# UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

# FACULDADE DE VETERINÁRIA

# PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

# "DINÂMICA DE INFECÇÃO E CONTROLE DO VÍRUS DA INFLUENZA A EM GRANJAS DE SUÍNOS"

ANNE CAROLINE DE LARA

PORTO ALEGRE

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**"DINÂMICA DE INFECÇÃO E CONTROLE DO VÍRUS DA INFLUENZA A EM GRANJAS DE SUÍNOS"**

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Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias.
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**Título:** "Dinâmica de infecção e controle do vírus da Influenza A em granjas de suínos".


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**RESUMO**

O vírus da influenza (IAV) é endêmico nos rebanhos brasileiros e causa doença respiratória aguda em suínos. Pouco se conhece sobre a dinâmica de infecção em fluxos de produção no Brasil e também novos métodos para controle da transmissão do vírus utilizando desinfetantes. Os objetivos deste trabalho foram acessar a dinâmica de infecção do vírus em granjas brasileiras, desenvolver ferramentas de controle para reduzir transmissão entre leitegadas e avaliar métodos de amostragem para testar a sensibilidade de detecção do vírus. O primeiro estudo longitudinal analisou três fluxos de produção, desde desmame até o abatedouro com relação ao comportamento do IAV. Para isso, 1800 leitões desmamados de 3 granjas de matrizes foram incluídos na avaliação, e foram distribuídos em grupos e alojados em 3 creches. Após 70 dias de vida, os mesmos foram transferidos para 3 unidades de terminação. Foram coletados suabe nasal, fluido oral e sangue para avaliação do status para o IAV. Foram observados altos percentuais de leitões positivos ao desmame, também diagnosticadas ao menos duas ondas de excreção do vírus e alta variabilidade genética nos 3 fluxos de produção estudados. Buscando entender e desenvolver ferramentas de controle, um segundo estudo avaliou o tempo que o vírus da influenza pode persistir no aparelho mamário de porcas contaminados. O vírus da influenza A foi detectado no aparelho mamário de fêmeas em 90% e 46,7%, 3 e 48 horas após o desmame, respectivamente. Neste mesmo estudo foi desenvolvido um protocolo para desinfecção do aparelho mamário visto o risco de disseminação do vírus em granjas que utilizam a prática de mães de leite. Para isso, após o desmame dos leitões, foi utilizado um desinfetante via spray cobrindo toda a superfície do aparelho mamário das porcas. Lenços umedecidos em meio de transporte para o IAV foram utilizados para coleta antes e 30 minutos após o procedimento. A utilização do desinfetante reduziu significativamente os níveis de contaminação do IAV no aparelho mamário, enquanto os grupos controles mantiveram os níveis do IAV iniciais. Considerando os diferentes produtos químicos disponíveis para controle do IAV em superfícies, o terceiro estudo avaliou 4 desinfetantes comerciais com relação a sua eficácia para inativar o IAV no momento da diluição dos produtos e 72 horas após. Apenas um produto avaliado não inativou o vírus da influenza A. Um produto a base de fenol teve seu efeito reduzido 72 horas após a diluição, quando comparada à diluição preparada no momento do uso. O quarto e último estudo avaliou o efeito de fazer *pool* de amostras de lenços de aparelho mamário de porcas sobre a sensibilidade analítica e detecção por RT-PCR para o IAV. Conclui-se que podem ser analisadas *pool* de até 3 amostras sem perda significativa da sensibilidade, otimizando desta forma custo sem prejudicar a probalidade de detecção do agente. Ainda, neste

trabalho, conclui-se que o número de diluições possíveis está relacionado à contaminação inicial da amostra, demonstrada pelo ciclo de treshold (Ct).

**Palavras-chave:** desinfecção**,** leitegadas, longitudinal, ondas de excreção, transmissão.

*ABSTRACT*

*Influenza (IAV) virus is endemic in Brazilian herds and cause acute respiratory disease in pigs. Information about infectious dynamics in 3-sites of production in Brazil and methods to prevent transmission of the virus using disinfectant are scarce. The aims of this study were to assess the infectious dynamics of IAV in Brazilian farms, to develop control tools to decrease transmission among litters and to evaluate sampling methods to assess the sensitivity of the RT-PCR assay. The first was a cohort study in three production flows, that evaluated IAV behavior from weaning to slaughter age. Therefore, 1800 piglets were enrolled at weaning, from three different sow farms and were distributed and housed in three nursery farms. Pigs were transferred to three finishing farms approximately at 70 days of age. Nasal swabs, oral fluids and sera were sampled to assess the IAV status. There was a high number of positive piglets at weaning, at least two waves of shedding were detected during the study and there was high genetic variability among and within farms. To understand and to develop control alternatives, a second study evaluated how long IAV can persist on the udder skin of sow. The IAV was detected in 90% and 46.7% of the udder skin at 3 and 48 hours after weaning, respectively. A protocol to disinfect the udder skin was also developed in this study, due to the risk of IAV spreading in farms where nurse sows are routinely used. After piglets were weaned-off, a disinfectant was sprayed in udder skin, covering the underline of the sow. Udder skin wipes were sampled before and 30 minutes after the procedure. There was a significant decrease in the levels of IAV in the group where the disinfectants was applied, while no reduction was observed in the control groups. Considering the different disinfection products available for IAV surface control, a third study evaluated 4 commercial disinfectants to inactivate IAV post dilution and 72 hours post dilution. One product tested did not inactivate IAV, while three others were effective. One phenol disinfectant decreased efficacy 72 hours post dilution when compared to fresh disinfectant solution. The fourth and last study evaluated the effect of pooling udder skin wipes to detect IAV by RT-PCR. In conclusion, there is no effect on the sensitivity, when pooling up to 3 samples together. This information can help producers to optimize sampling strategies, without compromising detection. Also, the number of possible dilutions that can be performed was calculated and was related to the initial viral amount, demonstrated by cycle threshold (Ct).*

**Key words:** *disinfection, longitudinal, piglets, shedding waves, transmission.*

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# SUMÁRIO

# 1. INTRODUÇÃO

Com a intensificação da produção de suínos ao longo dos anos, e por consequência o aumento da densidade associada à adoção de outras estratégias, como produção em sítios segregados com mistura de lotes de diferentes origens, as doenças respiratórias passaram a gerar um maior impacto com perdas no desempenho zootécnico e também com aumento de custo devido aos tratamentos com antimicrobianos (MORES et al., 2015). O complexo de doenças respiratórias é constituído por diversos agentes virais e bacterianos, sendo que após a emergência do vírus H1N1 pandêmico em 2009 (H1N1pdm09), houve uma mudança na dinâmica das doenças respiratórias nas granjas de suínos no Brasil, visto que este vírus passou a ser diagnosticado com maior frequência (RECH et al., 2018). O vírus da Influenza A (IAV) é endêmico na produção de suínos, causando prejuízos econômicos e sanitários. Ao infectar o suíno, o vírus causa doença respiratória aguda e hipertermia, podendo atuar de forma única ou em conjunto com outros agentes no complexo de doenças respiratórias, o que pode agravar os sinais cínicos.

As perdas financeiras por Influenza podem variar desde U$3,23 por suíno terminado, quando o vírus atua sozinho, bem como, atingir U$10,41 por suíno terminado quando a infecção é associada ao *Mycoplasma hyopneumoniae* (HADEN et al., 2012), agente também frequentemente presente nas pneumonias em suínos criados de forma intensiva. Além do impacto econômico na produção de suínos, a doença causada pelo IAV é caracterizada como zoonose, sendo uma grande preocupação em saúde pública. A Organização Mundial da Saúde (OMS) estima aproximadamente 300 mil óbitos de pessoas por ano causados pelo vírus da Influenza sazonal em todo o mundo. A transmissão homem-suíno também é descrita (FORGIE et al., 2011) demonstrando a importância em controlar a transmissão entre espécies e evitar rearranjos virais que podem aumentar a diversidade genética dos vírus nas populações humana e suína (NELSON et al., 2012, NELSON & VINCENT, 2015).

Estudos longitudinais para avaliar a dinâmica da infecção do IAV foram realizados no EUA (ALLERSON et al., 2014) e Canadá (FERREIRA et al., 2017) demonstrando que existe mais de uma onda de excreção do vírus, sendo a primeira normalmente entre oito e 11 dias após o alojamento na creche, enquanto que a segunda onda de excreção variou consideravelmente entre os estudos, demonstrando diferença na dinâmica de infecção, bem como nos subtipos virais circulantes. No Brasil, estudos longitudinais com relação ao comportamento do vírus da influenza em produção de suínos são escassos. É de grande relevância entender como circulam

os vírus nas granjas, especialmente em diferentes sítios, como ocorre a produção de suínos no Brasil.

Ferramentas de controle e medidas de biosseguridade devem ser adotadas para reduzir a excreção e circulação do agente, e, consequentemente, sua disseminação. O desmame de leitões negativos é um ponto chave no controle do vírus e algumas práticas de manejo já foram descritas como um potencial risco para transmissão do vírus. A utilização de mães de leite está relacionada à maior transmissão do vírus entre leitegadas (GARRIDO-MANTILLA et al., 2020), aumentando a pressão de infeção deste agente no setor de maternidade e ainda contribuindo para aumento de leitões positivos ao desmame, o que impacta nas fases subsequentes. Por se tratar de informações bastante recentes, ainda não há estudos que avaliem possíveis intervenções nessas fêmeas utilizadas como mãe de leite.

O IAV tem alta morbidade e a transmissão ocorre principalmente por contato direto entre animais, porém aerossóis e fômites também são formas importantes de transmissão. Ainda, a adoção do manejo todos-dentro todos-fora (*all-in all-out*) e adequado programa de limpeza, desinfecção e vazio sanitário são medidas importantes e eficientes para controlar as doenças respiratórias. O IAV é relativamente sensível à maior parte dos desinfetantes químicos comerciais disponíveis, devido à presença do envelope lipídico em sua estrutura (PRINCE & PRINCE, 2001). No entanto, variações nos programas de desinfecção como doses dos princípios ativos, qualidade da água utilizada, matéria orgânica residual, tipo de superfícies, tempo de contato do desinfetante, estabilidade do produto, entre outras situações, podem interferir diretamente na atuação do produto desinfetante frente ao vírus (MARZOUK et al., 2014; CHANDLER-BOSTOCK &. MELLITS, 2015).

Dada a importância do suíno no controle sistemático do IAV, a proposta deste projeto foi compreender a dinâmica de infecção em diferentes fluxos de produção e identificar ferramentas e protocolos que possam contribuir no controle do vírus.

## 2. Capítulo I - REVISÃO BIBLIOGRÁFICA

### 2.1 Etiologia

É um vírus envelopado pertencente à família *Orthomyxoviridae*, RNA fita simples, composto por 8 segmentos, sentido negativo e classificado em tipo, subtipo e genótipo. A classificação em tipos A, B ou C baseia-se nas características da proteína da matriz (M) e nucleoproteínas (NP; VAN REETH et al., 2012). O IAV infecta humanos e várias outras espécies, incluindo os suínos, apesar das aves aquáticas serem a fonte original dos vírus encontrados nas demais espécies (WRIGHT et al., 2007). Influenza tipo A é o único que causa doença clínica em suínos. Os subtipos são definidos pela combinação das duas glicoproteínas presentes na superfície do envelope viral lipídico: a hemaglutinina (HA) e a neuraminidase (NA). Atualmente são descritas 18 HA e 11 NA (RAJÃO et al., 2019), o que gera um alto número de subtipos, e por consequência aumenta a variabilidade dos vírus (VAN REETH et al., 2012). O vírus da Influenza possui características genéticas específicas, que durante o processo de replicação permitem algumas trocas de nucleotídeos no momento da transcrição, e devido a essa característica, constantemente ocorrem modificações graduais em seu material genético (*antigenic drift*). Outra situação que pode ocorrer é quando o hospedeiro é infectado por mais de um subtipo viral simultaneamente, predispondo ao rearranjo viral (*reassortment*) (TORREMOREL, 2012; RAJÃO et al., 2019), podendo gerar novas estirpes potencialmente patogênicas.

Informações sobre a sobrevivência do IAV isolados a partir de suínos são escassas, portanto, grande parte dos estudos foram realizados a partir de vírus isolados de aves e humanos. O vírus é mais estável em baixas taxas de umidade relativa (BUCKLAND & TYRRELL, 1962; SCHAFFER et al., 1976;) e temperatura (HARPER, 1961). Ainda, sobrevive mais eficientemente em superfície não porosa (BEAN et al., 1982; TIWARI et al., 2006, PERRY et al., 2016) e a presença de muco aumenta sua sobrevivência (THOMAS et al., 2008). Ainda, o vírus da influenza aviária pode sobreviver em fezes de aves por nove semanas quando a 5ºC e duas semanas a 20ºC (HAAS et al., 1995). A persistência do vírus em água é inversamente proporcional à temperatura, sendo possível recuperá-lo durante vários dias a 20º e 30ºC, por semanas à 10ºC, e por meses a 0ºC (NAZIR et al., 2010). Em outro estudo foi encontrada partícula viral infecciosa em água por até 207 dias quando a 17 ºC (STALLKNECHT et al., 1990).

## 2.2 Epidemiologia

O vírus é distribuído mundialmente e, provavelmente, está presente onde há criação de suínos, porém existe variação dos genótipos circulantes conforme a localização geográfica (GRAY & BAKER, 2011; VAN REETH et al., 2012; ANDERSON et al., 2016). A circulação do vírus ocorre durante todo o ano em suínos, porém normalmente ocorrem picos sazonais de doença clínica (CHAMBA-PARDO et al., 2017).

