viral replication, cell cycle arrest, apoptosis and inhibition of neural precursor cell differentiation, leading to microcephaly [89].

A fully immunocompetent humanized mouse model has been recently developed by knocking in (KI) the human STAT2 into the mouse Stat2 locus (hSTAT2 KI). Infection of pregnant hSTAT2 KI mice was carried with a highly virulent mouse-adapted ZIKV strain derived from the ZIKV-Dak-41525 strain. The infection resulted in viral spread to the placenta and foetal brain [90].

In addition to mouse age and lineage, the route of infection can directly affect the outcome of ZIKV infection. Significant lesions and cell death in the CNS were observed in newborn Swiss mice inoculated with ZIKV through either

the intracerebral or the subcutaneous route, which led to the development of myelopathy and encephalopathy, respectively. Further, animals inoculated by the subcutaneous route presented spinal cord injury [91]. A haematogenic infection model was conceived to evaluate the effects of ZIKV infection on embryonic and foetal development, using FBV/NJ and C57BL/6J mice. Early infections caused growth restriction and/or severe malformations in infected embryos, including hydrocephalus and dysraphia. Later exposure to ZIKV did not generate significant effects as foetal development progressed [92]. Remarkably, intravenous ZIKV infection of pregnant immunocompetent C57BL/6J pregnant mice led to profound placental pathology and a high frequency of foetal demise, in the absence of foetal infection. In this study, placental pathology rather than embryonic/foetal viral infection seemed to be a stronger contributor to adverse pregnancy outcomes in mice and direct viral infection of the embryo was not essential for foetal demise. [88]. Yockey *et al.* observed long-lasting infections and high rates of viral replication in C57BL/6 females infected intravaginally. In addition, foetuses of females inoculated by this route during pregnancy developed cerebral infection and intrauterine growth retardation [93].

Thus, although immunocompetent mice models present limitations regarding clinical manifestation of the disease, they are valuable to obtain evidences about viral pathogenesis under the full action of the innate and adaptive immune responses of the host to ZIKV.

Immune-deficient strains

In contrast to immunocompent mice, immunocompromised mice display signs of disease and high levels of viraemia, and can be infected even with low-passage viral strains (Table 4). The major disadvantage of immunocompromised mice is the lack of essential components of the immune response, which may underestimate the efficacy of some vaccine candidates and not model disease pathogenesis accurately in immunocompetent hosts. Nevertheless, these models have been successfully used for preclinical evaluation of vaccines and antivirals against ZIKV [94–97].

A129 mice lack the receptor for IFNα/β, making them unresponsive to type I IFNs. It was one of the first models used to characterize ZIKV infection, although it had already been used in studies about other viruses, such as chikungunya virus (CHIKV) and yellow fever virus (YFV) [87, 98]. On the other hand, the AG129 mouse strain is a double knockout for the IFNα/β and γ receptors and is more susceptible to ZIKV-induced disease than A129 mice, highlighting the role of IFN-γ in the outcome of the disease. Although the ZIKV infection kinetics in AG129 mice is similar to that in A129, the disease signs are more severe in AG129, probably because of the protective role of IFNγ [57, 71–73].

The A129 mice may also be useful in vaccine challenge studies since they remain susceptible to the induction of morbidity and mortality caused by ZIKV even when infected at 6 months of age. The detection of persistent infection foci in organs such as the brain, spinal cord, testes and ovaries even after the resolution of disease symptoms is an important finding in the study of the development of GBS and congenital infections (associated or not with microcephaly) and the occurrence of sexual transmission [73, 76, 87, 99]. Furthermore, Rossi and colleagues [73] characterized ZIKV infection in both A129 and AG129 mice. Intraperitoneal ZIKV infection of A129 mice resulted in clinical disease (tremors, lethargy and anorexia) and mortality in an age-dependent manner. The virus replicated in several organs, but the highest titres were found in the spleen, testes and brain. Overall, little difference was seen between the disease and virulence of ZIKV in A129 and AG129 mice, except for the severity of the neurological manifestations, which was more pronounced in the latter strain [73]. The neurovirulence of ZIKV in AG129 mice was endorsed by Aliota and coworkers, who reported significant histopathological lesions in the brain upon infection [71]. These observations suggest the relevance of these mice strains for studying disease pathogenesis in humans, including the Guillain–Barré Syndrome and microcephaly.

Another relevant model is characterized by Irf3$^{-/-}$ Irf5$^{-/-}$ Irf7$^{-/-}$ mice that are C57BL/6 triple knockout (TKO) for

Table 4. Major immunocompromised mouse strains used in the study of ZIKV infection

Strain	Background	Knockout	Applications	References
A129	129 Sv/Ev	IFNα and β receptors	Study of the spread of the virus in different organs. Evaluation of the immune response induced by vaccines	[118].
AG129	129/Sv	IFNα/β and γ receptors	Viral dissemination profile, lethality, mechanisms of neuropathogenesis and attenuation/virulence of viral strains using various inoculation routes	[57, 71–73].
Irf3$^{-/-}$Irf5$^{-/-}$ Irf7$^{-/-}$	C57BL/6	Transcription factors 3, 5 and 7 (interferon pathway)	Analysis of viraemia and lethality conferred by ZIKV. Important for findings related to infection of neural stem cells	[76, 100].
Ifnar1$^{-/-}$	C57BL/6	IFNα and β receptors	Characterization of infection. Mimics immunosuppressed individuals, neonates and elderly. Studies on placental infection, trans-placental transmission, neuroinvasion and consequences of neurological disease	[104]
LysMCre^{+} IFNAR1$^{fl/fl}$	C57BL/6 (H-2^{b})	Type I IFN in myeloid lineage cells	Demonstration of protection mediated by CD8+ T cells in the control of ZIKV infection. Promising for more complete studies on the cellular immune response and for the evaluation of control measures such as vaccines and antivirals	[108]
Rag1-/-	C57BL/6	T-cell and B-cell responses	Role of the adaptive cellular response in the control of ZIKV infection	[169]

the transcription factors interferon-regulatory factors 3, 5 and 7, respectively [76, 100]. This lineage has been used to study the impact of ZIKV in the CNS, where high levels of viral RNA were detected in tissues after infection and severe signs of neurological disease, such hindlimb weakness and paralysis, were observed [76]. Compared to Ifnar1$^{-/-}$, which is knockout for IFNα/β receptors, the TKO model was more susceptible to ZIKV infection and this difference points to a role for an IRF-3 dependent, IFN-α/β-independent mechanism. In fact, ZIKV infection blocks the induction of type I IFN by downregulating IRF3 and antiviral NF-κB-mediated signalling and targets STAT2 for proteasomal degradation [101].

Following the discovery that ZIKV evades the innate immune system by targeting STAT2 for degradation, Tripathi *et al.* infected *Stat2*$^{-/-}$ mice subcutaneously and showed that this strain is highly susceptible to ZIKV infection. They demonstrated that ZIKV spread systemically in this knockout mice strain and caused neurological disease [102]. Interestingly, it has been observed that SCID mice (deficient in T and B lymphocytes) develop more severe disease and are more resistant to infection than the AG129 model, suggesting that ZIKV infection in mice is mainly controlled by the innate immune response mediated by IFNs rather than the adaptive response mediated by T-cells. A systemic inflammatory response mediated by proinflammatory cytokines was also detected in the sera of AG129 mice [99, 103].

Different immunocompromised mice have also been applied to identify which cells of the nervous system are permissive to replication. Brain analysis of *Ifnar1*$^{-/-}$ mice revealed that astrocytes are one of the most susceptible types, while cerebral cortex neurons are the least permissive, being observed not only in the brain but also in other regions of the CNS, such as the cerebellum and spinal cord [104]. While in the *Ifnar1*$^{-/-}$ model the most permissive infection sites were the astrocytes, in *Rag1*$^{-/-}$ mice treated with anti-IFNAR, the most evident focal areas were neurons in the cerebral cortex and hippocampus regions [105]. The disagreement of these results reinforces the need to explore different models in order to characterize a strain that can mimic the infection in the CNS with more fidelity.

Case reports of ZIKV sexual transmission and viral persistence in the human genital tract are mounting. Murine models recapitulate these phenotypes and may provide bases for understanding the sexual transmission of ZIKV in humans. In fact, recurrent viral detection in the mouse testes has been achieved in experimental studies [73, 76, 106, 107]. In this sense, studies using females may also be informative for research about this route. Vaginal exposure of pregnant mice during early pregnancy resulted in foetal infection and intrauterine growth restriction [93]. The evaluation of different mice strains with attenuation of the innate immune response mediated by IFNs shows that the transcription factors IRF3 and IRF7 are required to block viral replication locally. In contrast, the adaptive immune response does not seem to play a critical role in the control of vaginal infection [93]. Tang and colleagues inoculated ZIKV into the vagina of both AG129 mice and LysMCre^{+} IFNAR$^{fl/fl}$ C57BL/6 mice, which lack IFNAR in myeloid cells, in diestrus-like and estrus-like phases after respective hormonal treatments [progesterone and pregnant mare serum gonadotropin (PMSG), respectively]. Whereas the mice infected during the estrus-like phase were resistant to vaginal infection, those infected during the diestrus-like phase developed disease following atraumatic intravaginal ZIKV administration. There was a strain-dependent susceptibility, in which LysMCre^{+}IFNAR$^{fl/fl}$ C57BL/6 mice experienced transient illness and AG129 mice succumbed to infection [108]. These models of venereal transmission will be useful to understand the pathogenesis of ZIKV through this route and will be a suitable challenge system for evaluating the protective efficacy of vaccine and antiviral candidates.

Chicken embryos

The chicken embryo is a well-established model in developmental biology and has advantageous features, including size, low cost, easy manipulation and the fact that it allows high-throughput *in vivo* screening of drugs. The model closely mirrors human foetal neural development and the sequencing of the chicken genome has opened up possibilities for uncovering the molecular basis of development and changes associated with viral infections [109, 110].

An early study using the MR766 ZIKV strain showed that cultured primary embryonic chicken cells were not susceptible to infection [111]. However, recent studies have demonstrated that the DF-1 chicken fibroblast cell line [30] and chicken embryos are susceptible to infection by contemporary ZIKV strains [110]. The infection of chicken embryos at embryonic days 2.5 and 5 resulted in dose-dependent mortality, virus replication in various organs, stunted brain growth and other malformations, such as enlarged ventricles, but not calcifications (Table 5). Thus, the chicken embryo proved to be a well-characterized, non-immunocompromised *in vivo* animal model capable of recapitulating some of the teratogenic manifestations of ZIKV in human foetuses [110]. The postnatal effects of ZIKV infection of chicken embryos warrant further investigation

Guinea pigs

Guinea pigs have been used as a model for infectious disease since the nineteenth century, including tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) and West Nile virus (WNV) [112–114]. Their small size and docility and the low cost of acquiring and maintaining them make them an attractive laboratory animal model, although the paucity of available immunological reagents is a limitation [115]. In contrast to mice and rats, they have a haemochorial placenta, through which maternal and foetal circulation is separated by a single layer of trophoblasts, making them useful for studying congenital infections [31]. Initial studies characterizing the pathogenicity of the MR766 strain shortly after the discovery of ZIKV by the

Table 5. Non-murine models for studying ZIKV biology

Animal Species	Age	ZIKV strain (country/year)	Route of infection/dose	Major findings	Reference
Guinea pigs	200–250 g*	PRVABC59 (Puerto Rico/2015)	Subcutaneous/ ~10^6 p.f.u.	Fever; lethargy; hunched back; ruffled fur; decreased mobility; seroconversion; viraemia; increase in the levels of multiple cytokines; chemokines and growth factors in the serum	[117]
	5 weeks old	GZ01 (China/2016) or FSS13025 (Cambodia/2010)	Subcutaneous, intranasal, or contact /10^5 p.f.u.	Infectious dose 50 % (ID_{50}) of ZIKV was calculated to $10^{3.5}$ p.f.u. by the subcutaneous route; no overt clinical signs; sustained viraemia; viral shedding in both saliva and tears; seroconversion; replication and pathology in multiple organs; 100 % contact transmission	[118]
	6 months old , at 18 to 21 days gestational age	H/PF/2013 (French Polynesia/ 2013)	Subcutaneous/ 10^7 p.f.u.	Non-pregnant animals had low-level viraemia and no clinical disease; pregnant animals had no overt clinical signs; no interference with normal pup development; no detectable viraemia and antigenaemia in pregnant animals; seroconversion in pup and dams	[31]
Hamsters	8 weeks old	FSS13025 (Cambodia/ 2010)	Subcutaneous or intradermal/~10^5 p.f.u.	No clinical disease; no viraemia; no seroconversion	[121]
	6–8 weeks old	P6-740 (Malaysia/ 1966)	Subcutaneous/5000, 500, and 50 $TCID_{50}$	Used *STAT2* knock-out hamsters; death; weight loss; viral load in the brain, testes, spinal cords, kidney, spleen; no histopathological lesions; infection of pregnant *STAT2* knock-out hamsters led to ZIKV infection in placental and foetus brains, but no detectable phenotype in pups; infection of wild-type hamsters did not result in placental or foetal brain infection	[122]
Chicken embryo	Embryonic days 2.5 and 5	MEX 1–44 (Mexico/ 2015)	*In ovo*/0.2 to 10^4 p.f.u.	Embryo mortality; virus replication in various organs; microcephaly-like phenotype; ventriculomegaly	[110]
Swine	1 day old	PRVABC59 (Puerto Rico/2015)	Intracerebral/~10^6 $TCID_{50}$	Seroconversion and low-titre viraemia, viruria and virus replication in internal organs. Two out of eleven pigs exhibited leg weakness, ataxia and tremor	[124]
	3 months old	FSS13025 (Cambodia/ 2010); PRVABC59 (Puerto Rico/2015)	Subcutaneous or intradermal/~10^8 p.f.u.	Subclinical infection without viraemia but with seroconversion	[121]
	Mid-gestation (gestation day 50)	PRVABC59 (Puerto Rico/2015)	*In utero* intraperitoneally +intraamniotic or intracerebrall /~10^5 $TCID_{50}$	Detection of ZIKV RNA in foetal membranes; persistent infection in porcine conceptuses; no brain lesions or histopathology in offspring; impaired health in offspring: weak piglets, delayed feeding, neonatal body length and weight lower than controls, seizures, splayed back leg, reduced growth rate, aggressive behavior	[125]
	Mid-gestation (gestation day 50)	PRVABC59 (Puerto Rico/2015)	*In utero* or intravenous / ~10^{5-6} $TCID_{50}$	Microcephaly in 2 out of 15 pigs inoculated *in utero* and mild to severe neuropathology, characterized by neuronal depletion in the cerebral cortices of various lobes, in all foetuses	[126]

*Corresponds to age between 11–13 days.

virologist George Dick found that guinea pigs (n=2) that had been inoculated intracerebrally had no signs of infection but died on the sixth day after inoculation with a low-passage mouse-adapted virus. Infection with highly mouse-adapted viruses did not result in lethal infection, suggesting that adaptation to mice reduces the virulence to guinea pigs (and also monkeys) [116].

The guinea pig model has been recently revisited using contemporary ZIKV strains (Table 5) [31, 117, 118]. The infection of juvenile animals (200–250 g) with ZIKV PRVABC59 strain (Puerto Rico/2015) via the subcutaneous route resulted in fever, lethargy, hunched back, ruffled fur and decreased mobility. Viraemia was detected at 2 and 3 days post-infection (p.i), but not at 5 days p.i. ZIKV RNA load was detected in the spleen (only at 2 days p.i.) and brain (at 2, 3 and 5 days p.i.) of these animals. Analysis of cytokines, chemokines and growth factors in the serum using multiplex immunoassay showed a marked increase in the levels of IL-2, IL-5, IL-18, IL-12 (p70), TNF-α, G-CSF, MCP-1, MIP-1α, LIX, fractalkine and VEGF, whose roles in ZIKV pathogenesis need to be further investigated [117]. Deng and coworkers [118] demonstrated that guinea pigs are susceptible to infection through the intranasal route and that the virus can be detected in the sera, saliva, tears, brain and parotid glands. Interestingly, the 2010 Cambodian ZIKV strain FSS13025 was less virulent than the 2016 Chinese ZIKV strain GZ01 FSS13025. Remarkably, close-contact transmission experiments showed that ZIKV is highly transmissible to co-caged animals. No overt clinical signs were

seen for any of the routes of infection used (intranasal, subcutaneous or direct contact). A third study evaluated the effect of ZIKV infection in non-pregnant and pregnant adult guinea pigs infected at mid-gestation (at 18 to 21 days gestational age) [31]. Clinical signs of disease were not observed in either non-pregnant or pregnant animals infected with the 2013 French Polynesian ZIKV strain. Naturally circulating ZIKV strains are not pathogenic to immunocompetent guinea pigs and do not interfere with normal pup development. Although pregnant guinea pigs and their pups seroconverted upon infection, no detectable viral levels were seen in maternal or pup blood, plasma or tissues. Of special interest, no significant adverse outcome was detected in the guinea pigs' offspring following challenge, suggesting that ZIKV is not pathogenic during the pregnancy of guinea pigs and does not interfere with normal foetal development in this species. The reason for the difference in overt clinical disease among these studies is not clear but might be related to the age of animals used and strain differences.

Hamsters

Syrian golden hamsters (*Mesocricetus auratus*) have been established as a model for some neurotropic flaviviruses, including WNV [119] and JEV [120]. Recently, the model has been evaluated for ZIKV (Table 5). Like guinea pigs, hamsters are small and relatively inexpensive to maintain, but there are few immunological reagents for this species. The infection of hamsters with a 2010 Cambodian ZIKV strain did not result in clinical disease, viraemia or seroconversion [121]. However, genetic ablation of the STAT2 gene, a key mediator of type I and type III IFN signal transduction pathway signalling, renders these animals susceptible to ZIKV infection [122]. Subcutaneous infection with 500 or 50 $TCID_{50}$ of ZIKV resulted in 37 and 42 % mortality, respectively, over a course of 30 days of infection. ZIKV replication was detected in the brain, testes, spinal cords, kidney and spleen, although no histopathological lesions were seen. Infection of pregnant STAT2 knockout (KO) hamsters, but not immunocompetent hamsters, led to ZIKV infection in placental and foetus brains, but no adverse phenotype in pups. Taken together, these studies indicate that wild-type hamsters are not susceptible to infection, but STAT2 KO animals are valuable for some studies.

Swine

Swine are used as a model for biomedical research because they share similarities in several aspects of human anatomy, physiology, genetics and immune response. Regarding their immunity, pigs closely resemble humans in more than 80 % of immunological parameters, as opposed to less than 10 % in mice [123]. In addition, pigs are susceptible to several flaviviruses, including dengue virus (DENV), WNV and JEV [124]. Based on this, Darbellay *et al.* experimentally infected neonatal piglets with a 2015 Puerto Rican strain of ZIKV using three different inoculation routes: intracerebral, intradermal and intraperitoneal. They found that 2 out of 11 piglets inoculated intracerebrally, a non-natural route of infection, exhibited leg weakness, ataxia and tremor. ZIKV infection of piglets also resulted in seroconversion and low-titre viraemia, viruria and virus replication in internal organs, demonstrating that newborn pigs can be used as models to study some aspects of ZIKV biology (Table 4). An independent study using 3-month-old pigs did not detect viraemia or overt clinical signs, albeit the animals did seroconvert [121]. From these initial studies, it seems that pigs can serve as reservoirs for ZIKV, but not as amplifying hosts, given the low viraemia following the infection. However, additional studies using a larger number of animals are needed to clarify the role of pigs in the epidemiology of ZIKV.

Experimental *in utero* infection of conventional swine with ZIKV at 50 gestation days (i.e. mid-gestation) resulted in persistent infection in porcine conceptuses and impaired health in porcine offspring characterized by weak piglets, delayed feeding, lower neonatal body length and weight than controls, seizure-like activities, splayed back leg, reduced growth rate and aggressive behaviour [125]. A recent study inoculated pregnant sows with ZIKV *in utero* at 50 gestation days and euthanized them at 4 weeks after inoculation, which is comparable to the end of the second trimester of human pregnancy, and examined the foetuses [126]. The sows remained healthy during the experiment, but at necropsy 2 out of 15 inoculated foetal piglets were found to have microencephaly and all inoculated foetuses presented mild to severe neuropathology, characterized by neuronal depletion in the cerebral cortices of various lobes (Table 5). Although useful, their large size and high cost, and the lack of disease signs exhibited by the majority of infected animals, limit the utility of swine for widespread use in ZIKV research.

Non-human primates

Nonhuman primates (NHPs) are the most closely related animals to humans and are therefore the preferred human surrogates in ZIKV studies. NHPs are natural hosts for ZIKV and recapitulate several aspects of the disease in humans. In fact, the first described ZIKV-susceptible animal model was the rhesus monkey, which had historical importance for the discovery of ZIKV in 1947. Mice intracerebrally inoculated with viraemic rhesus monkey sera developed clinical disease, but mice and monkeys inoculated with the same virus intraperitoneally had no overt disease [127]. Since the emergence of ZIKV in the Americas, several groups have carried out experiments in NHPs to understand pathogenesis and transmissibility and develop countermeasures [128–143]. NHP models are essential for advancing ZIKV vaccine and drug candidates into clinics. Table 6 summarizes the studies that have used NHPs for ZIKV. Although NHP models are the most similar to human beings, the need for special animal facilities and their high cost and limited availability, together with the ethical issues associated with their use, limit the applications of this model for ZIKV studies.

Rhesus macaque (*Macaca mulatta*)

Rhesus macaques have been the most widely used NHP model for ZIKV. Most studies have used needle infection by the subcutaneous route to mimic mosquito bites. In these experiments, ZIKV infection in non-pregnant animals resulted in either subclinical infection or mild clinical signs of disease, such as rash, fever and conjunctivitis. Viraemia and virus excretion in several body fluids, such as urine, saliva, tears, semen, vaginal secretions and cerebrospinal fluid, have been reported. ZIKV has also been detected in several organs, where it resulted in gross and histopathological changes, with a marked tropism to the CNS [128–135]. ZIKV can persist in the CNS, lymph nodes and other immune privileged sites of rhesus monkeys for weeks after virus has been cleared from peripheral blood, urine and mucosal secretions [144]. ZIKV infection resulted in robust innate and humoral and cellular adaptive responses in these animals, which were protected from subsequent challenge with homologous or heterologous strains [131–133, 136, 137]. This monkey species has been useful in characterizing the innate and adaptive immune responses after ZIKV infection. In one study, Hirsch and coworkers infected pregnant rhesus macaques and found that ZIKV causes placental dysfunction and immunopathology. Within 5 days p.i., all infected dams showed innate immune cell activation, as demonstrated by the presence of CD169+ staining within monocytes/macrophages, myeloid DCs and NK subsets. Infected dams displayed marked activation of DCs and NK cells which peaked at 70 and 85 days p.i. Proliferation of CD8 T-cell and B-cell was also detected, with maximum responses seen in the first two weeks post infection. ZIKV-specific maternal antibodies were detected as early as 6 days p.i. and the antibody titres increased through 28 days p.i. Serum collected from dams and foetuses at 85 days p.i. showed neutralizing activity [136]. The evaluation of three vaccine platforms (inactivated vaccine, plasmid DNA vaccine or a rhesus adenovirus serotype 52 vector-based vaccine) led to the development of neutralizing antibodies and full protection of the rhesus macaques against challenge [129]. The infection of pregnant animals resulted in long-lasting viraemia, vertical transmission and microcephaly in foetuses [137]. The viraemia in pregnant animals was longer than that for non-pregnant animals [128, 143]. A recent study demonstrated that the infection of pregnant rhesus monkeys early in pregnancy recapitulates many lesions that are characteristic of congenital Zika syndrome (CZS), including foetal loss, smaller brain size, and microscopic brain pathology characterized by microcalcifications, necrosis, vasculitis, haemorrhage, gliosis and apoptosis of neuroprogenitor cells. The viraemia in animals infected early in pregnancy was longer (28–70 days) than that in animals infected later in pregnancy (14–42 days) [143].

A single study that inoculated ZIKV intravenously reported no clinical disease, but the animals developed short-lived viraemia, viruria and virus excretion in saliva. ZIKV was present in the lymph nodes and spleen and in cardiopulmonary, gastrointestinal, integument and genitourinary tissues, in the absence of major histopathological changes [135]. Direct inoculation of high-dose ZIKV directly to the tonsils resulted in viraemia, but saliva from infected monkeys inoculated in the palatine tonsils or conjunctiva or nasally did result in infection, suggesting that the transmission risk from saliva is low [139]. Animals inoculated through either the vaginal or the rectal route had overt clinical disease, but viraemia was detected in 50 and 100 % of the macaques inoculated by these routes, respectively [138].

Cynomolgus macaques (*Macaca fascicularis*)

The ban on exporting rhesus monkeys from India has reduced the availability of these animals and stimulated research using other NHP species. Cynomolgus macaques (*Maaaca fascicularis*) are closely related to rhesus monkeys, but they are smaller and therefore easier to handle and maintain in animal facilities.

Similar to rhesus monkeys, subcutaneous inoculation of these animals with the PRVABC59 strain resulted in viraemia and viral detection in the lymph nodes, CNS and male (seminal vesicles, testes, prostate) and female genital tracts (uterus, ovaries) [132]. Intravaginal and intrarectal inoculation resulted in viraemia in 50 and 100 % of inoculated animals, respectively, and no clinical disease [138]. Infections carried with the Asian and African ZIKV strains suggested that Cynomolgus monkeys appear to be susceptible only to infection with ZIKV isolates of the Asian lineage [134], although these results have not been reproduced using different African isolates of ZIKV.

Pig-tailed macaque (*Macaca nemestrina*)

The pigtail macaque (*Macaca nemestrina*) has been known to be susceptible to several viruses from the family *Flaviviridae*, including DENV, JEV and HCV. ZIKV that was inoculated subcutaneously in pregnant pigtail monkeys did not cause overt clinical disease, but the foetuses developed brain lesions characterized by cerebral white matter hypoplasia, periventricular white matter gliosis, and axonal and ependymal injury, all of which are associated with the presence of ZIKV RNA [130].