No Brasil, o primeiro relato de Influenza A em suínos foi em 1974 (CUNHA et al., 1978), e até 2009 poucos relatos do vírus associado à doença clínica nos rebanhos suínos foram descritos. Após o surgimento dos casos clínicos envolvendo o vírus H1N1pdm09 na América do Norte, o vírus da Influenza também foi isolado a partir de pulmão com sinais clínicos de pneumonia no Brasil (SCHAEFER et al., 2011). Após a emergência do vírus H1N1pdm09, inquéritos epidemiológicos apresentaram soro prevalência variando de 46% a 100% nos rebanhos de suínos brasileiros (CARON et al., 2010; RAJÃO et al., 2013; CIACCI-ZANELLA et al., 2015). Após o evento de 2009, outros subtipos foram isolados a partir de suínos com doença respiratória, como por exemplo, um vírus recombinante H1N2 em leitões na fase de creche (SCHAEFER et al., 2015b).

A evolução dos vírus Influenza no Brasil foi estudada pelo grupo de pesquisa da Embrapa Suínos e Aves, onde foram analisadas amostras de secreção nasal e tecido pulmonar provenientes de 131 granjas localizadas em SC, PR, MS e RS, MG, MT, SP entre os anos 2009 e 2012. Por meio da análise de RT-PCR foram detectadas 5,93% de amostras positivas e em 16 amostras foram sequenciados os oito genes do vírus Influenza. A análise filogenética identificou os três subtipos virais (H1N1, H1N2 e H3N2) e foi evidenciada a importância da circulação dos vírus entre humanos e suínos, pois os subtipos H3N2 e H1N2 isolados a partir de casos clínicos em suínos foram relacionados àqueles que circulavam na população humana no final da década de 1990 e início dos anos 2000, respectivamente. Adicionalmente, entre as amostras H1N1pdm09 analisadas, foi possível concluir que houve no mínimo, oito diferentes eventos de transmissão entre humanos e suínos desde 2009, o que possibilitou a ocorrência de rearranjo genético com os vírus que já circulavam nas granjas (NELSON et al., 2015, SCHAEFER et al., 2015b). Em um outro estudo com amostras de soro suíno coletadas antes e após 2009, ficou evidenciado que não havia anticorpos para o vírus H1N1pdm09 antes da pandemia de 2009 (CIACCI-ZANELLA et al., 2011). Esses dados de estudos brasileiros demonstram a importância da transmissão de influenza A entre humanos e suínos e sua contribuição na evolução genética desses vírus.

Devido à grande variabilidade genética, dentro dos subtipos virais existe uma classificação para agrupar os vírus em clados que se assemelham geneticamente. Os critérios desta classificação variam conforme país e região geográfica. Nos Estados Unidos os vírus H1 são classificados em sete grupos com letras gregas: α (alfa), ϐ (beta), γ1 (gamma 1), γ2 (gamma 2), δ1a (delta 1a) δ1b (delta 1b), δ2 (delta 2) e mais o H1N1pdm09. Os vírus H3 são classificados em quatro clados, I, II, III, IV, sendo que no clado IV há sete subgrupos (A, B, C, D, E, F, G) descritos (CULHANE, 2019). Já o *European Surveillance Network for Influenza in Pigs* (ESNIP) classifica os vírus da Influenza H1 baseado no hospedeiro e/ou no histórico de introdução, por exemplo $H1_{av}N1$ são os vírus detectados em suínos com origem aviária. São descritas quatro linhagens, $H1_{av}N1$; $H1_{hu}N2$ (origem humano, recombinante); H1N1pdm09 e H1N1 (linhagem clássica suínos). Nesse sentido, Anderson et al. (2016) propuseram uma nomenclatura global padrão baseada no critério filogenético da evolução da hemaglutinina dos vírus H1 que resultou em 28 clados. Avaliando mais de 7 mil sequências HA H1, foi observado que 87% dos vírus obtidos entre 2010 a 2016 pertenciam a sete clados contemporâneos (ANDERSON et al., 2016).

A persistência do vírus nas granjas produtoras de leitões (UPL) ocorre principalmente devido à presença de leitões suscetíveis e sem imunidade materna (TORREMOREL et al., 2012, REYNOLDS, et al., 2016). Já em lotes de terminação e creche, a disseminação do vírus pode ser controlada nas instalações através do sistema *all-in all-out,* através da saída dos animais e da adequada limpeza, desinfecção e vazio sanitário entre lotes. Porém, esse manejo não impede nova introdução do vírus em lotes posteriores. Entre os fatores de risco, considerando-se as características do rebanho, destacam-se principalmente planteis grandes, alta taxa de reposição, alta densidade, ausência de barreira física entre baias de maternidade, granjas de ciclo completo e granjas sem restrição de acesso (POLJAK et al., 2008; SIMON-GRIFE et al., 2011).

### 2.3 Patogenia

Apesar do alto número de subtipos virais, os envolvidos em casos clínicos em suínos são H1N1, H3N2 e H1N2. Ao infectar o suíno, o IAV replica nas células epiteliais do sistema respiratório superior e inferior, limitando-se apenas a esses sítios. Dessa forma, a transmissão e excreção viral ocorrem unicamente por via respiratória nesta espécie. A transmissão ocorre principalmente através de contato direto entre animais, porém aerossóis também tem papel importante (CORZO et al., 2014), especialmente em situações de alojamento em alta densidade. A importância dos fômites na disseminação do vírus também já foi demonstrada por Allerson et al. (2013a), onde foi avaliada a transmissão indireta entre grupos de animais infectados e não infectados com o IAV, considerando processos com alto e baixo nível de biosseguridade,

incluindo troca de roupas e equipamentos de proteção individual. Ainda, a transmissão através da água também já foi descrita em suínos infectados com vírus de origem aviária (KARASIN et al., 2000).

O vírus pode ser isolado um dia após a infecção e torna-se indetectável depois de seis a oito dias (TORREMOREL, 2012; SCHAEFER et al., 2013). Estudos experimentais demonstram que a severidade das lesões no pulmão, sinais clínicos da doença e cinética da replicação viral variam de acordo com a via de inoculação e dose (VAN REETH et al., 2012). De Vleeschauwer et al. (2009) observaram infiltração de neutrófilos característica nos pulmões e uma carga de $1x10^8$ partículas virais por grama de pulmão, além de febre e letargia em animais inoculados experimentalmente pela via intra-traqueal.

Para causar a doença é necessário que o vírus infecte, replique e infecte novas células. Nesse processo, o primeiro passo é a ligação em receptores presentes nas células epiteliais do trato respiratório do hospedeiro. A glicoproteína HA é responsável pelo processo de ligação do vírus nos receptores das células. Considerando a grande diversidade de subtipos virais, a especificidade está relacionada à expressão de receptores nas células dos hospedeiros que contém ácido siálico (AS), sendo que os vírus mais adaptados aos humanos e outros mamíferos têm uma maior afinidade pelos receptores α 2,6 AS, enquanto os vírus adaptados às aves têm maior afinidade pelo α 2,3 AS (MEDINA & GARCIA-SASTRE, 2011; DE GRAAF & FOUCHIER, 2014). Os suínos e humanos expressam ambos receptores, com distribuição e frequência semelhante ao longo do trato respiratório, portanto apresentam susceptibilidade para se infectar tanto com vírus de origem humana como vírus originalmente aviário (DE GRAAF & FOUCHIER, 2014; RAJÃO et al., 2019). Outras características também são importantes para definir a especificidade dos vírus Influenza, como temperatura ótima de replicação da polimerase, balanço entre atividade de clivagem da neuraminidase e ligação da hemaglutinina do vírus junto às células do hospedeiro (RAJÃO et al., 2019).

Semelhante à outras doenças respiratórias, a infecção pelo vírus da Influenza em suínos também depende de outros fatores como idade, estado imune do hospedeiro, condições ambientais, pressão de infecção e presença de outros agentes concomitantes como *Mycoplasma hyopneumoniae, Pasteurella multocida, Glaesserella parasuis* e *Actinobacillus pleuropneumoniae* (VINCENT et al., 2008).

### 2.3 Imunidade

A resposta imune para o vírus da Influenza é rápida e eficaz, resultando em eliminação da infecção em até sete dias, sendo importante tanto a imunidade humoral quanto a celular.

Anticorpos podem ser detectados no soro de 7-10 dias pós exposição, atingem o pico em 2-3 semanas e duram até 8-10 semanas na espécie suína (VAN REETH et al., 2012; LARSEN et al., 2000). A resposta humoral é em grande parte, produzida para as proteínas M, NP, HA e NA. Os anticorpos contra a HA bloqueiam a ligação do vírus na célula do hospedeiro e neutralizam a infectividade viral (VAN REETH & VINCENT, 2019). Além da imunidade humoral sérica, o suíno produz IgA e IgG no tecido da mucosa nasal e brônquio-alveolar, sendo a IgA mais predominante (LARSEN et al., 2000). Já a resposta celular é composta pelos linfócitos T *helper*, diferenciado a partir de células T CD4+, o qual atua como facilitador da resposta celular e anticorpos. Outro componente importante é o linfócito T CD8+ ou citotóxico que atua eliminando as partículas virais no hospedeiro. A imunidade celular por linfócitos T é detectada sete dias após exposição do suíno ao IAV (KHATRI et al., 2010; LARSEN et al., 2000).

Após a primeira exposição, o animal permanece protegido caso ocorra reinfecção pelo mesmo genótipo viral ou por um vírus muito similar antigenicamente e, normalmente, não há reação sorológica cruzada entre os subtipos virais (JANKE, 2000; DE VLEESCHAUWER et al., 2011). Existem alguns relatos de imunidade parcial entre clusters, como é o caso dos vírus norte-americanos H1 cluster γ (gamma) e α (alfa), e pode ser explicado em parte pela combinação de anticorpos de mucosa IgA, imunidade celular mediada e reação cruzada entre os subtipos, porém esses mecanismos devem ser mais estudados (VAN REETH & VINCENT, 2019). A imunidade passiva protege os leitões contra a doença clínica frente a vírus homólogos, dependendo, portanto, da imunidade da porca, sendo que sua duração pode variar de 4-14 semanas. (LOEFFEN et al., 2003). Porém há de considerar que a imunidade materna pode interferir no desenvolvimento de imunidade ativa, seja por vacinação ou infecção (DEBLANC et al., 2018; VAN REETH & VINCENT, 2019).

A infecção pelo vírus da Influenza causa doença com alta morbidade, especialmente em rebanhos sem contato prévio com o agente, em que não há imunidade. ROMAGOSA et al. (2011) avaliaram a taxa de transmissão ($R_0$) em animais não vacinados, vacinados com vacina homóloga (H1 β) e vacinados com vacina heteróloga (H1 γ, H1 δ e H3) em estudo de inoculação experimental e encontraram $R_0$= 10,66; 0 e 1, respectivamente. Em outro estudo norte americano, também foi avaliada a taxa de transmissão do vírus da Influenza A, sendo observado $R_0$=7,1 no grupo de animais vacinados com vacina heteróloga (H1 β) e $R_0$= 0,8 no grupo em que foi utilizada vacina homóloga (H1 α), enquanto o grupo controle apresentou $R_0$=10,4 (ALLERSON et al., 2013b). Chamba-Pardo et al. (2019) observaram que altas taxas de

anticorpos homólogos no momento do desmame interferiram diretamente na dinâmica de infecção do vírus na fase de creche, reduzindo a excreção durante até seis semanas após o desmame.

### 2.4 Sinais Clínicos e Lesões

A doença é caracterizada por hipertermia (40,5 a 41,5ºC), anorexia, letargia, dispneia, espirros e tosse. O período de incubação dura de 1 a 3 dias, sendo alta a morbidade, chegando até 100% e baixa mortalidade, desde que não haja interação com agentes secundários associados. A infecção é autolimitante e, normalmente, os animais recuperam-se em seis a oito dias. Sinais clínicos como tosse, espirro e hipertermia são observados principalmente na fase de creche, mas também ocorrem na fase de terminação e coinfecções já foram descritas com *Mycoplasma hyopneumoniae*, *Glaesserella parasuis* e *Actinobacillus pleuropneumoniae* (RECH et al., 2018; POMORSKA-MÓL et a., 2017a; POMORSKA-MÓL et a., 2017b).

As lesões macroscópicas são caracterizadas por atelectasia e consolidação pulmonar e normalmente são limitadas aos lobos apicais e cardíacos, podendo haver edema interlobular. As lesões microscópicas incluem necrose e descamação do epitélio bronquiolar associado descamação de epitélio metaplásico das vias áreas condutoras de ar e infiltração de células inflamatórias, especialmente neutrófilos. Watanabe et al. (2012) avaliou 60 pulmões com histórico e lesões características de Influenza A e nas amostras com marcação positiva pela técnica de imunoistoquímica as lesões mais encontradas em pulmões foram bronquiolite necrótica, atelectasia, broncopneumonia purulenta e hiperemia. Amostras negativas na imunoistoquímica apresentavam principalmente hiperplasia dos pneumócitos tipo II, estruturas similares a pólipos em alvéolo e bronquíolo e pleurite, as quais são classificadas como lesões crônicas.

### 2.5 Diagnóstico

O diagnóstico é realizado através de coleta de amostras do trato respiratório para detecção e isolamento do vírus, uma vez que os sinais clínicos são inespecíficos. O isolamento viral pode ser realizado em células MDKC – *Madin-Darby Canine kidney* (SWENSON et al., 2018) ou em ovos embrionados de galinha e teste de hemaglutinação após 72 horas de incubação (SWENSON et al., 2018; ZHANG & GAUGER, 2014). Além do isolamento viral, técnicas moleculares podem ser utilizadas para detectar material genético viral, porém é importante que os reagentes utilizados (iniciadores e sondas) estejam atualizados com as sequências dos vírus circulantes.