Marmoset (*Callithrix jacchus*)

The common marmoset (*Callithrix jacchus*) is a small-sized primate from the Americas that has been regarded as a relevant and convenient experimental model for investigating ZIKV pathogenesis. Intramuscular infection of these animals with the MR766 strain (Uganda/1947) resulted in no overt clinical disease, but ZIKV persisted in body fluids such as semen and saliva for longer periods of time than in serum. Infection with this non-contemporary African strain elicited strong neutralizing antibodies and antiviral responses, and complete protection against a heterologous challenge with a recent Asian lineage strain isolated in Brazil [140].

A second study by the same research group in pregnant marmosets reported no clinical disease in these animals upon infection with the SPH2015 strain (Brazil/2015). However, the animals developed viraemia, viruria and had

Table 6. Non-human primate models for ZIKV

Animal species	ZIKV strain (country/year)	Route of infection/dose	Major findings	Reference
Rhesus macaque (*Macaca mulatta*)	H/PF/2013 (French Polynesia/2013)	Subcutaneous /10^4 to 10^6 p.f.u.	Viraemia in non-pregnant and pregnant animals for 21 days and for up to at least 57 days, respectively. Virus was present in saliva, urine and cerebrospinal fluid. Homologous challenge at 10 weeks after the initial infection resulted in protection from disease and virus replication	[128]
	Brazil/ZKV/2015 (Brazil/2015)	Subcutaneous/ 10^5 p.f.u.	Animals were immunized with either an inactivated vaccine, a plasmid DNA vaccine or a rhesus adenovirus serotype 52 vector-based vaccine. Vaccinated animals developed neutralizing antibodies and were fully protected from challenge	[129]
	GZ01/2016 (China/2016)	Subcutaneous /10^5 p.f.u.	Fever, viraemia and ZIKV RNA excretion in urine, saliva and lacrimal fluid. Necropsy of two infected animals revealed systemic infections involving the CNS and visceral organs. Robust humoral and cellular response was detected in infected animals	[131]
	PLCal_ZV (Canada/2014) and PRVABC59(Puerto Rico/2016)	Subcutaneous/ 10^6 p.f.u.	High-level viraemia and viral detection in saliva, urine, cerebrospinal fluid (CSF) and semen (but only transiently in vaginal secretions); increase in the levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and creatinine phosphatase. Animals were protected from heterologous ZIKV infection	[132]
	MR766 (Uganda/1947) and H/PF/2013 (French Polynesia/2013)	Subcutaneous/ 10^4 to 10^6 p.f.u.	Immunity elicited by African lineage ZIKV protects against subsequent infection with Asian lineage ZIKV; robust cellular and humoral immunity to ZIKV	[133]
	PRVABC59 (Puerto Rico/2016)	Subcutaneous/ 10^4 to 10^6	Transient plasma viraemia and viruria from 1 to 7 days p.i.; rash, fever and conjunctivitis; strong adaptive immune response to ZIKV, although systemic cytokine response was minimal; virus detection in peripheral nervous tissue, multiple lymphoid tissues, joints, and the uterus of the necropsied animals at 7 days p.i.	[136]
	H/PF/2013 (French Polynesia/2013)	Subcutaneous or oral/10^6 p.f.u.	Application of high-dose ZIKV directly to the tonsils resulted in viraemia. Animals infected either to the palatine tonsils, conjunctivae or nasal passages with saliva from monkeys infected subcutaneously did not become infected	[139]
	Brazil/ZKV/2015 (Brazil/2015)	Intravenous/ 10^5 p.f.u.	Animals had no clinical disease; short-lived viraemia that cleared as neutralizing antibody developed; ZIKV RNA detected in urine and saliva; virus present in lymph nodes, spleen, cardiopulmonary, gastrointestinal, integument and genitourinary tissues	[135]
	H/PF/2013 (French Polynesia/2013)	Subcutaneous or oral/10^6 p.f.u.	Pregnant macaques had viraemia from 11 to 70 days p.i.; head circumference (HC) in all foetuses was between one and three SD below the mean and ZIKV RNA was detected in tissues from all foetuses, suggesting consistent vertical transmission	[137]
	PRVABC59(Puerto Rico/2016) or Brazil/ZKV/2015 (Brazil/2015)	Subcutaneous/ 10^5 to 10^6 f.f.u.	Persistence in central nervous system, lymph nodes and colorectal tissues for weeks after virus has been cleared from peripheral blood, urine and mucosal secretions	[144]
	ArD 41525 (Senegal/1984)	Intravaginal or intrarectal/10^7 p.f.u.	No overt clinical signs; viraemia was detected in 50 and 100 % of macaques inoculated intravaginally or intrarectally, respectively	[138]
	Brazil/ZKV/2015 (Brazil/2015)	Subcutaneous/ 10^5 p.f.u.	Infection of pregnant monkeys recapitulates many lesions that are characteristic of CZS, including consistent neuropathology in the foetal brain and spinal cord. Pregnant animals infected early in pregnancy exhibited prolonged viraemia (28–70 days) compared with dams infected later in pregnancy (14–42 days)	[143]
Cynomolgus macaques (*Macaca fascicularis*)	PRVABC59 (Puerto Rico/2016)	Subcutaneous/ 10^5 $TCID_{50}$	Viraemia, viral detection in lymph nodes, CNS and male (seminal vesicles, testes, prostate) and female genital tracts (uterus, ovaries)	[132]
	ArD 41525 (Senegal/1984)	Intravaginal or intrarectal/10^7 p.f.u.	No overt clinical signs; viraemia was detected in 50 and 100 % of macaques inoculated intravaginally or intrarectally, respectively	[138]
	PRVABC59(Puerto Rico/2016) or FSS13025 (Cambodia/2010) or IBH30656 (Nigeria/1968)	Subcutaneous/ 10^4 to 10^6 p.f.u.	RVABC59 strain induced viraemia detectable up to day 10 and moderate viral in testes, urine and saliva. FSS13025 strain induced shorter and lower viraemia compared to PRVABC59 and had detectable viral loads in testes but not in urine and saliva. IBH30656 failed to establish infection	[134]

Table 6. cont.

Animal species	ZIKV strain (country/year)	Route of infection/dose	Major findings	Reference
Pig-tailed macaque (*Macaca nemestrina*)	PSS13025 (Cambodia/2010)	Subcutaneous/ 10^7 p.f.u.	The pregnant inoculated animal did not develop clinical disease; foetal necropsy revealed ZIKV in the brain, and marked cerebral white matter hypoplasia, periventricular white matter gliosis, and axonal and ependymal injury	[130]
Marmoset (*Callithrix jacchus*)	MR766 (Uganda/1947)	Intramuscular/ 10^5 p.f.u.	No overt clinical signs; virus persistence in body fluids such as semen and saliva for longer periods of time than in serum; strong neutralizing antibodies and antiviral responses; MR766-infected animals rechallenged with the SPH2015 Brazilian strain were fully protected.	[140]
	SPH2015 (Brazil/2015)	Intramuscular/ 10^5 p.f.u.	Pregnant animals had no overt clinical signs; seroconversion; induction of type I/II interferon-associated genes and proinflammatory cytokines; persistent viraemia and viruria; spontaneous pregnancy loss was observed 16–18 days p.i., with extensive active placental viral replication and foetal neurocellular disorganization similar to that seen in humans	[141]

spontaneous pregnancy loss at 16–18 days p.i., with extensive active ZIKV replication in the placenta and foetal neurocellular disorganization indicating a disruption in the development and neuronal migration patterns of the cerebral cortex, as seen in humans [141]

Other vertebrate animal models

Early studies with the prototype MR766 strain in rabbits and cotton rats has shown that these animals do not develop clinical signs of infection after intracerebral inoculation of late-passage mouse brain virus, but do seroconvert [116]. In addition to serological evidence for natural infection, some African bat species supported replication and developed clinical signs of disease upon experimental infection with ZIKV. However, studies with contemporary ZIKV strains in bat species from the New World are needed to better elucidate the role of bats in ZIKV ecology [145–147].

Recently, Ragan *et al.* infected several animal species from North America with ages ranging from neonates to adults. The species investigated included armadillos, cottontail rabbits, goats, mink, chickens, pigeons, groundhogs, deer mice, cattle, raccoons, ducks, Syrian golden hamsters, garter snakes, leopard frogs, house sparrows and pigs. Animals were infected with ZIKV by the subcutaneous and intradermal routes with a high virus dose (10^5 p.f.u.). Low-level viraemia was found only in frogs and armadillos. Neutralizing antibodies were detected in goats, rabbits, ducks, frogs and pigs, suggesting these animals are unlikely to act as animal reservoirs or good models to reproduce ZIKV disease [121].

Mosquito infection models

ZIKV is thought to be transmitted mainly by mosquito vectors and most of the available ZIKV strains have been recovered from mosquito species collected in nature. Several species of mosquitoes have been detected that are naturally infected by ZIKV – primarily *Aedes* species, which are vectors for YFV, DENV and other arboviruses.

Before the ZIKV epidemics reported in Yap Island (2007) and in French Polynesia (2013), very few studies registered experimental infections in mosquitoes. These studies were mainly performed to demonstrate the vectorial competence of mosquito species in order to identify or incriminate a mosquito species as potential vectors. *Ae. aegypti* has been used as the major experimental model, as it is a mosquito species that is easy to colonize and maintain in insectaries. It was the first mosquito species to be used in an artificial feeding system for studying ZIKV infections (148Boorman and Porterfield, 1956). *Ae. aegypti* was employed to feed directly on a Zika patient through the exposure of the left forearm to a batch of mosquito females in order to demonstrate ZIKV transmission by mosquitoes [149]. Most of the studies use an artificial membrane feeder to infect mosquitoes and study the spread of ZIKV in the mosquito organs. Usually, the midgut and salivary glands or mosquito saliva are collected to detect and quantify the presence of ZIKV particles. In addition, ZIKV can be inoculated via intrathoracic injection [150]. *Ae. aegypti* artificially infected with ZIKV through blood meal successfully transmitted ZIKV to BALB/c mouse upon feeding on the ears of these animals, an approach that allows evaluation of the development of infection in and transmission from mosquitoes using a transmission process that resembles the natural process [151]. *Ae. aegypti* that were allowed to feed on infected AG129 mice acquired ZIKV in their midguts, but transmission from these mosquitoes depended largely on the colony of *Ae. aegypti* used, suggesting that genetic differences play an important role in vector competence for ZIKV [150].

A recent debate about the possible role that another mosquito species, *Culex quinquefasciatus*, could play in ZIKV transmission [152] inspired several groups to investigate the vector competence of mosquito species from the genera *Aedes* and *Culex*. Several studies using different combinations of ZIKV strains and mosquito colonies or populations failed to demonstrate ZIKV transmission by *Culex* species [153–155]. However, Guo *et al.* successfully demonstrated

the transmission of ZIKV by *Cx. quinquefasciatus* to mice [156]. In addition, Guedes *et al.* photographed ZIKV mature particles in the salivary glands of this mosquito species after 7 days p.i. [157]. Further field studies reported *Cx. quinquefasciatus* naturally infected with ZIKV [158], confirming that it is possible that other species besides *Ae. aegypti* are transmitting ZIKV in urban environments. Further investigations are required to determine the primary, secondary or occasional vectors of ZIKV during outbreaks. Care should be taken in interpreting transmission studies using laboratory-reared mosquitoes. Furthermore, although the virus maybe found in certain mosquito species, this does not mean the the virus will be transmitted by that species under natural conditions.

Thus, mosquito models fed using the available systems of artificial infection could be better exploited, for instance in studies to understand the pathogen–vector–host interactions, in order to identify the immune response trigged by distinct mosquito species against the viral strains. This response, together with receptor molecules, is the basis for a mosquito that is refractory to viral infection and could be used in future studies to develop new strategies for ZIKV control.

MODELS TO STUDY ANTIBODY-DEPENDENT ENHANCEMENT

Antibody-dependent enhancement (ADE) is a phenomenon by which non-neutralizing antibodies enhance viral entry and replication in host cells, resulting in increased viral load and exacerbation of clinical disease. ADE is a well-studied mechanism in DENV pathogenesis and is thought to occur through the formation of infectious virus–antibody immune complexes (ICs) that bind to Fc gamma receptors (FcγR) expressed on permissive cells such as DCs and monocytes/macrophages [159]. The enhancement of ZIKV infection by DENV antiserum was first described in 1987 [160], and has been evaluated by many groups recently, given the possible impact of ADE on ZIKV pathogenesis, vaccine development and immunotherapy [17, 161–165].

Typical *in vitro* assays to study ADE consist of incubating ZIKV with serially diluted test serum (or antibodies) and then infecting a cell line that expresses the FcγR. ADE is then defined as a significant increase in virus titres obtained after incubation with test serum relative to controls. Several cell lines have been used to study ZIKV ADE activity, including the murine macrophage-like P388D1 cell line [160], FcγR-expressing BHK cell lines [166], the human erythroleukemic K-562 cell line [162, 165, 167], the human macrophage cell line U937 [17], the human monocytic cell line THP-1 [161] and primary human macrophages [168].

ADE of ZIKV infection has been evaluated *in vivo* using immunodeficient mice and monkeys. Bardina *et al.* were able to reproduce ADE *in vivo* by using Stat2$^{-/-}$ knockout C57BL/6 mice and convalescent plasma from DENV- and WNV-infected individuals and then infecting these animals 2 hours later with the ZIKV strain PRVABC59. They found that pretreatment with anti-DENV or anti-WNV plasma resulted in more elevated body temperature, higher viraemia and viral burden in the spinal cord and testes (but not in the brains, ovaries and eyes), increased weight loss, higher mortality and enhanced clinical symptom scores relative to controls. Their study also showed that *in vivo* ADE occurs optimally at low concentrations of ZIKV-reactive IgG, while high levels may be protective [167]. In fact, treatment of type I interferon receptor-deficient (IFNAR$^{-/-}$) mice with a cross-reactive dengue human monoclonal antibody (mAb) protected adult non-pregnant mice from ZIKV-induced weight loss and mortality. Treatment of pregnant mice infected with ZIKV with mAb also significantly reduced the viral titres in the placenta and foetal organs and abolished virus-induced foetal growth retardation [165].

Recently, a study using two groups of rhesus monkeys (one naïve to flavivirus infection and the other previously exposed to DENV almost 3 years earlier) demonstrated that while ADE could be confirmed *in vitro*, preexisting DENV immunity did not result in exacerbated ZIKV disease [163]. These findings were supported by a human cohort study of ZIKV-infected patients previously exposed to dengue [164]. However, more studies using a larger number of animals and including patients from different epidemiological settings are needed to better establish the role of ADE on ZIKV-induced disease.

CONCLUSION AND PERSPECTIVES

Tremendous advances have been made in the development of *in vitro* and *in vivo* models that recapitulate many aspects of ZIKV biology and disease in humans. Infection by contemporary ZIKV strains has been characterized in a variety of cell culture systems and animal models, and this has already contributed to advancements in the fields of viral pathogenesis, epidemiology, vaccinology and antiviral discovery. However, each of these models has limitations that must be considered in the design and interpretation of experiments, and in the extrapolation of experimental results to humans. Sharing of these research models among different laboratories will ensure data reproducibility and accelerate the discovery and development of new products and processes. The continuous development and characterization of research models is essential for a better understanding of ZIKV biology and the translation of research findings from the bench to the clinic.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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APÊNDICE B - ARTIGO PUBLICADO

Development and Validation of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) for Rapid Detection of ZIKV in Mosquito Samples from Brazil

Artigo anexo na página seguinte.

A doutoranda e co-autora desse artigo publicado na *Scientific Reports*, foi responsável pela produção dos sequenciamentos e análises dos resultados do eletroferogramas do RT-LAMP. Resumidamente, os amplicons da reação RT-LAMP foram purificados usando o Kit de Purificação illustra GFX PCR DNA (GE) de acordo com as instruções do fabricante. Amplicons purificados foram sequenciados usando o BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, EUA), conforme estabelecido pelo fabricante e executado em um sistema capilar ABI Prism 3100 Automatic. As sequências dos fragmentos foram analisadas no software Bioedit v7.0.5, e submetidas banco de dados NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) para identificar a linhagem ZIKV mais próxima.

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Development and Validation of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) for Rapid Detection of ZIKV in Mosquito Samples from Brazil

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The rapid spread of Zika virus (ZIKV) represents a global public health problem, especially in areas that harbor several mosquito species responsible for virus transmission, such as Brazil. In these areas, improvement in mosquito control needs to be a top priority, but mosquito viral surveillance occurs inefficiently in ZIKV-endemic countries. Quantitative reverse transcription PCR (qRT-PCR) is the gold standard for molecular diagnostic of ZIKV in both human and mosquito samples. However, the technique presents high cost and limitations for Point-of-care (POC) diagnostics, which hampers its application for a large number of samples in entomological surveillance programs. Here, we developed and validated a one-step reverse transcription LAMP (RT-LAMP) platform for detection of ZIKV in mosquito samples. The RT-LAMP assay was highly specific for ZIKV and up to 10,000 times more sensitive than qRT-PCR. Assay validation was performed using 60 samples from *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes collected in Pernambuco State, Brazil, which is at the epicenter of the Zika epidemic. The RT-LAMP had a sensitivity of 100%, specificity of 91.18%, and overall accuracy of 95.24%. Thus, our POC diagnostics is a powerful and inexpensive tool to monitor ZIKV in mosquito populations and will allow developing countries to establish better control strategies for this devastating pathogen.

Zika virus (ZIKV) is a mosquito-borne flavivirus, first identified in a rhesus monkey from Uganda in 1947 and isolated from *Aedes africanus* mosquitoes in 1948[1]. For nearly 60 years few ZIKV cases in human have been reported. However, in 2007 a large ZIKV outbreak occurred in the Yap Island, Federated States of Micronesia. In 2013, the virus was detected in French Polynesia and rapidly spread throughout the Pacific[2,3]. In these outbreaks, most ZIKV infections have been asymptomatic and, when present, symptoms include rash, fever, headache, and arthralgia[4]. However, the unprecedented epidemics of developmental defects first reported in 2015 in newborns from Brazil and neurological complications associated with the infection such as Guillain-Barré syndrome (GBS) mobilized public health officials and scientists around the world to fill knowledge gaps of this until then overlooked pathogen[5–7].

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ZIKV is an arbovirus member of the genus *Flavivirus* in the family *Flaviviridae*. The ZIKV genome consists of a single positive-sense single-stranded RNA (+ssRNA), with approximately 11 Kb in length. Other important viruses within this genus include yellow fever virus (YFV), dengue 1–4 virus (DENV 1–4), Japanese encephalitis virus (JEV) and West Nile virus (WNV)[8].

Mosquitoes from the genus *Aedes* are widespread in tropical and subtropical regions of the world and have been postulated as the main vector for ZIKV[9]. However, different studies have suggested that the southern house mosquito *Culex quinquefasciatus* mosquitoes could act as another important ZIKV vector[10–13]. Moreover, many ZIKV strains have been isolated from *Anopheles*, *Mansonia*, *Culex* and *Aedes* mosquitoes[14]. Non-vector-borne transmission of ZIKV can occur through blood transfusion, transplacentally, perinatally and sexually[15]. Given the lack of vaccines and antivirals against ZIKV, vector control remains the most effective manner to limit virus spread and the size of outbreaks[16].

ZIKV surveillance in insect vectors is an important tool for identifying viral circulation and potential entry points, therefore contributing to prevent outbreaks of disease[17]. This virus has spread rapidly particularly in developing countries that lacks good sanitation infrastructure and harbors several mosquito species competent for ZIKV transmission. In these areas, improvement in mosquito control needs to be a top priority, but occurs inefficiently in ZIKV-endemic countries, such as Brazil[17–19]. Surveillance of ZIKV in mosquitoes sheds lights into virus dynamics and allows early detection of new introductions before the virus become widespread in vector and host populations. In addition, surveillance data allows the evaluation of trends and the impact of vector control programs[20].

Currently, quantitative reverse transcription PCR (qRT-PCR) is the gold standard for molecular diagnostic of ZIKV in both humans and mosquito samples[21,22]. However, qRT-PCR is expensive, requires highly specialized manpower, and involves costly and sophisticated equipment for amplification and detection of the viral genome. These drawbacks make the technique unsuitable for large-scale applications in low-resource settings areas, which negatively impact the establishment of effective disease control programs[23,24].

Point-of-care (POC) molecular diagnostic platforms may address these concerns and increase the diagnostic capacity of ZIKV-affected countries. RT-LAMP is a promising tool that allows rapid, simple and practical diagnosis of a number of pathogens[25–27]. Considering the advantages of rapid amplification, simple operation, low cost, high sensitivity and specificity, RT-LAMP has potential applications for clinical diagnosis as well as for surveillance of infectious diseases in developing countries[28]. Differently from the qRT-PCR assay, detection of RT-LAMP amplification products can achieved by naked eye analysis through color change of the reaction tube[29]. For this purpose, different LAMP assays have been developed for detecting the ZIKV since its emergence in the Western hemisphere[30–38]. However, most ZIKV LAMP assays developed to date evaluated only handful mosquito samples, which raise concerns about their fitness for ZIKV detection in the field. Moreover, many of the developed ZIKV LAMP assays still require special equipments for virus detection, which limits its applicability in low-resource scenarios.

In the present study, we developed and validated a one-step reverse transcription LAMP (RT-LAMP) platform for detection of ZIKV in both laboratory and wild-caught mosquitoes. The RT-LAMP assay described here enables the diagnosis of ZIKV in mosquito samples as fast as 20 minutes even in the absence of RNA isolation from the samples. In addition, it does not require highly trained workforce and does not involve expensive and sophisticated equipment for amplification and virus detection. Our point-of-care test is a powerful and inexpensive tool to monitor ZIKV in mosquito populations and will allow developing countries to establish better and timely decisions regarding ZIKV control strategies.

Results

Detection of ZIKV in *Aedes aegypti* under controlled conditions. First, we determined the ability of RT-LAMP to detect ZIKV in *A. aegypti* under controlled conditions. To this end, crude lysate of uninfected mosquitoes were spiked to result in either a high (1×10^6 PFU/mL) or low viral load (1×10^3 PFU/mL) in order to mimic physiological concentrations of ZIKV in these vectors. Spiked samples were processed for RT-LAMP without RNA isolation. RT-LAMP assay for ZIKV were positive in both viral loads tested. As expected, non template control (NTC) samples (water) and negative control (crude lysate of uninfected *A. aegypti*) tested negative (Fig. 1A–C). RNA extraction did not improve RT-LAMP detection (data not shown). RT-LAMP results were confirmed by qRT-PCR, through which the Ct value was 12.1 and 26.8, for high viral and low viral load, respectively (Figs 1 and S4). The same results were obtained with viral spike in *C. quinquefasciatus* homogenates (data not shown).

In order to mimic a real world scenario of ZIKV surveillance in mosquitoes, we determined the capacity of the RT-LAMP to detect ZIKV in *A. aegypti* mosquitoes experimentally infected by oral feeding on rabbit blood spiked with ZIKV. In this study, mosquitoes fed on unspiked rabbit blood were also included as controls. Crude mosquito lysates were used for RT-LAMP assay without RNA isolation. After incubation, the RT-LAMP was able to detect ZIKV only in infected mosquitoes, but not controls (Fig. 1D–F), suggesting the test may be useful for ZIKV detection in entomological samples. RNA extraction did not improve RT-LAMP detection (data not shown).

Analytical specificity of RT-LAMP for detection of ZIKV. To evaluate the specificity of the RT-LAMP assay to detect only ZIKV, we tested crude lysate of *A. aegypti* spiked with several arboviruses circulating in Brazil: DENV-1 (PE/97-42735), DENV-2 (PE/95-3808), DENV-3 (PE/02-95016), DENV-4 (PE/10-0081), YFV (17DD), and CHIKV (PE2016-480) (Table 1). Only the *A. aegypti* lysate spiked with ZIKV was positive in RT-LAMP reaction, as determined by naked eye analysis, visual observation under UV light or agarose gel electrophoresis (Fig. 2). Thus, these results suggested that RT-LAMP assay described here is highly specificity for detection of ZIKV.

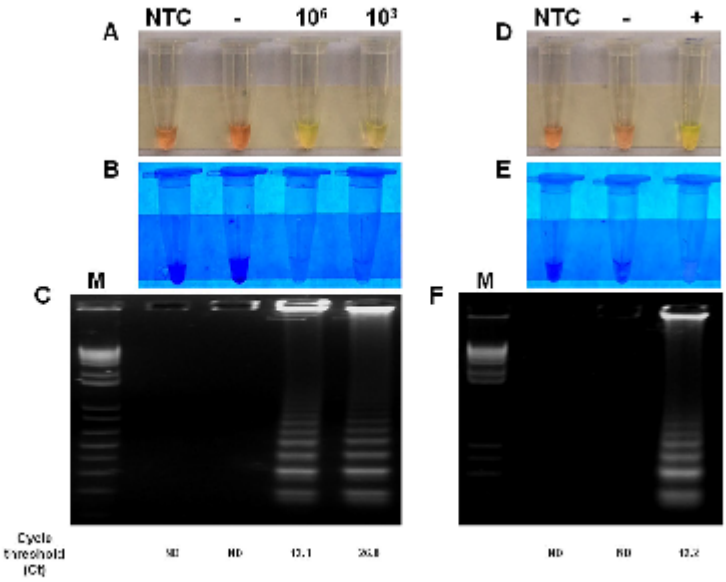

Figure 1. Detection of ZIKV in virus-spiked mosquito samples and crude lysate of experimentally infected *Aedes aegypti*. Crude lysates of uninfected *A. aegypti* were spiked with ZIKV to result in either a high (1×10^6 PFU/mL) or low viral load (1×10^3 PFU/mL) and processed for RT-LAMP without RNA isolation (**A–C**). (**D–F**) Represents RT-LAMP results of experimentally infected mosquitoes. RT-LAMP amplicons were observed by visual color change of the products and gel electrophoresis. The amplification products were observed by naked eye under natural light (**A,D**), under UV irradiation (**B,E**) and agarose gel electrophoresis (**C,F**). Legends in (**A–C**) are: NTC (non-template control): water; (−): macerate of uninfected *Aedes aegypti*; (10^6): macerate of *Aedes aegypti* spiked with 10^6 PFU; (10^3): macerate of *Aedes aegypti* spiked with 10^3 PFU. Legends in (**D–F**) are: NTC (non-template control): water; (−): macerate of uninfected *Aedes aegypti*; (+):macerate of *Aedes aegypti* experimentally infected with ZIKV. M: molecular weight marker.