A RT-PCR é a técnica mais comumente utilizada para detectar o vírus e apresenta alta sensibilidade, especificidade, além de menor custo e maior agilidade em relação ao isolamento viral. A escolha do animal é fundamental, pois devido ao curto ciclo do vírus da Influenza A, animais que não estiverem na fase aguda podem resultar negativos. Deve-se, portanto, coletar amostras de animais febris, que estejam na fase de aguda da infecção (SCHAEFER et al., 2013). Os materiais de eleição para isolamento e detecção viral são suabe nasal, pulmões, brônquios ou traqueia. Para a coleta, deve-se utilizar suabes de Rayon ou Dracon, para preservar o material genético e o vírus adequadamente.

Outro método de coleta para amostragem de grupo de animais que vem sendo amplamente utilizado é o fluido oral (PRICKETT & ZIMMERMAN, 2010). O mesmo consiste em suspender uma corda de algodão em altura adequada para que os animais possam mastigá-la e depositar saliva e transudato mucoso oral. Após manter a corda em contato com os animais por aproximadamente 20 a 30 minutos, é realizada uma compressão da extremidade umedecida em sacos plásticos estéreis para obtenção do líquido, e posterior transferência para tubos estéreis (WHITE et al., 2014). Este método apresenta algumas vantagens em relação às coletas individuais, principalmente quando o objetivo é verificar se o lote é positivo para o IAV ou se os animais estão em fase de excreção viral (GARRIDO-MANTILLA et al., 2019). Além disso não é considerado invasivo, pois por instinto e curiosidade os animais acessam a corda e depositam o material, não havendo necessidade de contenção. Porém para isolamento e sequenciamento viral, esse tipo de amostra não é recomendada, devendo, portanto, ser realizado a partir de amostras de suabe nasal ou tecido pulmonar (GARRIDO-MANTILLA et al., 2019).

Para a colheita de leitegadas em lactação foi desenvolvido um método em aparelho mamário de fêmeas lactantes (GARRIDO-MANTILLA et al., 2019). Isso porque os leitões se infectam com o vírus e ao acessar o aparelho mamário da fêmea suína, os mesmos depositam o vírus da influenza na superfície. A colheita é realizada utilizando lenços umedecidos em meio de transporte específico para o IAV, esfregando toda superfície do aparelho mamário e retornando o lenço ao meio de transporte (GARRIDO-MANTILLA et al., 2019).

Após a coleta, as amostras devem ser acondicionadas em meio de transporte sob refrigeração (2-8ºC) e serem analisadas em até 48h. Do contrário, devem ser armazenadas a -70º C, pois o vírus não é estável a -20ºC (SCHAEFER et al., 2013). A identificação dos subtipos virais pode ser realizada pelo método da inibição da hemaglutinação, utilizando antissoros específicos para os subtipos H1, H2, H3, N1 e N2 ou ainda, por meio de métodos moleculares, utilizando a RT-PCR (CHOI et al., 2002; GOECKE et al., 2018, HAACH et al., 2019).

A análise sorológica detecta anticorpos para Influenza e não diferencia resposta vacinal de infecção natural. A técnica de ELISA (*Enzyme-Linked Immunosorbent Assay)* é um teste que pode ser utilizado para definir o *status* imunitário do rebanho ou como triagem, pois normalmente os *kits* desenvolvidos são baseados em proteínas bem conservadas do vírus (CIACCI-ZANELLA et al., 2010), não evidenciando necessariamente imunidade protetiva. Também estão disponíveis *kits* comerciais de ELISA específicos para um subtipo, por exemplo, H1N1 ou H3N2. Outro teste bastante utilizado é a inibição da hemaglutinação (HI), o qual é baseado em antígenos específicos e nesse caso, níveis de anticorpos maternais em leitões e resposta imune pós vacinação podem ser determinados para avaliar imunidade homóloga protetiva (CHAMBA PARDO et al., 2019).

### 2.6 Controle

O controle do IAV é baseado em medidas de manejo que contribuem para a melhoria nas condições de criação dos animais, aumento de biosseguridade e utilização de vacinas com o objetivo de reduzir a excreção e circulação viral (TORREMORREL et al., 2012). Nos EUA e Europa, a vacinação, tanto comercial quando autógena, é uma ferramenta amplamente utilizada visto que reduz excreção viral, severidade das lesões e transmissão entre os animais (CHOI et al., 2004; LEE et al., 2007, ROMAGOSA et al., 2011, ALLERSON et al., 2013b, CHAMBA-PARDO et al., 2019).

Vacinas com vírus atenuado replicam no hospedeiro induzindo além da imunidade humoral, imunidade celular e também de mucosa, sendo que foram detectadas IgG e IgA em mucosa nasal e linfonodos traqueobrônquicos (VAN REETH & VINCENT, 2019; HOLZER et al., 2019). Há de se considerar que os vírus presentes em vacinas atenuadas podem sofrer rearranjos com outros vírus presentes nas granjas, aumentando a variabilidade genética. Vacinas utilizando vírus inteiro inativado induzem produção de altos níveis de anticorpos neutralizantes, principalmente para as duas glicoproteínas de superfície, hemaglutinina e neuraminidase, os quais atuam reduzindo a ligação do vírus na célula do hospedeiro e na clivagem durante replicação viral, respectivamente (HOLZER et al., 2019). Importante considerar que há limitada reação cruzada entre subtipos virais (DE VLEESCHAUWER et al., 2011; JANKE, 2000), portanto o correto diagnóstico na granja faz-se necessário antes de utilizar a vacinação. Ainda, doença respiratória potencializada pela vacinação (*Vaccine associated enhanced respiratory disease* -VAERD) é relatada em casos onde o suíno é vacinado com vírus inteiro inativado e, posteriormente, entra em contato com um vírus heterólogo, porém

com a mesma HA (GAUGER et al., 2011; RAJÃO et al., 2016). Este mecanismo ainda não é bem entendido, porém acredita-se ser imuno-mediado com altos níveis de anticorpos não neutralizantes cobrindo o domínio da HA (HOLZER et al., 2019). O fenômeno VAERD resulta em potencialização dos sinais clínicos, prolonga o período febril, causando severa pneumonia intersticial e bronquiolite necrosante e desregulando a imunidade celular. O tipo do adjuvante pode modular a VAERD, sendo que adjuvantes oleosos causam lesões mais severas quando comparados com compostos de gel polímero ou nano emulsão (SOUZA et al., 2018).

No Brasil, atualmente apenas uma vacina comercial é registrada pelo Ministério da Agricultura Pecuária e Abastecimento (MAPA) para uso em suínos e existem poucos dados científicos disponíveis sobre os resultados. Ainda, há o uso de vacinas comerciais autógenas, aprovadas pelo Ministério da Agricultura Pecuária e Abastecimento (MAPA) desde o ano de 2017.

A imunidade passiva é importante para o controle da doença, uma vez que o leitão é o principal hospedeiro suscetível que mantem o vírus no rebanho (TORREMORELL, 2012; REYNOLDS et al., 2016). Cador et al. (2016) observaram que leitões com imunidade passiva apresentam melhor proteção frente a um primeiro desafio quando comparados ao grupo sem imunidade passiva. Por outro lado, a taxa de transmissão ($R_0$) do vírus nos leitões com imunidade passiva foi superior a 1, evidenciando que ainda ocorre transmissão e, por consequência, o vírus permanece no rebanho por mais tempo. Rose et al. (2013), em um estudo longitudinal, verificaram que leitões com imunidade passiva, quando entram em contato com o vírus próximo das oito semanas de idade, não desenvolvem imunidade humoral adequadamente. O mesmo foi observado por Deblanc et al. (2018) em estudo com inoculação experimental realizado às sete e onze semanas de idade, sendo que leitões com imunidade passiva apresentaram desenvolvimento de imunidade humoral e celular mais tardia quando comparado ao grupo de leitões sem imunidade passiva.

A correção dos fatores de risco relacionados às doenças respiratórias é uma forma importante de minimizar o impacto da influenza nas granjas. Um estudo realizado em 21 rebanhos contemplando 404 matrizes no sul do Brasil, demonstrou que a presença de tela anti-pássaro e procedimento adequado de aclimatação de leitoas antes da introdução ao plantel implicou em menores índices de animais soropositivos para Influenza (SILVA et al., 2019). Outros estudos e observações sugerem a eficácia de medidas como controle de visitantes com troca de roupa e processo de lavagem e assepsia das mãos, restringir a entrada de pessoas com sinais clínicos respiratórios e vacinação dos funcionários (TORREMOREL et al., 2012).

O vírus é relativamente sensível aos desinfetantes comerciais disponíveis, devido à presença do envelope lipídico, porém as informações sobre sensibilidade de vírus isolados a partir de suínos são escassas, já que a maioria dos experimentos foram realizados com vírus isolados de aves com características de alta patogenicidade (SUAREZ et al., 2003; MARZOUK et al., 2014) ou isolados de humanos.

**3.      Capítulo II – Primeiro artigo científico**

**Influenza A infection dynamics in pigs raised in a 3-site system: A cohort study perspective**

Artigo a ser submetido para a Veterinary Research (formato conforme as normas da revista)

Influenza A infection dynamics in pigs raised in a 3-site system: a cohort study perspective

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**ABSTRACT**

The aim of this study was to assess the IAV dynamics in three production flows with 3-sites, from weaning to market. A total of 1,800 piglets were enrolled in the study one day prior to weaning (20 days-old ±3) from 3 different sow farms. At weaning, piglets were ear tagged and transported to 3 separate nursery farms and 600 pigs were placed in 10 pens of 60 pigs per pen in each nursery farm. At 70 ±3 days of age, pigs were transferred to 3 finishing farms, following the same flow as nursery farms. Pen integrity was kept from weaning until the end of the study. Oral fluids and nasal swabs were collected at weaning, every 10 days in the nursery and every 20 days on finishing farms for RT-PCR. Pigs were bled at 20, 40, 60, 80, 120 and 160 days old and sera were tested by ELISA and hemagglutination inhibition (HI). Sixty four percent (IC 52.2-76.6%) of oral fluids and 17.8% (IC 9.9-25.7%) of nasal swabs from piglets were RT-PCR positive for IAV at weaning. More than 60% of the oral fluids at weaning tested positive for IAV and there was a first IAV shedding wave at about ~ 20d to 30d of age and the second at about ~ 60 days of age. Results from finishing barns indicated that farms 1 and 2 had active infections while there was no detection of positive IAV samples in farm 3. The number of times that IAV was detected in the same pig ranged from none up to four times and also, there was no IAV detection by RT-PCR in 16.6% of the pigs across all the 10 sampling events. Seroprevalence by ELISA resulted in 82.2% (C.I. 74.3-90.1%) of positive piglets at weaning. Seroprevalence at 40 days old decreased in all 3 nursery farms, as expected in the case of maternal antibodies. In conclusion, this study demonstrated that influenza can circulate during all phases of production and there is high virus genetic variability within and among farms.

# BACKGROUND

Influenza A virus (IAV) is widespread and endemic in pig farms worldwide. IAV causes acute respiratory disease, characterized by sneezing, coughing and fever and can be associated with other pathogens in the porcine respiratory disease complex (Torremorell et al., 2011). When IAV is associated with other respiratory diseases, the economic losses can reach U$10/pig (Haden et al, 2012). There are also implications in public health, due to the IAV capability to transmit between species, including humans (Rajão et al., 2019).

The main challenge to control IAV in pig farms is related to the high genetic variability of the virus. IAV is an Orthomyxoviridae with a segmented RNA, therefore this characteristic can often lead to drift or reassortments (Nelson & Vincent, 2015). Different virus strains can persist concomitantly in sow farms and keep reassorting and generating new strains (Diaz et al., 2017). H1N1, H3N2 and H2N1 are the most prevalent subtypes in pig farms, but also the variability within each subtype is relevant to protective immunity (Allerson et al, 2013).

IAV is a limited infection when there is no association with secondary pathogens. Nevertheless, IAV is often endemic because of the piglets that maintain the virus in sow farms (Diaz et al, 2015). As new piglets are born regularly, there is recurrent susceptible subpopulation, so the virus can infect, persist at the sow-farm (Reynolds et al., 2014) and spread to other sites after weaning (Diaz et al., 2015). Diaz et al (2017) demonstrated two epidemic waves of IAV in a longitudinal study, using one wean-to-finish site production in USA after piglets were weaned positive for IAV. A different study performed in Canada during nursery, diagnosed three waves of infection (Ferreira et al., 2017).

In order to contribute with strategies and control measures for IAV infection, it is relevant to understand the infectious dynamics in different types of pig production. The use of three different sites for pig production is common in some countries, including Brazil. Our hypothesis is that the production flow in 3 sites may impact in the dynamics of diseases, since facilities and labor are customized for each category, but at the same time, there is more movement of the pigs and a greater risk of introduction of new pathogens. Therefore, the aim of this cohort study was to assess the IAV dynamics in three production flows with 3-sites, from weaning to slaughter age.

## MATERIALS AND METHODS

*Farms facilities*

Farms A, B and C were 1,500-sow farrow-to-wean farms, with similar facilities that weaned piglets of 21 (±3) days of age. At weaning, pigs were moved to nursery farms, with similar facilities, which included 44 pens each, with temperature, humidity and negative pressure ventilation control systems. Pigs stayed in the nursery for approximately 7 weeks and after that, they were transported to finishing farms. Each finishing farm was composed of 13 pens (9.5m x 4.85m) and 4 smaller pens used for housing sick or fall behind pigs. Finishing barns had natural ventilation and housed pigs for 15 weeks approximately. Farms belonged to a single production company and followed similar management protocols. All farms were located in Southern Brazil, in a region of high-density pig production. Biosecurity measures included changing clothes and boots provided by the farm before accessing the facilities. Only farm workers and members of the research team were allowed to visit the farms during the study. Nurseries and finishing farms used all-in/all-out pig production protocols and followed cleaning and disinfection programs and a downtime of 4 days before housing pigs for this study. Farms included in the study had confirmed diagnostic cases of IAV that caused respiratory disease and had no history of Influenza A vaccination. Pigs in these farms had a history of *Mycoplasma hyopneumoniae* infections but they were negative to porcine reproductive and respiratory syndrome virus.