Family	Genus	Species	Strain	GenBank access code	Result of RT-LAMP
Flaviviridae	*Flavivirus*	Zika virus	PE-243	KX197192	+
		Dengue virus serotype 1	PE/97-42735	EU259529	–
		Dengue virus serotype 2	PE/95-3808	EU259569	–
		Dengue virus serotype 3	PE/02-95016	KC425219	–
		Dengue virus serotype 4	PE/10-0081	Unpublished	–
		Yellow fever virus	17DD	DQ100292	–
Togaviridae	*Alphavirus*	Chikungunya virus	PE2016-480	Unpublished	–

Table 1. Viruses used in this study.

Analytical sensitivity of RT-LAMP for detection of ZIKV. First, we sought to optimize the RT-LAMP assay conditions, reactions were performed at temperatures ranging from 59 °C to 75 °C following an incubation that ranged from 10 min to 60 min. The best amplification results were obtained at 72 °C for 40 min, but incubation time as short as 20 minutes was sufficient for detecting positive samples. Therefore, all assays were carried out using 40-min incubation time. The analytical sensitivity (limit of detection) of RT-LAMP was determined in crude lysate of *A. aegypti* spiked with a 10-fold serial dilution of ZIKV ranging from 10^5 PFU to 10^{-7} PFU without RNA isolation. RT-LAMP was able to detect a broad range of virus concentration (from 10^5 to 10^{-5} PFU), including viral loads found in naturally infected mosquitoes[39]. Considering 10 independent replicates per protocol developed, the probit regression analysis revealed that the limit of detection at 95% probability for each RT-LAMP was −2,98 $\log_{10}$ PFU of ZIKV (~1/1000 PFU) with confidence interval from −3,62 to −1,64 (Table 2 and Fig. S6). Additionally, viral RNA extracted from the same dilutions tested by RT-LAMP was assayed by the widely used ZIKV qRT-PCR method developed by Lanciotti[40]. For qRT-PCR assay, the lower detection limit was

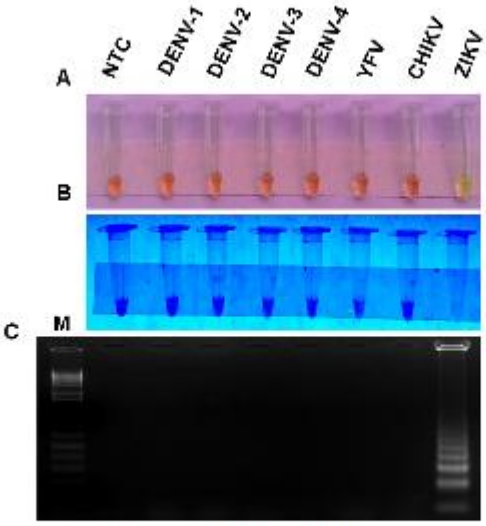

Figure 2. Analytical specificity of ZIKV RT-LAMP in mosquito samples. Crude lysates of uninfected *A. aegypti* were spiked with different arboviruses (DENV 1–4, YFV, CHIKV and ZIKV) circulating in Brazil so the final concentration would be 10^6 PFU per reaction. Spiked samples were then assayed by ZIKV RT-LAMP. The amplification products were observed by naked eye under natural light (**A**), under UV irradiation (**B**) and agarose gel electrophoresis (**C**). M: molecular weight marker. NTC (non-template control): water.

ZIKV Concentration (PFU)	No. of Replicates	No. of positive results	Hit rate in %
10^6	10	10	100
10^4	10	10	100
10^3	10	10	100
10^2	10	10	100
10^1	10	10	100
10^0	10	10	100
10^{-1}	10	10	100
10^{-2}	10	10	100
10^{-3}	10	9	90
10^{-4}	10	7	70
10^{-5}	10	6	60
10^{-6}	10	0	0
10^{-7}	10	0	0

Table 2. Detection limit of the ZIKV RT-LAMP assay[a]. [a]Probit regression analysis was calculated using MedCalc software (version 18.11), giving a C_{95} value (concentration detectable 95% of the time) of −2,98 $\log_{10}$ PFU of ZIKV. This indicates that the limit of detection is about −3 $\log_{10}$ (1/1000) PFU/reaction and that samples containing that concentration would be detected 95% of the time.

10^1 PFU ZIKV with Ct value 37.2 (Fig. 3). Taken together, the limit of detection was thus slightly than the gold standard technique for the diagnosis of ZIKV.

Diagnostic performance of ZIKV RT-LAMP for mosquito samples. A total of 60 mosquito samples from *A. aegypti* (n = 32) and *C. quinquefasciatus* (n = 28) were obtained from the Entomology Department[39,41] and tested for ZIKV by RT-LAMP assay. Samples with Ct values of ≤38.0 in duplicate wells were considered positive for ZIKV infection[42]. Of these, 31 samples were ZIKV negative as determined by qRT-PCR and 29 were positive, including naturally and experimentally infected mosquitoes (Table 3). The Ct value in these samples ranged from 27.0 to >40.0. From the total of 60 samples, the RT-LAMP assay was able to detect ZIKV in 32 samples,

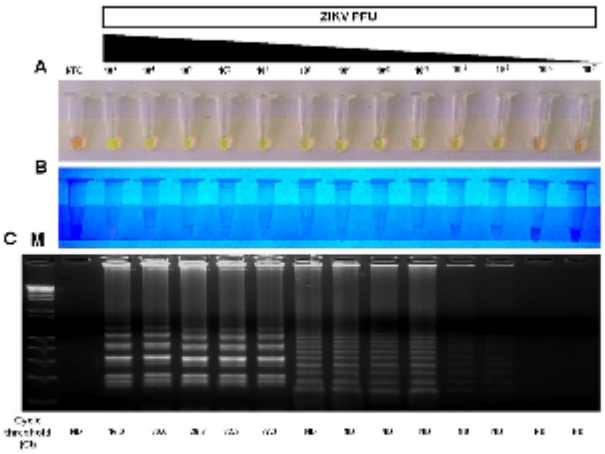

Figure 3. Analytical sensitivity of ZIKV RT-LAMP in mosquito samples. The sensitivity of RT-LAMP was determined by spiking different amounts (10^5 PFU to 10^{-7} PFU) of ZIKV in crude lysates of uninfected *Aedes aegypti* mosquitoes and then testing by RT-LAMP directly without RNA extraction. The amplification products were observed by naked eye under natural light (**A**), under UV irradiation (**B**) and agarose gel electrophoresis (**C**). M: molecular weight marker. NTC (non-template control): water. ND (Not detected). To compare the results of RT-LAMP with a gold standard technique, viral RNA was extracted from the same dilutions using Trizol reagent and assayed qRT-PCR.

including the 29 samples already determined to be positive by qRT-PCR (Fig. 4). Moreover, samples that were at the detection threshold by qRT-PCR (Ct values ranging from 37.5 to 40.3) were tested positive by the RT-LAMP assay result (Fig. 5), highlighting the sensitivity of the test in mosquito samples.

The diagnostic performance of ZIKV RT-LAMP for mosquito samples was determined by statistical analysis using qRT-PCR as the gold standard technique. The overall ZIKV prevalence in the samples was 46.03% (95% CI 33.39% to 59.06%). The RT-LAMP assay had a diagnostic sensitivity of 100% (95% CI 88.06% to 100.00%) and diagnostic specificity of 91.18% (95% CI 76.32% to 98.14%). The positive predictive value, which is probability that the virus is present when the test is positive, was 90.62% (95% CI 76.64% to 96.61%), whereas the negative predictive value, which indicates the probability that the virus is absent when the test is negative, was 100%. The overall accuracy of the RT-LAMP test was determined to 95.24% (95% CI 86.71% to 99.01%) (Table 4), highlighting the practical value of RT-LAMP for ZIKV detection in entomological samples.

To confirm the identity of ZIKV RT-LAMP positive samples, we sequenced positive samples from field-caught *Aedes spp.* and *Culex spp.* mosquitoes by the Sanger method. Sequencing results and BLAST analysis demonstrated that ZIKV RT-LAMP amplicons match 100% with virus circulating in Brazil (Fig. 6), confirming the specificity of the RT-LAMP for ZIKV.

Together, these results indicated that our ZIKV RT-LAMP assay represents a robust and affordable diagnostic platform that can be used as a surveillance tool for mosquitoes infected with ZIKV.

Discussion

The rapid detection of ZIKV in mosquito samples can help to understand the dynamics of the disease in areas that have favorable conditions for virus transmission[20]. In this context, we developed a rapid molecular test for the detection of ZIKV in mosquito samples that may be a valuable tool for vector surveillance. The RT-LAMP assay described here is straightforward, inexpensive, and enables ZIKV detection even in the absence of RNA extraction. To our knowledge, this is the first validation of a ZIKV RT-LAMP assay using experimentally and naturally infected *A. aegypti* and *C. quinquefasciatus* mosquitoes collected at the epicenter of the Zika epidemic in Brazil.

Currently, the gold standard technique for detection of ZIKV in mosquito samples is qRT-PCR. This assay is specific for detecting the virus in both human and mosquito samples[21,40]. However, its prohibitive cost makes qRT-PCR unfit for testing a large number of mosquitoes collected in entomological surveillance programs[41]. Another potential limitation of qRT-PCR is the inability to detect low viral titers, which may occur especially during interepidemic periods. The limit of detection for the assay described by Faye was 0.05 plaque forming unit (PFU) or 32 genome-equivalents and the one developed by Lanciotti was 25 RNA copies[21,40]. Recently, other research groups have developed methodologies using the LAMP approach for the detection of ZIKV using mosquito samples[34–36]. However, these studies used only a handful of mosquito samples and the lowest virus concentration detected was 10^3 PFU. Our RT-LAMP was evaluated using 60 and revealed to be about 10,000 fold more sensitive than the qRT-PCR, detecting virus concentrations as low as 10^{-5} PFU. The large amount of infectious and non-infectious ZIKV RNA released into the culture supernatant explains the ability of RT-LAMP to detect

Sample (ID)	Ct value	ZIKV PFU/mL equivalent	Mosquito sample	Source	Result of RT-LAMP
1	27.0	6.20×10^{5}	*Culex quinquefasciatus*	Field sample	+
2	29.0	3.97×10^{5}	*Culex quinquefasciatus*	Field sample	+
3	29.0	3.97×10^{5}	*Aedes aegypti*	Laboratory sample	+
4	30.0	1.50×10^{5}	*Culex quinquefasciatus*	Field sample	+
5	30.0	1.50×10^{5}	*Culex quinquefasciatus*	Field sample	+
6	30.0	1.50×10^{5}	*Aedes aegypti*	Laboratory sample	+
7	30.5	5.31×10^{4}	*Aedes aegypti*	Field sample	+
8	30.6	1.45×10^{5}	*Aedes aegypti*	Laboratory sample	+
9	30.6	1.45×10^{5}	*Aedes aegypti*	Laboratory sample	+
10	31.0	8.23×10^{4}	*Aedes aegypti*	Field sample	+
11	31.0	8.23×10^{4}	*Aedes aegypti*	Field sample	+
12	31.0	8.23×10^{4}	*Culex quinquefasciatus*	Field sample	+
13	31.0	8.23×10^{4}	*Aedes aegypti*	Laboratory sample	+
14	32.0	3.91×10^{4}	*Aedes aegypti*	Field sample	+
15	32.0	3.91×10^{4}	*Aedes aegypti*	Field sample	+
16	32.0	3.91×10^{4}	*Culex quinquefasciatus*	Field sample	+
17	32.0	3.91×10^{4}	*Culex quinquefasciatus*	Field sample	+
18	33.0	2.27×10^{4}	*Culex quinquefasciatus*	Field sample	+
19	34.0	9.97×10^{3}	*Aedes aegypti*	Field sample	+
20	34.0	9.97×10^{3}	*Aedes aegypti*	Field sample	+
21	34.5	5.17×10^{3}	*Aedes aegypti*	Field sample	+
22	35.3	3.23×10^{3}	*Aedes aegypti*	Field sample	+
23	35.5	3.00×10^{3}	*Aedes aegypti*	Field sample	+
24	35.5	3.00×10^{3}	*Aedes aegypti*	Field sample	+
25	36.5	1.41×10^{3}	*Aedes aegypti*	Field sample	+
26	36.5	1.41×10^{3}	*Aedes aegypti*	Field sample	+
27	37.5	6.00×10^{2}	*Culex quinquefasciatus*	Field sample	+
28	38.0	5.60×10^{2}	*Aedes aegypti*	Field sample	+
29	38.0	5.60×10^{2}	*Aedes aegypti*	Field sample	+
30	38.6	5.00×10^{2}	*Aedes aegypti*	Field sample	+
31	39.0	4.15×10^{2}	*Culex quinquefasciatus*	Field sample	+
32	40.3	0	*Culex quinquefasciatus*	Field sample	+
33	>40.0	0	*Aedes aegypti*	Field sample	–
34	>40.0	0	*Aedes aegypti*	Field sample	–
35	>40.0	0	*Aedes aegypti*	Field sample	–
36	>40.0	0	*Aedes aegypti*	Field sample	–
37	>40.0	0	*Aedes aegypti*	Field sample	–
38	>40.0	0	*Culex quinquefasciatus*	Field sample	–
39	>40.0	0	*Culex quinquefasciatus*	Field sample	–
40	>40.0	0	*Culex quinquefasciatus*	Field sample	–
41	>40.0	0	*Culex quinquefasciatus*	Field sample	–
42	>40.0	0	*Culex quinquefasciatus*	Field sample	–
43	>40.0	0	*Culex quinquefasciatus*	Field sample	–
44	>40.0	0	*Culex quinquefasciatus*	Field sample	–
45	>40.0	0	*Culex quinquefasciatus*	Field sample	–
46	>40.0	0	*Culex quinquefasciatus*	Field sample	–
47	>40.0	0	*Culex quinquefasciatus*	Field sample	–
48	>40.0	0	*Culex quinquefasciatus*	Field sample	–
49	>40.0	0	*Culex quinquefasciatus*	Field sample	–
50	>40.0	0	*Culex quinquefasciatus*	Field sample	–
51	>40.0	0	*Culex quinquefasciatus*	Field sample	–
52	>40.0	0	*Culex quinquefasciatus*	Field sample	–
53	>40.0	0	*Culex quinquefasciatus*	Field sample	–
54	>40.0	0	*Culex quinquefasciatus*	Field sample	–
55	>40.0	0	*Culex quinquefasciatus*	Field sample	–
56	>40.0	0	*Aedes aegypti*	Laboratory sample	–