*Study design*

A total of 1,800 piglets were enrolled in the study one day prior to weaning (20 days-old ±3) from 3 different sow farms (n=1100 from farm A, n=500 from farm B, n=200 from farm C). Piglets were randomly selected from 30, 20 and 9 litters from sow farms A, B and C, respectively. At weaning, piglets were ear tagged and transported to 3 separate nursery farms according to Figure 1. At arrival into the nursery, 600 pigs were placed in 10 pens of 60 pigs per pen in each nursery farm. Flow 1 received pigs from farm A only, while flow 2 received pigs from farms A and B and to form pen group, pigs from different sow farm source were allocated proportionally, with 30 pigs per pen from sow farm A and 30 pigs per pen from sow farm B. Flow 3 received pigs from farm A, B and C and each pen was formed with 20 pigs from each sow farm.
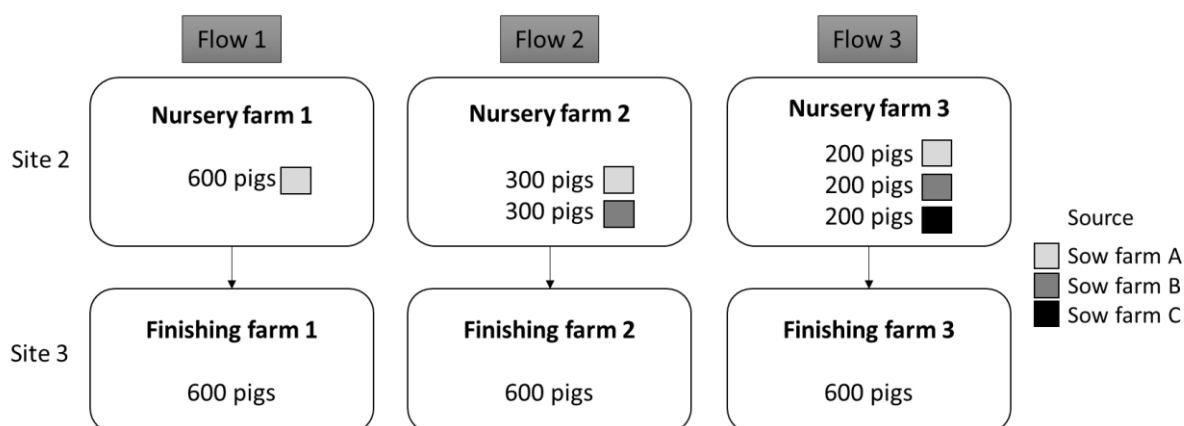
Figure 1. Diagram describing the pig flows for the study based on sow farm source and placement into the nurseries and finishing facilities.

Colored ear tagged was used to monitor and to ensure no moving between pens during the study, so pen integrity was kept from weaning to the end of the study. Out of these 600 pigs per flow, 30 pigs were randomly selected and identified for individual cohort sampling, to ensure that the same pig was sampled multiple times, as outlined below (Figure 2). At 70 ±3 days of age, pigs were moved to 3 finishing farms, following the same flow as nursery farms (Figure 1), regarding the same 10 groups of pigs in each farm.

The study was conducted between April and November of 2019 (fall-winter-spring).

*Management practices:*

Since it was an observational longitudinal study, all farms followed standard routine procedures, including vaccination for *Mycoplasma hyopneumoniae* and circovirus type 2 at weaning and re-vaccination 3 weeks later. Feed was provided *ad libitum* and pigs with clinical signs were evaluated and treated following standard practices. All intervention in pigs enrolled in the study were recorded and pigs that needed treatments to recover from illness, were moved to another dedicated pen for intensive care and were excluded from sampling and the study. No new pigs were enrolled in the study after pigs had been placed in the nursery and finishing facilities. Pigs were sent to slaughter under official veterinary inspection around 180 days of age.

*Sampling and diagnostic tests:*

Sampling size: To identify IAV at a group level, 10 groups of pigs were selected per flow (10 pens) to detect a prevalence of 25%. For individual sampling, 30 pigs were selected per flow under the assumption that there was a 10% prevalence. In both cases, the level of confidence was set at 95%.
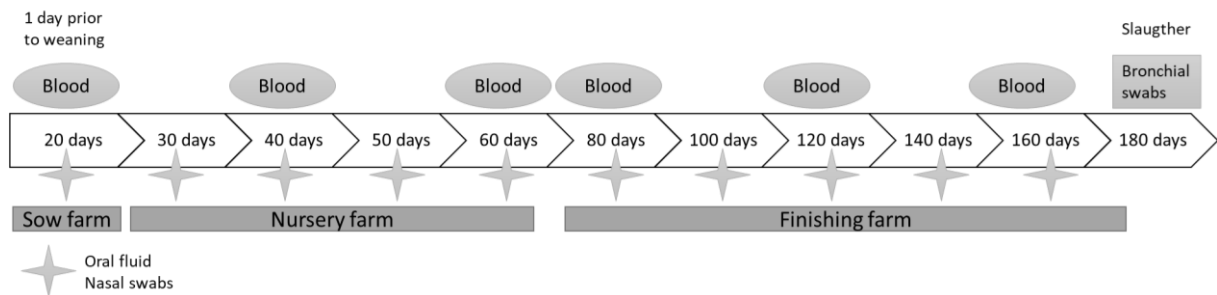


Figure 2. Timeline of sampling events indicating type of sample collected, age and location of the animals. Collection of oral fluid and nasal swab samples are indicated with stars.

Sample collection: to obtain IAV litter prevalence at weaning, one oral fluid was collected from each litter enrolled in the study (n=30 litter farm A, n=20 litter farm B, n=9 litter farm C). Additionally, one or two pigs enrolled in the study were sampled in each litter for nasal swabs. At nursery and finishing farms, in order to obtain IAV status of a group level, oral fluid was collected from 10 pens, in all sampling events (Figure 2). For individual samples, nasal swabs were collected from the same 30 pigs in each farm (n=90) in all sampling events. The same 90 pigs were bled at 20, 40, 60, 80, 120, 160 days of age (Figure 2). Oral fluids were collected using a cotton rope as previously described by Pricket et al (2008). Nasal swabs were collected from individual pigs using rayon swab (BBL CultureSwab™ Sterile, Single Swab), inserting the swab in both nostrils and rotating it smoothly. After sampling, swabs were placed in a 15 mL Polystyrene Conical Falcon Tube (Corning Science, Tamaulipas, Mexico) with 2 mL of transport medium (Brain Heart Infusion supplemented with antibiotics: Penicillin G 20.000 UI, Streptomicin 10.000µg, Gentamicin 2.000µg, Kanamicin Sulfate 1.300µg, Anfotericin B 1.300µg) and kept at -70°C until processed at the laboratory. Blood samples were collected in tubes (BD Vacutainer®) and were left at room temperature for serum separation. Serum was extracted in a new 2 mL microtube (INLAB; Diadema; SP; Brazil) and stored at -20°C until serological analysis. Bronchial swabs were collected from lungs at the slaughterhouse. Using a sterile scissors, the cardiac lobe was sectioned, and a rayon swab (BBL CultureSwab™ Sterile, Single Swab) was inserted in a large bronchus and harvested. Swabs

were placed in a 15 mL Polystyrene Conical Falcon Tube (Corning Science, Tamaulipas, Mexico) with 2 mL of transport medium (Brain Heart Infusion supplemented with antibiotics) as described above.

Assays: Nasal swabs, oral fluid and bronchial swabs were processed for viral RNA extraction using the magnetic particle processor procedure using the MagAtrackt® 96 Cador® Pathogen Kit (QIAGEN GmbH, Hilden, Germany) and tested by real time RT-PCR to detect the IAV matrix gene (Zhang & Harmon, 2014). Samples were tested individually and samples with cycle threshold (Ct) value <38 were considered positive and Ct≥38 were considered negative. Positive nasal swabs samples were subtyped by using a second real time RT-qPCR (Haach et al., 2019), and were further submitted to viral isolation (VI) in SPF embryonated chicken eggs (ECE) or in Madin-Darby canine kidney (MDCK) cells (Zhang & Gauger, 2014). The allantoic fluids collected from ECE and supernatants from MDCK cells were evaluated by hemagglutination (HA) test, and VI was confirmed by real time RT-PCR (Zhang & Harmon, 2014).

For sequencing, viral RNA was extracted from the isolated viruses and the eight gene segments were amplified by RT-PCR using primers forward 5´-CTGGATACGCCAGCRAAAGCAGG-3´ and reverse 5´-GACCTGATGCGGAGTAGAAACAAGG-3´. Reaction was run using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen™; Thermo Fisher Scientific®) following manufacture's guideline (PCR amplification of influenza A genomic segments for whole-genome sequencing, Ion Torrent sequencing application guide; Thermo Fisher Scientific®). DNA libraries were prepared and submitted for sequencing using Ion Torrent (Thermo Fisher Scientific®) system. Influenza genomes were assembled using Newbler v.2.9.

Sera were tested to detect antibodies using an IAV nucleoprotein-based ELISA (IDEXX Swine Influenza Virus Ab test; IDEXX Laboratories, Inc.) according to manufacturer's guidelines. Samples with results of S/N ratio ≥ 0.6 were considered negative and S/N ratio < 0.6 considered positive. The three lowest S/N values sera, from pigs of each evaluated phase (20, 40, 60, 80, 120 and 160 days old), were selected for hemagglutination inhibition (HI) assay, totalizing 54 samples from the three farms. The HI assay followed the protocol established by Kitikoon et al. (2014), using as reference viruses the IAVs isolated in this study or recently isolated from field cases of influenza in pigs in Brazil; H1N1 (1A.3.3.2), H1N2 (1B2.2) and

H3N2. HI antibody titers <40 were considered negative and titers ≥1:40 were considered positive.

Data analyses: The proportion of IAV positive samples and 95% confidence intervals were calculated over time based for both, RT-PCR of individual and group samples. Ct value distribution were displayed by boxplot. For ELISA results, S/N ratios were displayed by boxplot and the proportion of samples with IAV antibodies were tested using Kruskal Wallis to compare farms. P-value <0.05 was considered statistically significant. The proportion of IAV seroprevalence by ELISA was calculated for each sampling event.

## RESULTS

*IAV detection and subtyping*

A proportion of 38/59 (64.4%; IC 52.2-76.6%) of oral fluid and 16/90 (17.8%; IC 9.9-25.7%) of nasal swabs from piglets were RT-PCR positive for IAV at weaning. IAV prevalence varied among sow farms (Table 1).

Table 1. Number of influenza A virus positive oral fluid and nasal swab samples by RT-PCR ( ) and 95% C.I. [ ] collected from piglets at weaning (~ 20 days of age) by sow farm.

|  | Farm A | Farm B | Farm C |
|---|---|---|---|
| Oral fluid | 28/30 (93.3%) [84.4-100%] | 4/20 (20.0%) [2.46-37.5%] | 6/9 (66.7%) [54.3-79.0%] |
| Nasal swab | 14/56 (25.0%) [18.5-31.5%] | 2/24 (8.3%) [0-24.3%] | 0/10 (0%) [0-30.1%] |

IAV RT-PCR results from samples at weaning and throughout the nursery period are shown in Figure 3. More than 60% of the oral fluids at weaning tested positive and there was a first peak of infection at about ~ 20d to 30d of age and the second at about ~ 60 days of age (Figure 3a). Nasal swabs had the same pattern, with 2 waves of detection. IAV subtyping indicated presence of an H3N2 virus during the first peak at 20-30 days and a H1N2 virus during the second peak at 60 days old. The level of IAV in oral fluids and nasal swabs are shown by Ct value distribution in Figure 4.
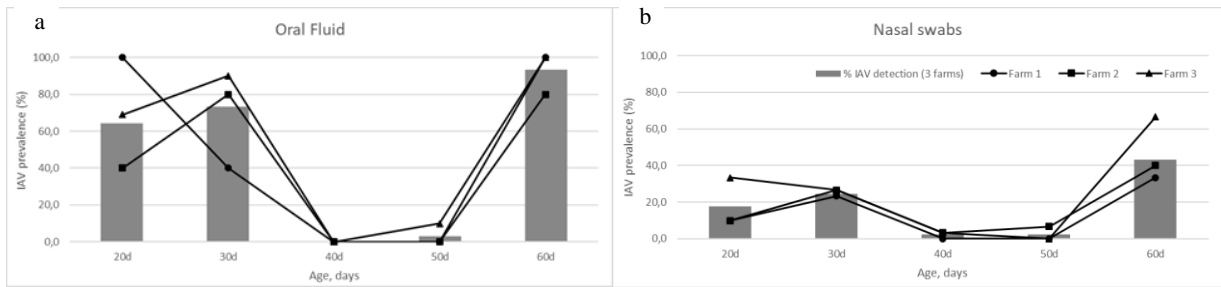
Figure 3. Influenza A virus prevalence in oral fluids (a) and nasal swabs (b) cohort at weaning and throughout the nursery period. Grey bars indicate prevalence (percentage of RT-PCR positives) of pigs from the 3 farms together, black lines represent prevalence of pigs by farm (circle Farm 1, square farm 2, triangle farm 3).
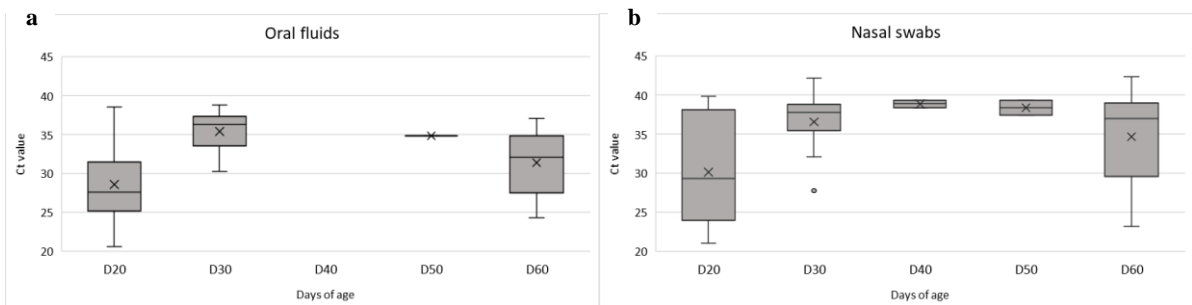


Figure 4. Cycle threshold (Ct) values distribution of RT-PCR for influenza A virus in oral fluids (a) and nasal swabs (b) at weaning and throughout the nursery period.