Continued

Sample (ID)	Ct value	ZIKV PFU/mL equivalent	Mosquito sample	Source	Result of RT-LAMP
57	>40.0	0	*Aedes aegypti*	Laboratory sample	–
58	>40.0	0	*Aedes aegypti*	Laboratory sample	–
59	>40.0	0	*Aedes aegypti*	Laboratory sample	–
60	>40.0	0	*Aedes aegypti*	Laboratory sample	–

Table 3. Mosquito samples used for RT-LAMP validation.

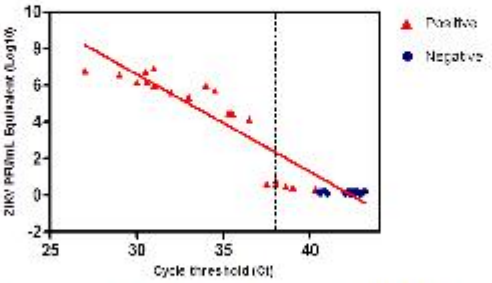

Figure 4. Diagnostic of mosquito samples by RT-LAMP. A total of 60 mosquito samples from *Aedes aegypti* (n = 32) and *Culex quinquefasciatus* (n = 28) were tested for ZIKV by RT-LAMP assay. Of these, 29 were positive for ZIKV and 31 were negative as determined by qRT-PCR. Dashed line represents the qRT-PCR cycle threshold (Ct value) value for ZIKV positivity (Ct ≤ 38). Red triangle indicates samples positive by RT-LAMP and blue circle are samples negative by RT-LAMP.

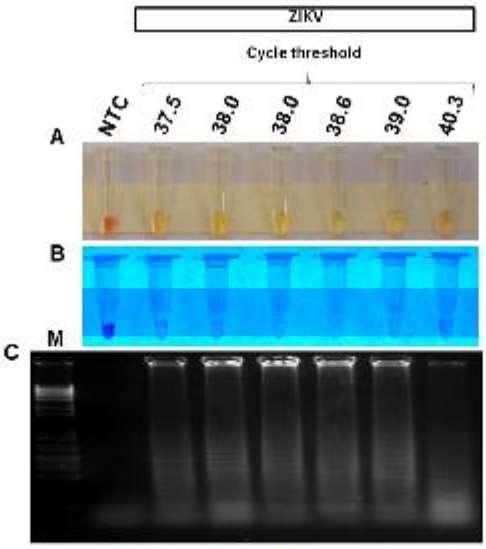

Figure 5. ZIKV detection by RT-LAMP in mosquito samples at the qRT-PCR detection limit. Mosquito samples at the detection threshold by qRT-PCR (Ct values ranging from 37.5 to 40.3) were assayed by RT-LAMP and all reactions showed a positive result. The amplification products were observed by naked eye under natural light (**A**), under UV irradiation (**B**) and agarose gel electrophoresis (**C**). M: molecular weight marker. NTC (non-template control): water.

	qRT-PCR +	qRT-PCR –	Total
RT-LAMP +	29	3	32
RT-LAMP –	0	31	31
Total	29	34	
Sensitivity	**100%** (95% CI 88.06% to 100.00%)		
Specificity	**91.18%** (95% CI 76.32% to 98.14%)		
ZIKV prevalence	**46.03%** (95% CI 33.39% to 59.06%)		
Positive Predictive Value	**90.62%** (95% CI 76.64% to 96.61%)		
Negative Predictive Value	**100%**		
Accuracy	**95.24%** (95% CI 86.71% to 99.01%)		

Table 4. Diagnostic performance of ZIKV RT-LAMP for mosquito samples.

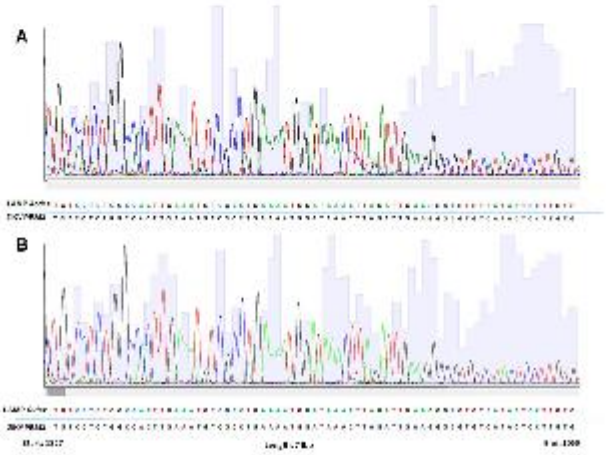

Figure 6. Electropherogram of ZIKV RT-LAMP detected in field-caught *Aedes spp.* and *Culex spp.* mosquitoes. Amplicons from RT-LAMP reaction from field-caught *Aedes aegypti* (**A**) and *Culex quinquefasciatus* mosquitoes (**B**) were sequenced using Sanger method to confirm the identity of ZIKV. The region amplified was genome position 1827 to 1900. The obtained sequences were aligned against the ZIKV PE243 reference strain.

less than 1 PFU even without RNA extraction[43]. The analytical sensitivity of both our qRT-PCR and RT-LAMP differed from previously published studies which developed the primers[31,40]. There are a number of reasons that might have accounted for this variation, including differences in kits and research suppliers, viral strains, type of biological samples, and detection systems.

Several mosquito-borne arboviruses, including ZIKV, DENV and CHIKV, are endemic and co-circulate throughout the Northeast Brazil[44,45]. One possible limitation of diagnostic tests for ZIKV is the possibility of cross-reactivity with other flaviviruses, particularly DENV[40,46,47]. Here, we showed no cross-reactions with other arboviruses including four serotypes of DENV, YFV or CHIKV and sequencing of RT-LAMP amplicons from naturally infected *A. aegypti* and *C. quinquefasciatus* confirmed ZIKV identity.

We validated the RT-LAMP assay using samples obtained from experimentally and naturally ZIKV-infected *A. aegypti* and *C. quinquefasciatus*. The RT-LAMP had a sensitivity of 100%, specificity of 91.18%, and overall accuracy of 95.24% as compared to qRT-PCR. Importantly, the ZIKV RT-LAMP could undoubtedly detect ZIKV RNA in mosquito samples that had been previously tested as negative by qRT-PCR. These samples were at the detection threshold by the qRT-PCR with Ct value ranging from 38.6 to 40.3. In contrast with our findings, some studies have reported that the analytical sensitivity of the RT-LAMP assay is lower when compared to the gold standard diagnostic test (qRT-PCR)[32,38]. However, recently published studies have corroborated our findings that the analytical sensitivity of the RT-LAMP assay is superior than qRT-PCR[36,48].

The RT-LAMP assay can be performed through either a two-step assay or one-step protocol. Two-step RT-LAMP requires the addition of the reverse transcriptase (RT) enzyme together with the DNA polymerase enzyme, which may be wild-type Bst DNA polymerase or Bst 2.0 polymerase 2.0 WarmStart. Several studies report the need for RNA extraction before performing the RT-LAMP assay and the use of the two-step RT-LAMP[49–51]. However, the two step protocol is longer, more expensive, and requires additional sample

handling, which increases the chances of pipetting errors and contamination. The use of Bst 3.0 Polymerase 3.0 WarmStart overcomes these concerns. This enzyme possesses high activity of reverse transcriptase and polymerase in a single-temperature incubation which allows the assay to be performed in a one-step. Additionally, the Bst 3.0 DNA polymerase is a robust enzyme capable of maintaining its activities even in the presence of inhibitors[33]. This is especially relevant for viral survey in entomological samples which are notorious to harbor amplification inhibitors[52].

Recently, Yaren *et al.* reported a diagnostic test based on RT-LAMP for detection of ZIKV in mosquito samples[35]. Nonetheless, the need for RNA extraction limits its applications for POC diagnostics. In another study, Lamb *et al.* reported a low-cost molecular diagnostic test method based on RT-LAMP for detection of ZIKV in mosquito samples without RNA isolation[36]. However, the authors tested only five experimentally infected *A. aegypti* and did not validate the technique using naturally infected mosquitoes.

Other groups have also developed several technologies for molecular detection of ZIKV[30–33,35,50,51,53–58]. However, many of these technologies still have limitations for POC diagnostic applications, including the need for RNA isolation or the use of sophisticated and proprietary hardware and software, which limits its applicability in the developing world.

The main advantages of the RT-LAMP assay described here is the ability to detect ZIKV without the need for pretreatment or RNA extraction from the mosquito samples. Importantly, positive samples can be diagnosed in just 20 minutes and the result can be easily interpreted visual examination. Given its simplicity, the assay can be run by individuals without specialty training. The cost per sample was inferior to $1, which is considerably lower than qRT-PCR. These advantages suggest that our diagnostic assay to detect ZIKV is suitable for use in viral surveillance in mosquitoes in remote areas or low resource countries affected by the ZIKV epidemics or at risk of viral introduction.

Conclusion

We have developed a low cost, point-of-care diagnostic platform based on the RT-LAMP assay to detect ZIKV in mosquito samples collected at the epicenter of the Zika epidemics in Brazil. The test is a robust, fast and inexpensive tool for surveillance of ZIKV in mosquito populations and will enable developing countries to establish better viral surveillance in vectors and improve the efficacy of control programs. Our results provide a potential new molecular diagnostic test for ZIKV in mosquito samples as a novel straightforward and inexpensive method for detection of ZIKV in arthropod vectors.

Methods

Cells and viruses. Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Gibco) and 100 U/mL penicillin/streptomycin (Gibco) at 37 °C in 5% CO_2. The ZIKV strain PE243 (GenBank access code: KX197192.1) used in this work was isolated in C6/36 cell line using serum sample of a Brazilian patient infected by ZIKV in 2015. After isolation, the virus was propagated and stored at −80 °C until use. Other arboviruses, including DENV-1 (PE/97-42735), DENV-2 (PE/95-3808), DENV-3 (PE/02-95016), DENV-4 (PE/10-0081), YFV (17DD) and CHIKV (PE2016-480) were similarly propagated in Vero cells and used to determine the specificity of the RT-LAMP. All viruses were titrated in Vero cells by the standard plaque assay method and resulted in titers ranging from 10^6 to 10^7 PFU/mL. With the exception of YFV (17DD), which is a vaccine strain, all other viruses were isolated from humans in Pernambuco, Brazil.