Results from finishing barns indicated that farms 1 and 2 had active infections while there was no detection of positive IAV samples in farm 3. On farm 1, there was a peak of infection of IAV at 120d of age, when 100% of oral fluids (10/10) and nasal swabs (30/30) resulted positive for IAV, with Ct mean of 29.3 and 31.6 for oral fluid and nasal swabs, respectively. Samples were subtyped as H1N1pdm09. Results from farm 2 showed a detection of 50% (5/10) at 160d only in oral fluids with Ct mean of 35.1., thus, it was not possible to obtain a subtype.

The number of times that IAV was detected the same pig ranged from none up to four times (Figure 5). There was no IAV detection by RT-PCR in 16.6% of the pigs across all the 10 sampling events. In flow 1, all pigs tested positive at least once with one pig testing positive 4 times. In flow 2, 66.7% of the pigs resulted positive for IAV at least once, while this proportion in flow 3 was 83.3% (Figure 5).

| Flow | Pigs | Site 1 | Site 2 | | | | Site 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 20d | 30d | 40d | 50d | 60d | 80d | 100d | 120d | 140d | 160d |
| Flow 1 | 1 | | | | | | | | ■ | | |
| | 2 | | | | | ■ | | | ■ | | |
| | 3 | | | | | | | | ■ | | |
| | 4 | | | | | ■ | | | ■ | | |
| | 5 | | | | | ■ | | | ■ | | |
| | 6 | | | | | | | | ■ | | |
| | 7 | ■ | | | | | | | ■ | | |
| | 8 | | | | | | | | ■ | | |
| | 9 | | | | | | | | ■ | | |
| | 10 | ■ | ■ | | | ■ | | | ■ | | |
| | 11 | | ■ | | | | | | ■ | | |
| | 12 | | | | | | | | ■ | | |
| | 13 | | | | | ■ | | | ■ | | |
| | 14 | | | | | | | | ■ | | |
| | 15 | | | | | | | | ■ | | |
| | 16 | | | | | | | | ■ | | |
| | 17 | | | | | | | | ■ | | |
| | 18 | | | | | | | | ■ | | |
| | 19 | | | | | | | | ■ | | |
| | 20 | | | | | ■ | | | ■ | | |
| | 21 | | ■ | | | | | | ■ | | |
| | 22 | | | | | | | | ■ | | |
| | 23 | | ■ | | | | | | ■ | | |
| | 24 | | ■ | | | | | | ■ | | |
| | 25 | | | | | | | | ■ | | |
| | 26 | | | | | | | | ■ | | |
| | 27 | | ■ | | | | | | ■ | | |
| | 28 | | | | | | | | ■ | | |
| | 29 | | | | | | | | ■ | | |
| | 30 | ■ | | | | | | | ■ | | |
| Flow 2 | 31 | | | | | ■ | | | | | |
| | 32 | | ■ | | | ■ | | | | | |
| | 33 | | | | | ■ | | | | | |
| | 34 | | | | | ■ | | | | | |
| | 35 | | ■ | | | ■ | | | | | |
| | 36 | | | | | ■ | | | | | |
| | 37 | | | | ■ | | | | | | |
| | 38 | | ■ | ■ | | ■ | | | | | |
| | 39 | | | | | | | | | | |
| | 40 | | | | | | | | | | |
| | 41 | ■ | | | | | | | | | |
| | 42 | | | | | ■ | | | | | |
| | 43 | | | | | ■ | | | | | |
| | 44 | ■ | | | | | | | | | |
| | 45 | ■ | ■ | | ■ | | | | | | |
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| | 47 | | | | | | | | | | |
| | 48 | | | | | | | | | | |
| | 49 | | ■ | | | ■ | | | | | |
| | 50 | | | | | ■ | | | | | |
| | 51 | | | | | ■ | | | | | |
| | 52 | | ■ | | | | | | | | |
| | 53 | | ■ | | | | | | | | |
| | 54 | | ■ | | | | | | | | |
| | 55 | | | | | | | | | | |
| | 56 | | | | | | | | | | |
| | 57 | | | | | | | | | | |
| | 58 | | | | | ■ | | | | | |
| | 59 | | | | | ■ | | | | | |
| | 60 | | | | | | | | | | |
| Flow 3 | 61 | | | | | | | | | | |
| | 62 | | | | | | | | | | |
| | 63 | | | | | ■ | | | | | |
| | 64 | | | | | | | | | | |
| | 65 | | ■ | | | ■ | | | | | |
| | 66 | | | | | ■ | | | | | |
| | 67 | | | | | ■ | | | | | |
| | 68 | | | | | ■ | | | | | |
| | 69 | | | | | | | | | | |
| | 70 | | | | | ■ | | | | | |
| | 71 | ■ | | | | ■ | | | | | |
| | 72 | ■ | | | | ■ | | | | | |
| | 73 | | | | | ■ | | | | | |
| | 74 | ■ | | ■ | | ■ | | | | | |
| | 75 | | ■ | | | ■ | | | | | |
| | 76 | | | | | ■ | | | | | |
| | 77 | ■ | | | | ■ | | | | | |
| | 78 | ■ | | | | ■ | | | | | |
| | 79 | ■ | | | | | | | | | |
| | 80 | ■ | | | | | | | | | |
| | 81 | ■ | | | | ■ | | | | | |
| | 82 | ■ | | | | ■ | | | | | |
| | 83 | | ■ | | | ■ | | | | | |
| | 84 | ■ | | | | ■ | | | | | |
| | 85 | | ■ | | | ■ | | | | | |
| | 86 | | | | | | | | | | |
| | 87 | | ■ | | | | | | | | |
| | 88 | | ■ | | | | | | | | |
| | 89 | | | | | | | | | | |
| | 90 | | | | | ■ | | | | | |

Figure 5. Detection of influenza A virus in nasal swabs from pigs at weaning and throughout the nursery and finishing period. Flow 1 (pig 1-30), flow 2 (pigs 31-60) and flows 3 (pig 61-90). Black square [ ■ ]: positive result; white square [ □ ]: negative result.

All bronchial swabs sampled at slaughter were negative for IAV by RT-PCR.

*IAV sequencing*

Influenza virus subtype H1N2 isolated from nasal swab in flow 3 at 60 days-old was classified as 1.B.2.2 for hemagglutinin H1 (Figure 6a) following the universal classification (Anderson et al., 2016). The neuraminidase segment (N2) is demonstrated on Figure 6b. Virus H3N2 isolated from nasal swab in flow 2 at 20 days of age is demonstrated in Figure 6c and is similar to other H3N2 Brazilian viruses isolated in the 1990's.

*Serology*

Seroprevalence by ELISA resulted in 82.2% [95% C.I.; 74.3-90.1%] of positives in the piglets at weaning. There was no statistical difference (p=0.2; Kruskal-Wallis test) in the geometric mean titer among piglets housed in flows 1, 2 and 3 (Figure 7).

Seroprevalence at 40 days old decreased in all 3 nursery farms (Figure 8). Pigs from flow 3 presented a slight increase at 60 days of age and later on the sampling event at 80 days old, but a decrease of seroprevalence during the rest of the finishing period (Figure 8). In contrast, flows 1 and 2 showed a stability or decrease of the seroprevalence during the nursery period, but a significant increase in the prevalence of IAV antibodies during the finishing period at 80d-160d (Figure 8).

a.



b.

c.
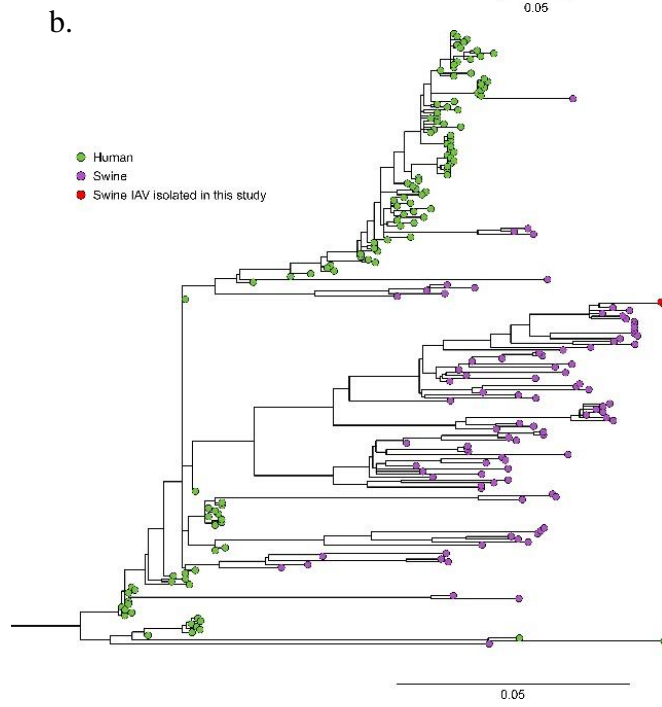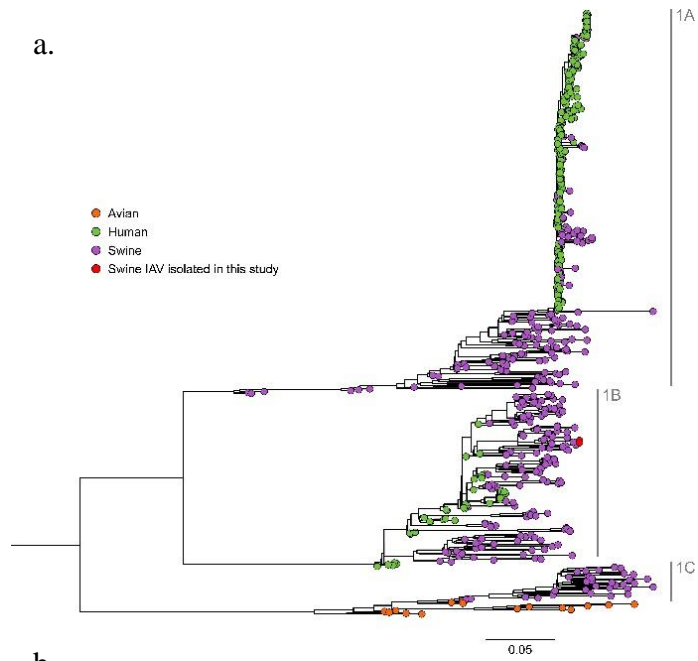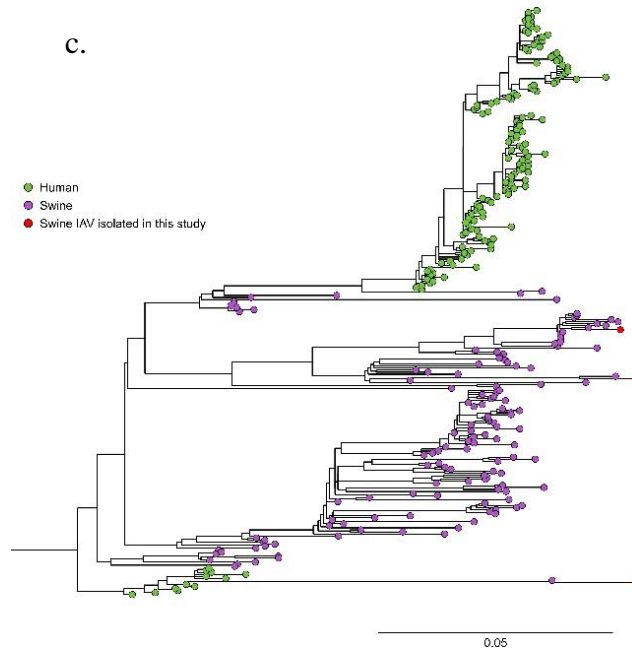


Figure 6. Phylogenetic tree based on nucleotide sequence of the hemagglutinin (6.a.) and neuraminidase (6.b.) gene of H1N2 viruses detected in this study and other viruses sequence representing cluster from 1A, 1B and 1C. Figure 6c represents phylogenetic tree based on nucleotide sequence of the hemagglutinin gene of H3N2.
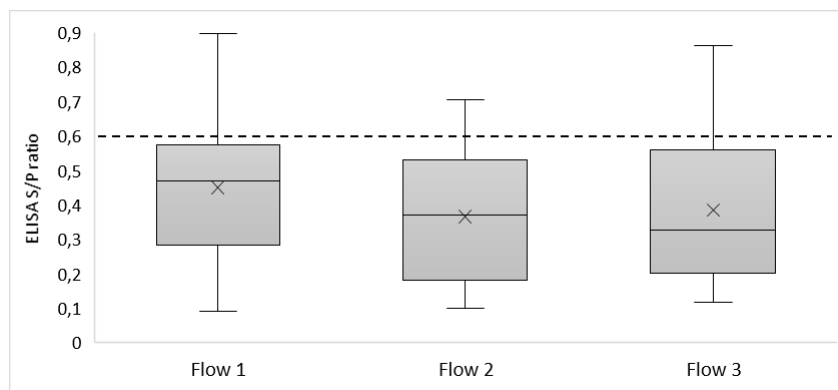


Figure 7. Distribution of ELISA S/N ratio in piglets sampled at 20 days old by pig flow. S/N ratio>0.6: negative result; S/N ratio<0.6: positive result. Boxplots contain the values of minimum, first quartile, median, third quartile and maximum S/P values.
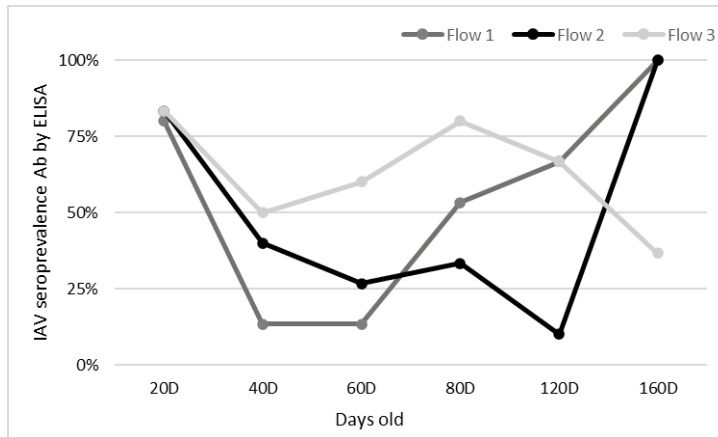
Figure 8. Influenza A virus seroprevalence by ELISA by pig flow from weaning to slaughter.

Two viruses isolated in this study were used as antigen for HI assays, a H1N2 [A/swine/Brazil/350-19-10/2019 Genbank accession number: MW772715 (HA), MW772717 (NA)] isolated from a nasal swab (pig n. 68) of a pig from flow 3 at 60 days of age and a H3N2 [A/swine/Brazil/350-19-70/2019 Genbank accession number: MW772937 (HA)] isolated from a nasal swab at 20 days old (pig n. 44) from flow 2. For the H1N2 [1B.2.2.] virus isolated in this study, there was no positive samples on flow 1 (Figure 9) and only one (1/3) positive serum in flow 2 at 60 days, while in flow 3 where this sample was isolated, almost all samples (11/12) tested positive after 60 days of age and titers ranged between 1:40 and 1:360 (Figure 9). Antibodies against the H3N2 virus were detected in the 3 flows studied (Figure 9). In flow 1, antibodies were detected after 60 days of age, while in flow 2, pigs in all sampling events tested positive. Lastly, sera from flow 3 were positive for IAV antibodies starting at 40 days of age and until 120 days-old (Figure 9). Other 5 influenza virus isolated in 2010 and 2018 were tested as antigen for the sera collected for this study, 3 H1N2 (H1N2 A/swine/Brazil/281-18-1/2018 [other human 1B.2.]; H1N2 A/swine/Brazil/276-18/2018 [1B.2.2.]; H1N2 A/swine/Brazil/091-18-14/2018 [1B.2.2.]) and 2 H1N1 (H1N1 A/swine/Brazil/062-18-4/2018 [1A.3.3.2]; H1N1 A/swine/Brazil/107-10-3a/2010 [1A.3.3.2]) and results are shown on Figure 9.
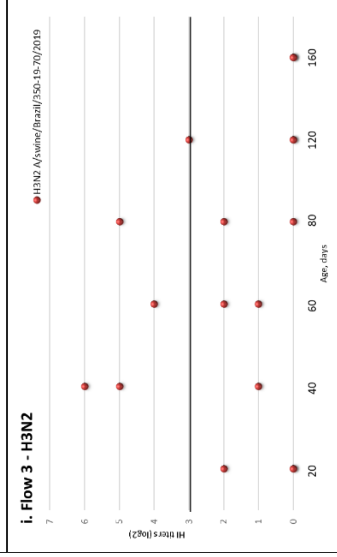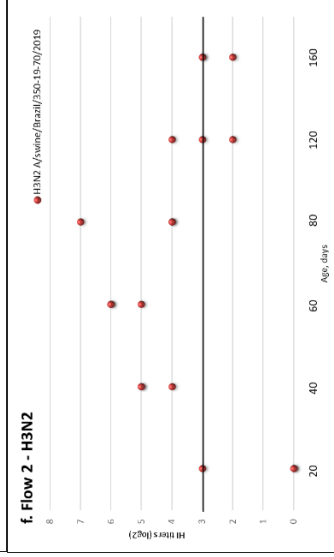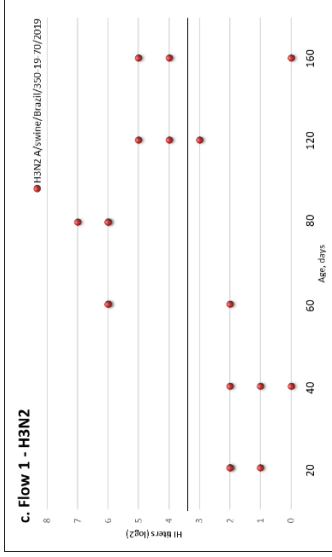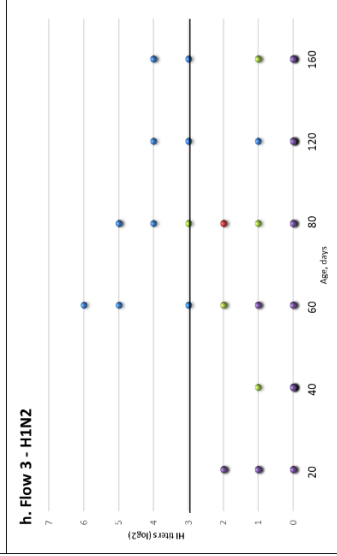
a. Flow 1 - H1N1
b. Flow 1 - H1N2
c. Flow 1 - H3N2
d. Flow 2 - H1N1
e. Flow 2 - H1N2
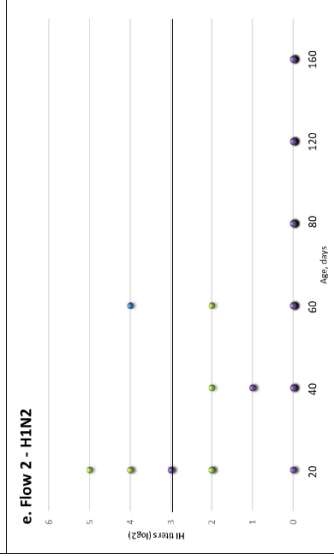f. Flow 2 - H3N2
g. Flow 3 - H1N1
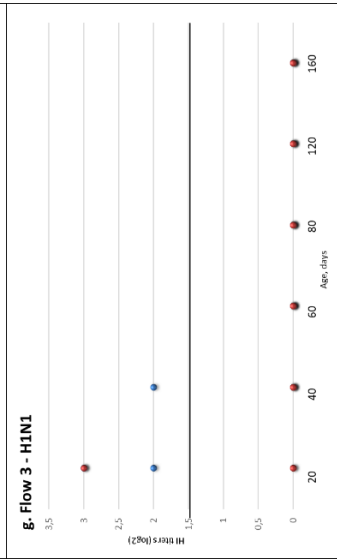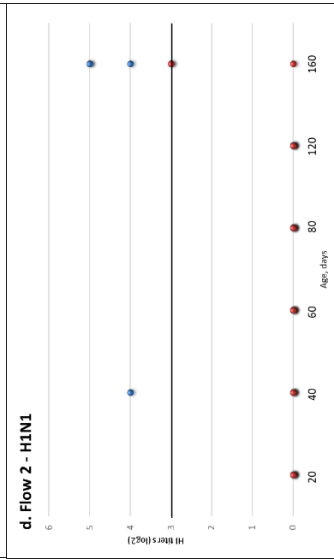h. Flow 3 - H1N2
i. Flow 3 - H3N2
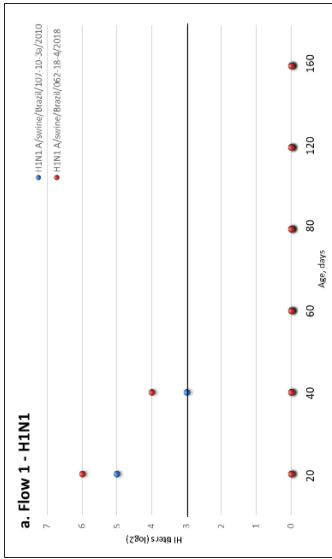
Figure 9. Distribution of samples with hemagglutination inhibition (HI) titers against [H1N1 A/swine/Brazil/107-10-3a/2010, H1N1 A/swine/Brazil/062-18-4/2018 (Figures 6a, 6d, 6g); H1N2 [A/swine/Brazil/350-19-10/2019 Genbank accession number: MW772715 (HA), MW772717 (NA)], H1N2 A/swine/Brazil/281-18-1/2018 , H1N2 A/swine/Brazil/091-18-14/2018, H1N2 A/swine/Brazil/276-18/2018 (Figures 6b, 6e, 6h); H3N2 [A/swine/Brazil/350-19-70/2019 Genbank accession number: MW772937 (HA)] (Figures 6c, 6f, 6i) in flows 1, 2 and 3. Titers are presented in log2, starting at 1:10 and titers ≥1:40 were considered positive.

## DISCUSSION

Understanding disease behavior is essential for control efforts, particularly with pathogens that can interact or associate with others. In this study, we assessed the infection dynamics of IAV from weaning to slaughter age and we observed more than two waves of shedding in at least 2 out of the 3 flows studied. We also demonstrated that pigs were challenged with different genotypes of IAV during the study, which supports high genetic variability in pig farms.

The IAV prevalence in groups of piglets at weaning varied from 40% to 100% among sow-farms. The IAV status of the piglet at weaning is relevant, since this could shape how IAV can spread to subsequent phases (Diaz et al., 2015). Thereby, it is also important to consider the piglet status of the different sow-farms before mixing different sources of piglets to compose a new batch at the nursery.

In our study, the waves of IAV detected during nursing were similar to those found in other studies developed in USA (Allerson et al., 2014; Diaz et al., 2015) and Spain (Simon-Grifé et al., 2012), although we observed fewer detection than a Canadian study (Ferreira et al., 2017). We identified two waves, the first about 20-30 days of age and a second one around 60 days of age by RT-PCR and results from serology by ELISA support those two waves. In flows 2 and 3 where 40% and 69% of the oral fluids were positive at 20 days of age, respectively, the prevalence at 30 days increased probably due to the negative subpopulation that was contaminated during the movement and mixing to nurseries. Whereas farm 1 had 100% of oral fluid positive at weaning, there was a decrease in the IAV positive results 10 days later, probably due to the cycle of the disease. The second wave detected at 60 days of age was very similar within the 3 flows, although same virus was not present in the 3 nurseries, as demonstrated by HI results. In flow 3 we detected a H1N2 1B.2.2., while sera from flows 1 and

2 showed no reaction to this same virus. We can assume that the source of the IAV that caused the second wave was not the same for the 3 farms, although it seems there was a pattern on the IAV dynamics during nursing phase.

The IAV dynamics varied among farms during the finishing period. In finishing farm 1 there was an outbreak of a pandemic H1N1 at 120 days of age and we hypothesized there was a human-to-swine transmission. We were not able to confirm this because it was not possible to isolate and sequence the virus. In the finishing farm 2 there was a detection of a virus infection at 160 days of age, although it was not possible to subtype the virus because the Ct was too high, due the fact that sampling event missed the acute phase of the disease. Results from HI suggest that it was caused by a virus antigenically similar to a pandemic H1N1 (1A.3.3.2.) virus isolated in 2010. Different biosecurity measures adopted have been already described as potential risk for influenza transmission (Allerson et al., 2013). We can suppose that different biosecurity events occurred among the 3 farms studied, which contributed for the IAV status during the finishing period.

We demonstrated that the same pig shed IAV up to 4 times, while 16% of the pigs we could not detect any shedding. It is important to note that the sampling was periodically and we could miss some shedding due to the short period of the IAV cycle, particularly at finishing phase where the gap was 20 days between sampling events. Nevertheless, the serology by ELISA corroborated with the PCR detections, demonstrating there was no IAV detection missing, at least on oral fluid. Also, oral fluid detected more positive IAV samples than nasal swabs. The likelihood of detecting positive pigs for IAV at a group level is higher than individual samples (Garrido-Mantilla et al., 2020).

Seroprevalence by ELISA was high since the first sampling event where pigs were ~20 days-old (Figure 8), which could indicate the circulation of the virus either by natural infection or maternal immunity. There were antigenically similar antibodies to H1N1 virus isolated in 2010 and to another isolated in 2018 in flows 1 and 3 and antigenically similar to H1N2 isolated in 2018 in flow 2. In flow 3, at 60 days old, there was a challenge with other H1N2 strain, that was isolated in this study (1.B.2.2). The serological profile against H3N2 virus was very similar in all 3 flows, starting a seroconversion around 40-60 days that increased by 60-80 days and then decreased over time. This could be explained by the source of the piglets, that had at least one farm source in common. Seroprevalence by ELISA during finishing period from flow 1 and 2 corroborates with the waves of infection detected by PCR. Also, in flow 3 the prevalence

decreases overtime after 80 days-old, confirming there was no IAV challenge or natural infection during finishing period.