RT-LAMP assay. RT-LAMP reactions were carried out in triplicate in a total volume of 25 µL containing 1x Isothermic Amplification Buffer, 8 mM MgSO4, 4 U of Bst DNA polymerase [version 3.0 WarmStart; New England Biolabs (NEB)], 1.8 mM deoxynucleotide triphosphates (dNTPs) (ThermoFisher Scientific), 1.6 µM for FIP (5′-GGCGACATTTCAAGTGGCCAGAGAGCTCTRGAGGCTGAGA-3′), 1.6 µM for BIP (5′-AGGGCGTGTCATACTCCTTGTGAGTGTTTCAGCCGGGATCT-3′), 0.2 µM for F3 (5′-CAGTTCACACGGCCCTTG-3′), 0.2 µM for B3 (5′-TGTACCTCCACTGTGACTGT-3′), 0.4 µM for LF (5′-CCTTCCCTTTGCACCATCCA-3′), 0.4 µM for LB (5′-TACCGCAGCGTTCACATTCA) primers and 5 µL test sample (no template control (NTC), extracted RNA, or samples without RNA extraction). Theses primers have been previously described[30]. In order to visualize positive reactions and prevent contamination, 1 µL of SYBR Green I (ThermoFisher Scientific) diluted 1:10 dilution in RNase-free water (Promega) was added to the center of the tube caps before the reaction and mixing afterwards. Reactions were incubated at 72 °C for 40 min in a heat block, and then inactivated at 80 °C for 5 minutes. To evaluated the robustness of the assay for POC applications, all set-up and execution of RT-LAMP reactions were done in a conventional lab bench using designated pipettes and filter tips. Imaging analysis took place in separate rooms. All experiments were independently replicated at least six times.

After the incubation, the RT-LAMP products reactions were detected using three different methods. In the first, the products were observed by naked eye under natural light and photographed using a conventional smartphone camera. A color change from orange to greenish yellow was used to identify positive sample, while a negative sample remained orange. The second method was visual analysis of reaction tubes under UV light irradiation (UV wavelength of 302–312 nm) using a transilluminator (model UVB LTB 20 × 20 STV, Loccus Biotecnologia, São Paulo, Brazil) coupled with a camera and connected to a computer. In this method, negative samples were dark blue and positive reactions were light fluorescent. In the third method, the RT-LAMP amplicons were analyzed by agarose gel electrophoresis (2.0%) in 1x TAE buffer, followed by ethidium bromide staining and gel visualization using transilluminator. For electrophoresis analysis, 1 kb Plus DNA Ladder (ThermoFisher Scientific) was used as a DNA size marker.

Real time RT-PCR. Samples with ZIKV are tested for positivity of the infection by qRT-PCR, according to protocols established by the Centers for Disease Control and Prevention - CDC USA with minor modifications[40]. Briefly, RNA from samples was extracted using Trizol reagent (Invitrogen Carlsbad, USA) following the instructions of the manufacturer. qRT-PCR was conducted using the QuantiNova Probe RT-PCR Kit (QIAGEN, Valencia, CA, USA) with amplification in the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's protocol. The reaction mixture (total volume, 15 µL) contained 7.5 µL of QuantiNova Probe RT-PCR Master Mix 2×, 0.9 µM each primers Zika1087 (5′-CCGCTGCCCAACACAAG-3′), Zika1163C (5′-CCACTAACGTTCTTTTGCAGACAT-3′), 0.9 µM FAM-labelled 1108 (5′-AGCCTACCTTGACAAGCAGTCAGACACTCAA-3′) probe for ZIKV, 0.1 µL of QuantiNova RT Mix, 0.08 µL of QuantiNova ROX Reference Dye, 5 µL of the RNA samples and RNA-free water. Primers and probes were synthesized by IDT (Integrated DNA Technologies, Skokie, Illinois, USA). The reaction program consisted of a single cycle of reverse transcription for 15 min at 45 °C, followed by 5 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation, and then 45 cycles of 5 s at 95 °C and 45 s at 60 °C. The amount of viral RNA in each sample was estimated by comparing the cycle threshold values (Ct) to the standard curve made by serial 10-fold serial dilutions of previously titrated ZIKV BRPE243/2015.

Detection of ZIKV in Mosquito Samples Under Controlled Conditions. To evaluate the ability of RT-LAMP to detect ZIKV in mosquitoes, pools of *A. aegypti* or *C. quinquefasciatus* mosquitoes (n = 10) were homogenized in 300 µL of RNA-free water. Crude lysates were then spiked with 100 µL of ZIKV so the final viral concentration in the lysates was either 10^6 or 10^3 PFU/mL, thus simulating a situation of high and low viral load, respectively. After incubation at 37 °C for 1 hour, samples were directly assay by RT-LAMP without RNA extraction.

In order to assess ZIKV detection by RT-LAMP in infected mosquitoes, we used samples from experimentally infected female *A. aegypti* mosquitoes. In brief, the Rec-Lab colony was maintained under standard conditions (temperature, 26 °C ± 1 °C, relative humidity of 60 to 80% and photoperiod 12:12 h C/E) at the Entomology Laboratory of the Institute Aggeu Magalhães (IAM). For artificial feeding, cell supernatant containing 10^6 PFU of ZIKV were mixed in 1:1 defibrinated rabbit blood and provided to starving mosquitoes for for 90 minutes as previously described[39]. Whole female mosquitoes were collected at 18 days post-infection, homogenized in 300 µL of RNA-free and processed for RT-LAMP. Mosquitoes independently fed on non-infected culture cells mixed to the defibrinated rabbit blood was used as controls.

Analytical Specificity and Analytical Sensitivity of RT-LAMP. To test specificity of the RT-LAMP primers for ZIKV, primers were validated by testing the cross-reactivity with other arboviruses currently circulating in Brazil, including ZIKV (PE243), four different serotypes of dengue DENV-1 (PE/97-42735), DENV-2 (PE/95-3808), DENV-3 (PE/02-95016), DENV-4 (PE/10-0081), YFV (17DD) and CHIKV (PE2016-480). Crude lysates of uninfected *A. aegypti* were spiked with different arboviruses so the final concentration would be 10^6 PFU per reaction. Spiked samples were then assayed by ZIKV RT-LAMP.

To evaluate the analytical sensitivity (limit of detection) of the RT-LAMP assay, ZIKV strain PE243 was 10-fold serially diluted in crude lysates of uninfected *A. aegypti* mosquito. Virus concentration in spiked mosquito samples ranged from 10^5 PFU to 10^{-7} PFU. After dilution, samples were directly assayed by RT-LAMP without RNA isolation. To compare the results of RT-LAMP with a gold standard technique, viral RNA was extracted from the same dilutions using Trizol reagent (Invitrogen Carlsbad, USA) according the manufacturer's instructions and then assayed by the widely used ZIKV qRT-PCR method[40].

Validation of RT-LAMP for ZIKV detection in Mosquito Samples. To validate the performance of the RT-LAMP for the diagnosis of ZIKV relative to qRT-PCR, 60 samples from *A. aegypti* (n = 32) and *C. quinquefasciatus* (n = 28) previously assayed by qRT-PCR[11,41] were obtained from the Entomology Department and tested by RT-LAMP. The intrinsic diagnostic utility of the test was determined using several statistical parameters described below.

Sequencing of LAMP fragments. The genetic characterization of the LAMP fragments from two field positives samples from *A. aegypti* and *C. quinquefasciatus* was performed by the Sanger sequencing method. Amplicons from RT-LAMP reaction were directly purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE) according to the manufacturer's instructions and eluted in 30 µL of water. Purified amplicons were directly sequenced using the primer FIP and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,USA) as established by the manufacturer and run on an ABI Prism 3100 Capillary Automatic DNA Analyzer. Sequences of fragments were analyzed using the Bioedit software, v7.0.5 and submitted to NCBI BLAST database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to identify the most closely ZIKV strain.

Statistical analysis. Graphs were generated using the GraphPad Prism Software version 5.01 for Windows (GraphPad Software, La Jolla, California, USA). A probit regression was performed to calculate the limit of detection of the RT-LAMP for detection of ZIKV using MedCalc software (version 18.11, MedCalc Software, Ostend, Belgium). The estimation of the several diagnostic parameters (sensitivity, specificity, ZIKV prevalence, positive predictive value, negative predictive value and overall accuracy) of the RT-LAMP for detection of ZIKV was calculated using the web-based software MedCalc's Diagnostic Test Evaluation Calculator (https://www.medcalc.org/calc/diagnostic_test.php). This analysis was based on the results from 60 mosquito samples previously diagnosed by qRT-PCR.

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Author Contributions

L.P. conceived the work. Experiments were performed by S.S., M.P., D.G., L.K. and A.S. All authors were performed data analysis and interpretation. S.S. drafted the article. All authors critically revised the article and approved the final version.

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APÊNDICE C - ARTIGO PUBLICADO

Evolutionary study of potentially zoonotic hepatitis E virus genotype 3 from swine in Northeast Brazil

Artigo anexo na página seguinte.

A doutoranda e co-autora desse artigo publicado na Memórias do Instituto Oswaldo Cruz, participou ativamente no planejamento das coletas, identificação e processamento de amostras. Também foi responsável pela revisão do manuscrito.

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Evolutionary study of potentially zoonotic hepatitis E virus genotype 3 from swine in Northeast Brazil

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Hepatitis E virus (HEV), an emerging virus associated with acute hepatic disease, leads to thousands of deaths worldwide. HEV has already been reported in Brazil; however, there is a lack of epidemiological and molecular information on the genetic variability, taxonomy, and evolution of HEV. It is thus unclear whether hepatitis E is a neglected disease in Brazil or it has low relevance for public health in this country. Here, for the first time, we report the presence of HEV in Northeast Brazil. A total of 119 swine faecal samples were screened for the presence of HEV RNA using real-time polymerase chain reaction (RT-PCR) and further confirmed by conventional RT-PCR; among these, two samples were identified as positive. Molecular evolution analyses based on capsid sequences revealed that the samples had close proximities to HEV sequences belonging to genotype 3 and were genetically related to subtype 3f isolated in humans. Parsimony ancestral states analysis indicated gene flow events from HEV cross-species infection, suggesting an important role of pig hosts in viral spillover. HEV's ability for zoonotic transmission by inter-species host switching as well as its possible adaptation to new animal species remain important issues for human health.

Key words: zoonotic hepatitis E virus - swine - genotype 3

Hepatitis E is an emerging zoonotic disease caused by the hepatitis E virus (HEV). HEV is a member of the genus *Orthohepevirus* A and can be divided into eight major genotypes[1] and 43 subtypes (assigned and unassigned).[2] Among them, only HEV-3 and HEV-4 have been reported in humans and animals worldwide, and they are known to cause cross-species infection, posing a threat of zoonotic transmission.[3] In Brazil, HEV-3 has been detected in humans and in swine in the South, Southeast, and Central-West regions.[4,5,6,7,8] To date, HEV isolates have not been detected in Northeast Brazil. Swine production in this area is characterised by a low technical level and by poor investments in mechanisation, technology, and biosecurity practices. In this study, we screened for and performed phylogenetic analyses based on different genome segments of HEV strains circulating among domestic swine in Northeast Brazil.

A total of 119 faecal samples were individually collected in 2017 from two to six-month-old animals from eight farms using both intensive and extensive production systems located within a high HEV seroprevalence area.[9] Viral RNA was extracted from a 200 µL faecal suspension using the ReliaPrep™ Viral Total Nucleic Acid Purification Kit (Promega, Brazil) and screened by a previously described real-time polymerase chain reaction (RT-PCR) method[10] using the NextGeneration ECO One-Step HotStart RT-qPCR Kit (DNA Express Biotecnologia, Brazil). Conventional HEV RT-PCR, using a set of previously designed primers to amplify the ORF2 region,[11,12] was used to confirm the positive samples. Primers for sequencing the complete capsid region were designed based on the pertinent literature[13,14] and on an alignment using 156 partial genomic Brazilian and HEV-3 subtype reference sequences (Tables I-II). RT-PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, USA) according to manufacturer's instructions. Nested PCR was performed using PCR Master Mix 2X (Promega, Brazil), and the obtained PCR products were purified with the QIAquick Gel Extraction Kit™ (Qiagen) before being sent for sequencing.

Initially, sequences were aligned using MUSCLE v3.6 software.[15] Markov models of nucleotide substitution were chosen using jModelTest v.2 software.[16] Sequences were retrieved from GenBank, and the accession numbers are displayed on the phylogenetic trees. Evolutionary studies were conducted using the maximum likelihood (ML) and maximum parsimony (MP) [17] inference methods implemented in FastTree v.2.1.7[18] and Mesquite v.3.5.1[19] software. ML analysis was conducted with the standard default GTR + CAT with 20 gamma distribution parameters and a mix of the nearest-neighbour interchanges (NNI) and sub-tree-prune-

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TABLE I

Oligonucleotides used to amplify the hepatitis E virus (HEV) capsid region

Regions	Oligonucleotides	Obtained from	Genome position*
Cap1 Ext F	GCGCAGGTYTGTGTTGATGT	This study	4969 - 4988
Cap1 Ext R	TACTGGGCATRGTTRGAYGCCTC	This study	5702 - 5724
Cap1 Int F	GGGYTGGTRCATAACCTYATTGG	This study	5017 - 5039
Cap1 Int R	GCCATAATRTGTGTRTTGGTGCC	This study	5675 - 5697
Cap2 Ext F	TCACCGGCCCCYGAYAC	This study	5510 - 5526
Cap2 Ext R	ARSCGRTGGCGGGCTGT	This study	6146 - 6162
Cap2 Int F	TGCGACGACAGTATAAYYT	This study	5565 - 5583
Cap2 Int R	GTRTACCGRGATACACG	This study	6125 - 6141
Cap3 Ext F	TGGTGATGCTYTGYATTCATGG	This study	5994 - 6015
Cap3 Ext R	ACCARTCMAGAGARCGGG	This study	6702 - 6719
CAP3 Int F	CTTGAYTTYGCGYTAGARCTTGA	This study	6068 - 6089
Cap3 Int R	CCTGRGCCCCTGTTGCYA	This study	6678 - 6695
Cap4 Ext F	GAGTAYGAYCAGACTACGTATGG	This study	6605 - 6627
Cap4 Int F	TCCACCAACCCGATGTATGT	This study	6632 - 6651
15T-aTag	CCAACGACCGGGAGGCCATTTTTTTTTTTTTTTV	[34]	Poly-A tail
TAG	CCAACGACCGGGAGGCCA		-

*: based on GenBank accession code AF082843.

regraft (SPR). The MP method, in which the best phylogenetic tree is determined based on favouring the fewest evolutionary changes, was performed using the parsimony ancestral states algorithm with the SPR rearranger to trace the history of character evolution on 1.000 trees.[17] The reliability of the tree nodes was obtained from Shimodaira-Hasegawa (SH-like) test support values with 1000 replications.[20] Phylogenetic signals were assessed via likelihood mapping analysis using Tree-Puzzle v.5.2 software.[21,22] The pairwise homoplasy index (PHI) for the Recombination search was implemented in SplitsTree v.4.14.6 following the default settings.[23] Lastly, pairwise and patristic distances were inferred using MEGA v.7[24] and PATRISTIC[25] software, respectively.