In conclusion, this study demonstrated that influenza can circulate from weaning to market age and there is high virus genetic variability within and among farms. We also showed that the same pig was detected positive by RT-PCR four times since weaning until market age, demonstrating that a pig can be susceptible, even in the presence of antibodies. As reported before, in this study the likelihood to detect positive samples for IAV was higher at a group level than individual samples. Studies like this can help veterinarians and producers to understand the IAV infection dynamics, which is often circulating in all phases of production, and set practices to reduce the risks involved with IAV.

*Ethics approval and consent to participate*

This study was approved by the Animal Care and Ethic Use Committee from the Federal University of Rio Grande do Sul (UFRGS) under protocol number 37543. The company that owned the animals provided written consent to collect samples from pigs during the study, from weaning until the slaughter.

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**4. Capítulo III – Segundo artigo científico**

**Uso de desinfetantes para reduzir transmissão do vírus Influenza A em fêmeas ao desmame**

Artigo a ser submetido para a Porcine Health and Management

Persistence of Influenza A virus on the udder skin of sows over time and a disinfection protocol to reduce surface viral load

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## ABSTRACT

The aim of this study was to assess how long the Influenza A virus (IAV) can persist on the udder skin of sows after weaning and to develop a method using disinfectant to reduce the viral load of IAV on the udder skin of sows. In the first study, 30 sows were enrolled after weaning and were distributed in 3 groups. Udder skin wipes were collected once after weaning on sows from group one, 30 minutes after weaning on sows from group two, and in both sampling events on sows from group 3. In study two, 30 lactating sows were enrolled and were sampled at weaning, 3 hours and 48 hours after weaning. In study three, 78 sows were enrolled and distributed in three groups. In group 1, after the litter was weaned-off, disinfectant was sprayed on the udder skin surface. In group 2, a similar procedure was followed using only water and no intervention was applied to group 3. Udder skin of sows were sampled before and 30 minutes after each procedure. All samples from study one, two and three were run for IAV detection. There was no difference between the first and second sampling on udders skin of sows in study one. In study two, the prevalence on udder skin of sows at weaning, 3h and 48 hours after weaning were 80.0%, 90.0% and 46.7%, respectively. In study 3 the proportion of positive samples on the udder skin decreased from 52.0% to 24.0%, in group 1 (p=0.023). There was no difference in the prevalence from group 2 and 3. Our study confirms that sows at weaning can be a source of IAV and also indicate that topical disinfectants can help to reduce the prevalence of sows with contaminated udder skin.

**KEY WORDS:** Influenza, persistence, disinfectant, udder skin, pig

## BACKGROUND

Piglets are susceptible to influenza A virus (IAV) and are the main subpopulation responsible for maintaining IAV in sow farms[1]. The sow can transmit influenza to the piglets by shedding or contaminated udder skin, in the moment piglets are suckling[2]. Contaminated

udder skin is a very important route of transmission since it may spread rapidly due to the piglet behavior. To the author's knowledge there is no information about how long the virus can persist on contaminated udder skin of the sow.

There are some practices adopted in order to improve the piglet viability as the use of nurse sow. This practice consists in a lactating sow after weaning-off the piglets, to adopt new piglets and it is often implemented to ensure suitable milk intake of piglets. However, the use of nurse sow has been implicated in the transmission of IAV among litters prior to weaning[3].

The aim was to assess how long the IAV can persist on the udder skin of sows after weaning-off the piglets and also to develop a method using disinfectant to reduce the viral load of IAV on the udder skin of sows at weaning. Also, a pilot study (study one) was developed before, in order to assess if one sampling on the udder skin of sows could impact with the subsequent samplings.

## RESULTS

*Study one*

The proportion of positive udder skin wipes samples are demonstrated in Table 1.

Table 1 – Prevalence and mean CT values of IAV tested by RT-PCR on udder skin of weaned sows

| Group | 1st sampling event (0 hour) | | 2nd sampling event (0h30m) | |
| --- | --- | --- | --- | --- |
| | Pos/Total (%) | Mean Ct* | Pos/Total (%) | Mean Ct* |
| 1 | 7/10 (70%) | 32.64 | NS | - |
| 2 | NS | - | 4/9 (44%) | 34.76 |
| 3 | 9/10 (90%) | 31.80 | 10/10 (100%) | 31.88 |

NS- not sampled. *Mean Ct does not include negative samples

All 30 sow's nasal swabs were negative.

*Study two:*

Overall, only 2 sows were negative on udder skin wipe for IAV by RT-PCR on the 3 sampling events. At weaning, 24 of the 30 (80.0%) samples were positive, while the proportion of positive samples was 90.0% and 46.7% 3 and 48 hours after weaning, respectively. The levels of IAV on udder skin over time are demonstrate on Figure 1. Results from nasal swabs detected only one positive sample sow (3,3%) at 0 hour.
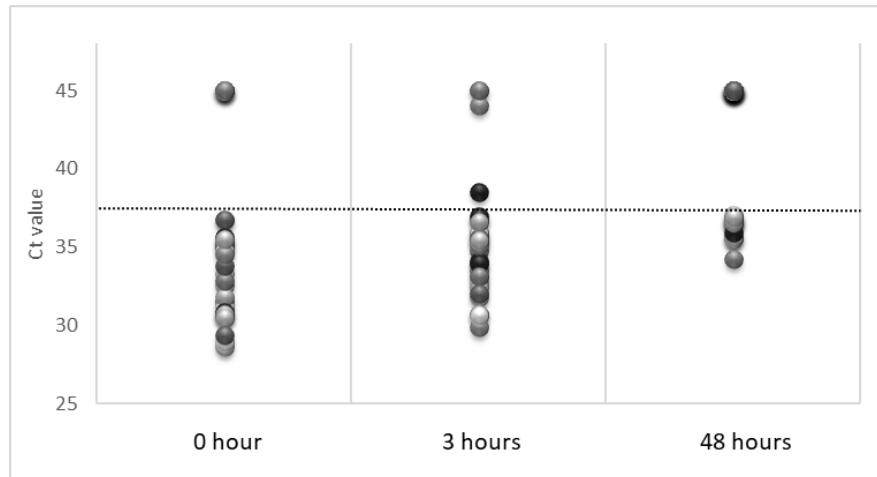
Figure 1. Distribution of Ct values for IAV on udder skin wipes by RT-PCR at 0, 3 and 48 hours after weaning.

*Study three*

The proportion of positive samples before and after each treatment are demonstrated on Figure 2. The proportion of IAV positive samples on the udder skin of the Group 1 decreased from 52.0% to 24.0%, after the skin was treated with disinfectant (p=0.023). There was no difference in the proportion of positive samples from group 2 and 3, before and after treatments (p>0.05; Figure 2).
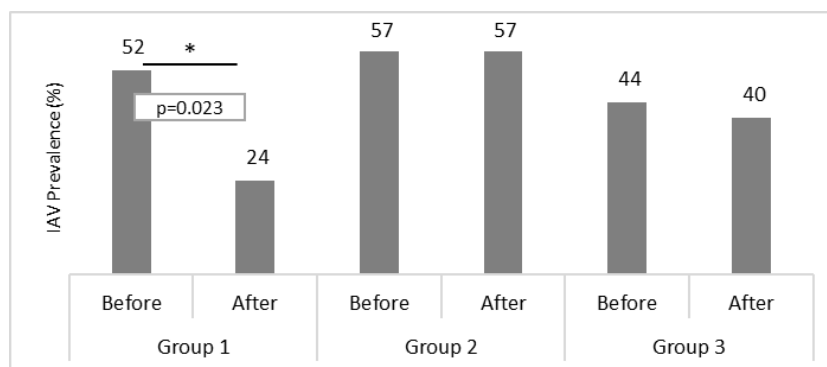


Figure 2. Prevalence of IAV on the udder skin of sows before and after treatment by group.

Ct values were higher after treating with disinfectant (p=0.001) indicating that the application of the disinfectant decreased the IAV load on the udder skin (Figure 3). Groups 2 and 3 had no difference in the Ct values before and after each treatment (Figure 3).
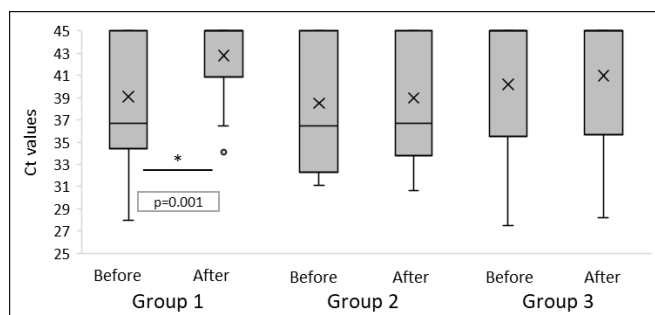
Figure 3. Cycle threshold (Ct) value distribution of IAV on the udder skin of sows before and after treatment by group.

Proportion of positive samples for nasal wipes were 16.0%, 39,1% and 16.0% on treatments 1, 2 and 3 respectively.

Five positive samples with Ct below 35 of each treatment were submitted to viral isolation and IAV was isolated from 4 samples, including one from groups 1 and 3 before treatment and one each from groups 2 and 3 after treatment.

**DISCUSSION**

In study one, the proportion of positive samples for IAV was high, even without sows shedding at weaning. There was no difference on the proportion of positive samples and levels of IAV, represented by Ct values, when we compare the first and second sampling on udder skin wipes. This data shows that the results of the second sampling is not affected by the first sampling.

The prevalence of IAV on udder skin wipes at weaning and 3 hours later was high in study two. Information about IAV persistence is important, particularly on farms that use nurse sows. For a practical perspective, the movement of the nurse sows usually is performed less than 3 hours after weaning-off the piglets, which represents a risk for IAV spreading between litters. Forty-eight hours after weaning, the proportion of positive udder skin wipes decreased significantly, demonstrating the reducing over time. It is unknow how this route of transmission could impact in the spreading to other sow, but this should be considered since sows are moved to the breeding barn after weaning-off piglets, and could be in contact with other sows, particularly with gilts.

On study three, the disinfection procedure decreased the levels of IAV, demonstrating that the virus is susceptible to the active ingredient and the protocol used in the study. Spraying only water had no effect to decrease IAV, demonstrating the mechanic procedure is not enough

to reduce IAV. Also, the control group demonstrated that 30 minutes is not relevant to reduce IAV, corroborating with results from study 2.

## CONCLUSIONS

Our study confirms prior findings that sows at weaning can be a source of IAV and also indicate that topical disinfectants can help to reduce the prevalence of sows with contaminated udder skin. To the knowledge of the authors, this is the first study that reports the use of a disinfection procedure to decrease the detection of IAV on the udder skin of sows. Additional studies are required to validate protocols and to evaluate whether such interventions will decrease transmission of IAV to piglets upon adoption.

## METHODS

The studies were run on a 3,000-sow farrow to wean farm located in Midwest, US.

*Study one*

Thirty sows were randomly enrolled after weaned-off piglets and were distributed in 3 groups. Udder skin wipes were collected in different times in order to detect if udder skin was contaminated by IAV. Sows from group 1 were sampled once right after weaning (hour 0). Sows from group 2 were sampled 30 minutes after weaning (hour 0h30m) and sows from group 3 were sampled on the 2 sampling events, hour 0 and 30 minutes after weaning. Udder skin wipes were collected as previously described (Garrido-Mantilla et al., 2019) using a $3 \times 3$ inches sterile gauze with 8 ml of DMEM-Dulbecco's Modified Eagle Medium Gibco™ (Grand Island, NY, USA) supplemented with 4% of 7.5% bovine serum albumin, fraction V, and 1X antibiotics Anti-Anti (100x) Gibco™ (Grand Island, NY, USA). Samples were kept under refrigeration and were sent to the laboratory.

*Study two*

In order to assess the persistence of IAV on udder skin, 30 lactating sows were enrolled randomly and udder skin wipes were collected multiple times. The population was considered the weekly group of weaned sows, under the assumption of 65% estimated prevalence, 7% accepted error and 95% confidence level. After the piglets were weaned-off, sows were sampled at weaning, 3 hours and 48 hours after weaning. Udder skin wipes were collected using a $3 \times 3$ inches sterile gauze with 8 ml of transport media as described above. Nasal swabs were

collected using a rayon-tipped swab applicators with Stuart's medium (BBL CultureSwab™ liquid, Stuart single plastic applicator; Becton, Dickinson and Com. Sparks, MD, USA) at weaning and 48 hours later, to detect if sows were shedding IAV during the study. Samples were kept under refrigeration and were sent to the laboratory.

*Study three*

To develop a disinfection protocol, 73 sows were enrolled randomly at weaning and distributed in three groups (Table 2).

Table 2. Distribution of sows on groups

| Group | n | Name | Intervention |
|---|---|---|---|
| 1 | 25 | Treatment 1 | Disinfectant |
| 2 | 23 | Treatment 2 | Water |
| 3 | 25 | Control | No intervention |

After the litter was weaned-off, udder skin wipes from all sows were sampled using a 3 × 3 inches sterile gauze with 8 ml of DMEM-Dulbecco's Modified Eagle Medium Gibco™ (Grand Island, NY, USA) as previously described. Nasal wipes were collected using a 3 × 3 inches sterile gauze with 8 ml of DMEM-Dulbecco's Modified Eagle Medium Gibco™ (Grand Island, NY, USA) as previously described (Garrido-Mantilla et al., 2019), wiping the exterior of the snout of the pigs. After the first sampling, each group of sows received a different procedure (Table 2). In Group 1 (n=25), a quaternary ammonia-based disinfectant (AgForte Pro; Atmosphere Global LLC, Chicago, IL) was sprayed on the udder skin surface for 20 seconds, covering the underline of the sow. After 30 minutes of contact time between disinfectant and udder skin, a second udder skin wipe was collected using a 3 × 3 inches sterile gauze with 4 ml of DMEM-Dulbecco's Modified Eagle Medium Gibco™ (Grand Island, NY, USA) supplemented with 4% of 7.5% bovine serum albumin, fraction V, and 1X antibiotics Anti-Anti (100x) Gibco™ (Grand Island, NY, USA) and 4ml of D/E Neutralizing Broth (Difco™). In Group 2 (n=23), a similar procedure was followed using only water instead of the disinfectant. Lastly, no intervention was applied to Group 3 which served as the untreated control (n=25). For group 2 and 3, sampling was performed using a 3 × 3 inches sterile gauze with 8 ml of DMEM-Dulbecco's Modified Eagle Medium Gibco™ (Grand Island, NY, USA) supplemented with 4% of 7.5% bovine serum albumin, fraction V, and 1X antibiotics Anti-Anti (100x) Gibco™ (Grand Island, NY, USA), 30 minutes after spraying water in sows of group 2 and 30 minutes after the first sampling event on sows from group 3. After each treatment and

sampling the udder skin, nasal wipes were collected from all sows enrolled in the study in order to assess if they were shedding IAV during the study. Samples were sent to the lab and were kept under refrigeration until testing.