From a total of 119 samples, two samples (1.68%) originating from three- (S67) and five-month-old (S26) clinically healthy piglets yielded 304-nucleotide (nt)-long DNA fragments corresponding to the HEV capsid region (Accession numbers: MH664123 and MH664124). The positive animals were from two distinct farms [Supplementary data (Fig. 2)]. Evolutionary analysis showed that both the S26 and S67 HEV sequences clustered within genotype HEV-3 together with the subtype 3f derived from a patient with acute hepatitis E in Japan who is known to have travelled to Bangladesh (see AB369387) (Fig. 1A). Additionally, we performed further analyses on a 1503-nt-long capsid sequence fragment isolated from sample S26. Analysis of this larger fragment along with 96 HEV-3 representative reference sequences retrieved using Boolean terms [Hepatitis E virus(Organism)] AND ORF2(Gene name) from GenBank, 43 of which had been previously assigned to clade 3e-3f-3g, thus confirming S26 to be phylogenetically related to clade 3f (see Fig. 1C clade two highlighted by the dotted lines, SH-like support ≥ 80%). These data were extrapolated from the consistent monophyletic origin and asymmetric phylogenetic tree backbone support [Fig. 1B, Supplementary data (Fig. 1)]. Intriguingly, although the sequence exhibited a high divergence, as demonstrated by the length of the tree branch (red line), we observed a proximity to the data obtained from the pairwise and patristic distances of HEV isolated from humans (KT591533.1 and KT591532.1) (Fig. 1B, Table II). Further gene flow analysis of S26 showed an ancestry degree and viral gene stream among HEV-3 virus populations typically found in human and animal hosts, suggesting both a switch of host direction and that this HEV-3 subtype 3f strain is probably of zoonotic origin (Fig. 1C). Similar patterns were also observed among strains of the HEV-e-f-g monophyletic clade, revealing a continuous flow of viral gene interchange and spread among sequences from human and animal hosts.

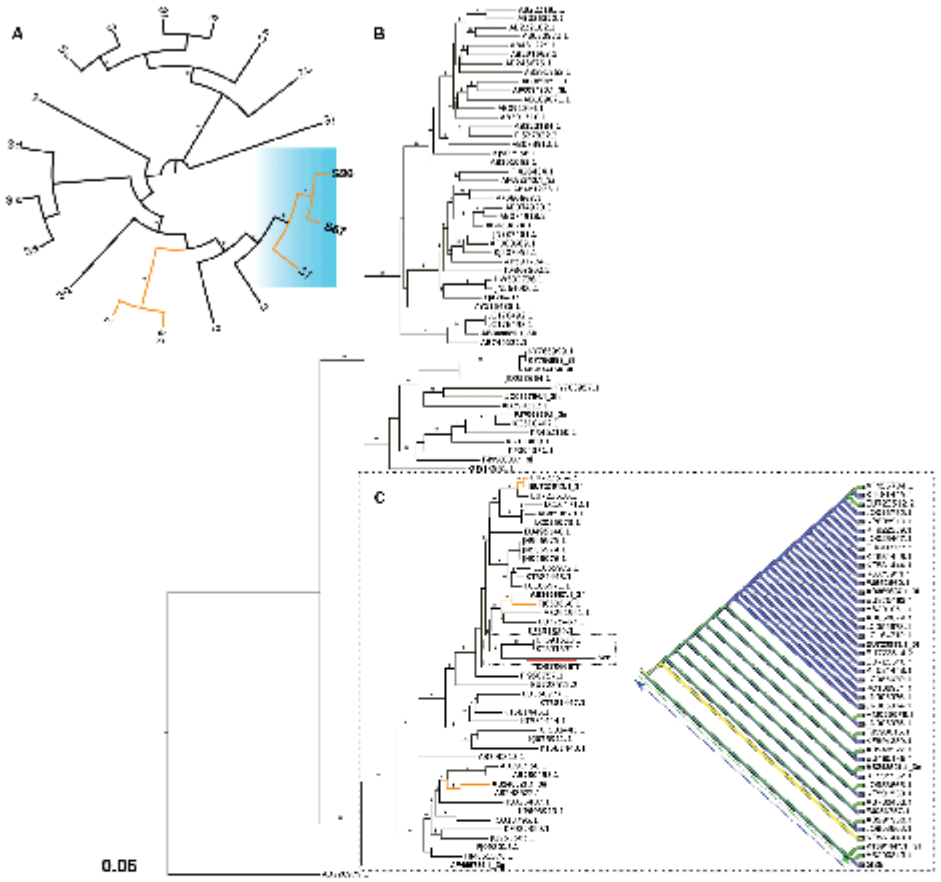
Although a human virus was recently suggested to be the most likely ancestor of *Orthohepevirus* A,[26] the human or animal origin of HEV remains under debate. In addition, with regard to its progressive host range expansion, it appears that host switching has played an important role during the evolutionary history of HEV and that genotype differences might have arisen via cyclical adaptation to different hosts.[27] Although there is a clear host range distinction among HEV genotypes, HEV-3 and HEV-4 viruses appear to be circulating between animal and human hosts.

TABLE II

Sequences comparison among hepatitis E virus 3 (HEV-3) e-f-g subtypes extracted from the well-supported monophyletic clade highlighted in Fig. 1

	Accession numbers	Countries	Hosts	Collection dates	Subtypes	Pairwise distances	Patristic distances
1	**AF455784**	Kyrgyzstan	Swine	1987-89(2004)	**3g**	0.226	0.3739
2	JQ013795	France	Human	2006	3e[§§]	0.215	0.3339
3	AB291958	Japan	Human	2004	3e	0.203	0.3607
4	AB780453	Japan	Wild boar	2011	3e	0.218	0.3757
5	AB248521	Japan	Swine	2006*	3e	0.188	0.3497
6	AB248522	Japan	Swine	2006	3e	0.187	0.3537
7	JQ026407	Japan	Monkey[§§]	2009	3e	0.211	0.3667
8	KP698919	Italy	Swine	2012	3e	0.206	0.3575
9	KF922359	France	Human	2009-10	3e	0.212	0.3691
10	JQ953665	France	Swine	2006	3e	0.214	0.3518
11	FJ998015	Germany	Wild boar	2007	3e	0.200	0.3505
12	HM055578	Hungary	Swine	2005	3e	0.202	0.3278
13	**AB290313**	Mongolia	Swine	2006	**3**	0.190	0.2762
14	EU723512	Spain	Swine	2009	3	0.189	0.2468
15	KJ873911	Germany	Human	2013	3	0.177	0.2695
16	KT581447	Sweden	Swine	2015*	3	0.184	0.2861
17	KT581444	Sweden	Swine	2015*	3	0.161	0.2740
18	KT581446	Sweden	Wild Boar	2015*	3	0.167	0.2387
19	**EU360977**	Sweden	Swine	2007*	**3**	0.175	0.2633
20	KT581443	Sweden	Swine	2015	3	0.205	0.2810
21	KT581445	Sweden	Wild boar	2015	3	0.174	0.2832
22	EU723516	Spain	Swine	2008*	3	0.134	0.1790
23	EU723514	Spain	Swine	2008*	3	0.141	0.1788
24	**EU723513**	Spain	Swine	2008*	**3**	**0.136**	**0.1767**
25	LC164712	Japan	Human	2007	3	0.142	0.1953
26	LC055973	Japan	Human	2008	3	0.149	0.1832
27	AB850879	Japan	Human	2012	3	0.140	0.1874
28	EU495148	France	Human	2008*	3f	0.139	0.1722
29	JN906975	France	Swine	2010	3f	0.111	0.1700
30	JN906974	France	Human	2010	3f	0.111	0.1700
31	JN906976	France	Swine	2010	3f	0.213	0.1700
32	KC166971	France	Human	2008	3f	0.114	0.1713
33	LC055972	Japan	Human	2012	3f	0.114	0.1827
34	KT581448	Spain	Swine	2015*	3f	0.118	0.1742
35	JQ953666	France	Swine	2008	3f	0.135	0.1499
36	FJ956757	Germany	Human	2005	3f	0.147	0.1899
37	KT591533	France	Human	2013	3f	0.124	0.1624
38	KT591532	France	Human	2013	3f	0.124	0.1624
39	KF891380	Italy	Swine	2013*	3f	0.131	0.1657
40	AB291961	Japan	Human	2004	3f	0.137	0.1810
41	EU375463	Thailand	Swine	2008*	3f	0.141	0.1752
42	**AB369687**	Japan**	Human	1998	**3f**	**0.123**	**0.1657**
43	FJ653660	Thailand	Human	2008	3f	0.148	0.1753
44	**S26**	Brazil	Swine	2017	**3f**	**0.000**	**0.0000**

HEV-3 subtypes references[23] are in bold. *: collection date was not available and GenBank deposition year is displayed; **: patient traveled to Thailand § classified as 3f on the GenBank §§ probably of human origin.



Phylogenetic analyses based on a 304 nt fragment (A) and 1503 nt (B and C) fragment of the hepatitis E virus 3 (HEV-3) capsid gene. Maximum likelihood (ML) phylogenetic tree showing the HEV samples in this study (blue gradient background: S26 and S69) clustering with subtype 3f isolated from humans (A). In (B), sequence S26 is in a rectangular dashed box. Subtype reference sequences[2] are in bold. The red line under a tree branch highlights the sequence divergence according to the tree bar scale. The branches in orange colour highlight the HEV 3f and 3e clades. The asterisks along tree branches represent SH-like support values of ≥ 0.75 (B). Parsimony ancestral states analysis inferring the character history of the sequences extracted from the second large clade from (B). Each colour represents HEV-3 isolated from different hosts, countries, and different sampling years (see Table II). The dotted blue and green arrows show our hypothesis about the continuous flow showing viral gene interchange and spread among HEV isolated from different hosts (C).

Our results are the first confirmation of HEV circulating among domestic swine farms in Northeast Brazil. The HEV RNA prevalence rate found here is markedly low considering the seroprevalence rates of up to 95% found in the same region.[9] Nevertheless, our findings are similar to those obtained in other countries such as Germany, China, Japan, and India.[13,28,29,30] Differences between serological and molecular prevalence rates are expected and likely to be related to animal age because HEV RNA is more easily detected in piglets that are two to six months old.[5]

Following the ICTV recommendation,[2] S26 was assigned to subtype 3f, which has already been detected in Brazil.[7] Although we were able to assign it successfully, a clear subtype separation based on pairwise or patristic distances was not possible (Table II). This divergence is likely related to differences in HEV-3 evolutionary rates among different subtypes, which seem to be higher among human-associated lineages.[27] In addition, the marked heterogeneity observed within the subtype 3f and 3e-3f-3g clade might be an evolutionary hallmark, for example resulting from the accumulation of mutations

positively selected through successful infection in different hosts.[27] Thus, these results illustrate that subtyping inside this clade can be challenging and might indicate the possibility of the future emergence of new subtypes if additional new heterogenic sequences appear.

The close relationship between HEV strains isolated from different host species points towards interspecies transmission. In addition, the gene flow study corroborated a possible evolutionary host switch origin and zoonotic potential. Thus, the molecular evidence indicates that the HEV strains identified here are potentially zoonotic and pose a threat regarding the infection of animal handlers, veterinarians, and consumers of non-cooked swine meat and pork products. Future studies should address HEV molecular epidemiology and explore the genetic variability and potential transmission among human and animal populations in Brazil.

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AUTHORS' CONTRIBUTION

EFOF - Study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript, critical revision; DRLS and RD-C - analysis and interpretation of data, drafting of manuscript, critical revision; AS, GBL, AFBBF and LJP - study conception and design, acquisition of data, critical revision; LHVGG - study conception and design, analysis and interpretation of data, drafting of manuscript, critical revision.

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