*Laboratory assays*

All samples were run individually and processed for RNA extraction using the Ambion® MagMAX™AM1835 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) and tested by RT-PCR to detect the IAV matrix gene[4]. Samples with cycle threshold (Ct) <37.5 were considered positive. Positive samples were cultured for virus isolation on MDCK cells.

*Data analyses*

In study one and two, the proportion of positive samples were calculated. In study three, the number of positive samples were analyzed using McNemar test before and after each treatment. Differences in Ct values were analyzed using the Wilcoxon signed rank test before and after each treatment. P-value greater than 0.05 were considered significant.

*Ethics approval and consent to participate*

This study was approved by the University of Minnesota IACUC, under the protocol 1705-34808A. The company responsible for the animals consented to run the studies and collect samples from pigs

*Competing interests*

The authors declare no conflict of interests.

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**5. Capítulo IV – Terceiro artigo científico**

**Efficacy of disinfectants to inactivate H1N1 influenza A virus isolated from pigs**

Artigo aceito para publicação na Pesquisa Veterinária Brasileira (formato conforme normas da revista)

Efficacy of disinfectants to inactivate H1N1 influenza A virus isolated from pigs[1]

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**ABSTRACT**

Lara A.C., Fernando F.S., Takeuti K.L., Bortolozzo F.P., de Barcellos D.E.S.N. 2021. **Efficacy of disinfectants to inactivate H1N1 influenza A virus isolated from pigs.** *Pesquisa Veterinária Brasileira, 2022.* Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Bairro Agronomia, Porto Alegre, RS 91540-000, Brazil. E-mail: davidbarcellos@terra.com.br

The aim of this study was to access the efficacy of four disinfectants to inactivate influenza A[H1N1] 0hour and 72hours after disinfectant dilution. A pandemic H1N1 influenza virus isolated from a pig with respiratory disease was used to obtain inoculums containing $6.4\log_{10}$ $EID_{50}$/mL; $5.4\log_{10}$ $EID_{50}$/mL; $4.4\log_{10}$ $EID_{50}$/mL and $3.4\log_{10}$ $EID_{50}$/mL. Suspension test was composed of 400µL of viral inoculum, 100µL of organic load and 500µL of each individually diluted disinfectant and incubated for ten minutes of contact time. After a neutralizing step, each mixture was filtered on a 0.22µm membrane and 0.2mL was inoculated in six 9-day-old embryo chicken egg through allantoic route. The allantoic fluid from eggs was harvest for RT-PCR and hemagglutination test. The experiment was repeated 72hours after disinfectant dilution. On the first assessment with fresh disinfectant, influenza virus was inactivated by oxidizing compost disinfectant and phenolic disinfectant in all virus concentrations, the quaternary ammonium compound (QAC) and glutaraldehyde association inactivated the virus up to a concentration of $5.4\log_{10}$ $EID_{50}$/mL. QAC disinfectant did not eliminate virus viability. Seventy-two hours after disinfectants were diluted, oxidizing compost disinfectant and QAC and glutaraldehyde association disinfectant demonstrated the same result as the evaluation with fresh disinfectant solution. Phenolic disinfectant inactivated viral inoculum up to a concentration of $5,4\log_{10}$ $EID_{50}$/mL. QAC had no effect on inactivating $3,4\log_{10}$ $EID_{50}$/mL of influenza virus. In conclusion, three of the four disinfectants tested were effective to inactivate pandemic H1N1 influenza virus in the presence of organic load. Test result performed 72hours after disinfectant dilution suggest a decrease in the effectiveness of one disinfectant.

**RESUMO.- [Eficácia de desinfetantes para inativar o vírus da influenza A H1N1 isolado de suínos.]** O objetivo deste trabalho foi avaliar a eficácia de quatro desinfetantes em inativar o vírus da influenza A [H1N1] 0-hora e 72-horas após a diluição dos produtos. Um vírus H1N1 pandêmico isolado previamente de um suíno com doença respiratória foi utilizado e foram obtidas quatro concentrações de inóculo contendo $6.4\log_{10}$ $EID_{50}$/mL; $5.4\log_{10}$ $EID_{50}$/mL; $4.4\log_{10}$ $EID_{50}$/mL and $3.4\log_{10}$ $EID_{50}$/mL. Para compor o teste em suspensão foram adicionados 400µL de inóculo viral, 100µL de matéria orgânica e 500µL de cada desinfetante diluído individualmente e a mesma foi incubada por 10 minutos. Após a etapa neutralizante, a suspensão foi filtrada em membrana 0.22µm e 0.2mL foi inoculado em seis ovos de galinha embrionados de 9 dias de incubação, via rota alantoide. O fluido alantoide foi colhido após 72 horas para testes de hemaglutinação e RT-PCR. O mesmo protocolo experimental foi repetido usando as soluções desinfetantes 72 horas após a diluição. O vírus da influenza foi inativado pelo composto oxidante e também pelo desinfetante fenólico em todas as concentrações virais testadas na hora 0 após diluição. O desinfetante com associação de amônia quaternária e glutaraldeído inativou o vírus na concentração de até $5.4\log_{10}$ $EID_{50}$/mL. O desinfetante a base de amônia quaternária não inativou o vírus. Os resultados 72 horas após a diluição não diferiram quando comparado com a hora 0, exceto o desinfetante fenólico, o qual inativou o vírus da influenza somente até a concentração $5,4\log_{10}$ $EID_{50}$/mL. Concluindo, três dos quatro desinfetantes testados foram efetivos ao inativar o vírus da influenza [H1N1] pandêmico na presença de matéria orgânica. Os resultados do teste com produtos diluídos após 72 horas sugerem redução da efetividade em pelo menos um desinfetante.

**INTRODUCTION**

Influenza A virus (IAV) is endemic in swine herds (Torremorel et al., 2012), causing health and economic losses and is an important pathogen involved in porcine respiratory disease complex (Rech et al., 2018). IAV is a zoonotic pathogen and its transmission among species is relevant to public health (Nelson & Vincent, 2015; Anderson et al., 2020). Some measures must be considered to control IAV in pigs, as increasing biosecurity, adopting practices to prevent the introduction of new strains in the herd by pigs or people, implementing all-in all-out system and following disinfection protocols. Influenza virus can persist in some surfaces as wipes, plastic and stainless steel (Bean et al., 1982, Perry et al., 2016), and in the presence of organic load and mucus, IAV can increase the viability period (Hauck et al., 2017, Hirose et al., 2017). Moreover, Neira et al. (2016) detected significant levels of IAV on barn surfaces and air during an outbreak of influenza in pigs. Even though the most common route of influenza transmission within pigs is direct contact with shedding individuals, indirect transmission by fomites has also been demonstrated (Allerson et al., 2013). Thus, disinfection procedures can reduce the contamination of surface facilities, equipment and fomites, reducing the risk of the pathogen spread.

Chemical disinfection is widely used in pig farms and there are several classes of disinfectants available including aldehydes, oxidizing agents, phenols and ammonia compounds (Dvorak, 2008). The action mechanism varies regarding each class of disinfectant, but the main mechanisms consist on viral protein or lipids denaturation, nucleic acid disruption and/or damage and changes on membranes (Dvorak, 2008; Prince & Prince, 2001). However, some other factors can influence the effectiveness of the disinfectant such as dose, time of contact, surface composition and characteristics, organic load, temperature and pH (De Benedictis et al., 2007).

In vitro tests can provide information regarding effectiveness of chemical disinfectants against specific pathogens and guide the appropriate choice for a disinfection program. Some studies have reported the susceptibility of H1N1 influenza virus isolated from humans after the pandemic event in 2009 (Jeong et al., 2010; Subhash et al., 2014; Perry et al., 2016), but usually the disinfectants tested are not used in livestock due to the cost and applicability. In addition, cleaning conditions, levels of organic load and type of surface can be a challenge to disinfection procedures in livestock. The aim of this study was to evaluate the efficacy of four disinfectants, commonly used on swine farms, to inactivate a pandemic H1N1 influenza virus using fresh disinfectant solution and 72hours after dilution.

## MATERIAL AND METHODS

**Influenza virus, isolation and titration**. A Brazilian field pandemic H1N1 influenza virus1 was previously isolated from a lung sample collected from a pig with clinical respiratory signs. The inoculum was diluted in PBS, 0.01M, pH 7.4, clarified by centrifugation at 12.000r.p.m. for 20 min at 4°C, filtered through a 0.22μm membrane (Milliplex™, Millipore corp., Bedford, USA) and inoculated in specific pathogen-free embryonated chicken eggs (ECE). Inoculated eggs were candled daily for seven consecutive days and any deaths during the first day were discarded as non-specific deaths. The isolated virus was titrated in ECE, and the 50% embryo-infective doses ($EID_{50}$) were determined (Reed & Muench, 1938). Three ten-fold dilution were performed in order to obtain four concentrations of inoculums containing $6.4\log_{10}$ $EID_{50}$/mL; $5.4\log_{10}$ $EID_{50}$/mL; $4.4\log_{10}$ $EID_{50}$/mL and $3.4\log_{10}$ $EID_{50}$/mL. All inoculums were tested by real-time reverse transcription-polymerase chain reaction (RT-PCR) to assess the initial Cycle threshold (Ct) and stored at -70°C until use.

***In vitro* evaluation of disinfectants**. Disinfectant concentration (%) followed the manufacturer's guidelines (Table 1), using a standard hard water prepared according to AOAC 960.09 E and F. In order to simulate a surface cleaning failure, organic load was included in the test. Pig feces were previously autoclaved at 121°C for 20 minutes and the sterility test was performed on blood agar, Sabouraud (Thermo Scientific, Waltham, US) and brain heart infusion (BHI; Merck KGaA, Darmstadt, Germany). BHI and blood agar were incubated for 72hours at 37°C and Sabouraud agar at 25°C for five days. After approval of sterility, feces were diluted at 1/3 (v/v) in PBS pH 7.4 and homogenized. An aliquot of 0.2mL was inoculated in ten 9-day-old ECE and eggs were candled daily to determine the viability of embryos for ten days.

On a safety cabinet, 400μL of viral inoculum were added to 100μL of organic load solution and 500μL of each individually diluted disinfectant, vortexed for 30s and incubated for ten minutes. At the end of the contact time, 1000μL of neutralizer solution (1.5g sodium thiosulfate [$Na_2S_2O_2$, Thermo Fisher Scientific, Waltham, US], 0.07g lecithin [Thermo Fisher Scientific, Waltham, US], and 0.1mL Tween 80 [Thermo Fischer Scientific, Waltham, US] in 100mL phosphate buffered saline [PBS, pH 7.2]) was added to the solution. To ensure that

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neutralizer solution was not toxic to the embryos, ten 9-day-old ECE were previously inoculated with the mixture of each disinfectant and neutralizer solution and eggs were candled daily for 10 days. The final suspension from each mixture of disinfectant and inoculum was filtered through a 0.22µm membrane (Milliplex™, Millipore corp., Bedford, USA) and 0.2mL inoculated in six 9-day-old ECE through allantoic route. Inoculated ECE were candled daily for 72 hours and observed to assess embryos viability, any deaths during the first 24 hours were discarded as non-specific deaths. Allantoic fluid from reminiscent eggs were harvest for RT-PCR and hemagglutination test.

**Table 1.** Disinfectants and dilutions tested against four concentrations of influenza A virus (IAV)

| Study group | Disinfectant concentration | IAV inoculum ($\log_{10}EID_{50}$/mL) | Organic load | Contact Time |
|---|---|---|---|---|
| Oxidizing agent[a] | 0.5% | 6.4<br>5.4<br>4.4<br>3.4 | Yes | 10 minutes |
| Phenol[b] | 0.4% | 6.4<br>5.4<br>4.4<br>3.4 | Yes | 10 minutes |
| Quaternary ammonium (QAC) and glutaraldehyde association[c] | 0.1% | 6.4<br>5.4<br>4.4<br>3.4 | Yes | 10 minutes |
| Quaternary ammonium compound[d] (QAC) | 0.1% | 6.4<br>5.4<br>4.4<br>3.4 | Yes | 10 minutes |

[a] Virkon™S, Lanxess, Germany, [b] Biophene, Neogen, US, [c] TH4, Theseo, Brazil, [d] Germon 80, Sanphar, Brazil.

The experiment was conducted simultaneously with negative and positive controls. A negative control consisted of ten 9-day-old ECE inoculated with 0.2mL of a mixture containing water (400µL), organic load solution (100µL) and neutralizer solution (1000µL). Another negative control was composed of ten 9-day-old ECE that were inoculated with 200µL of a mixture containing each disinfectant (400µL), organic load solution (100µL) and neutralizer solution (1000µL) to assess the toxicity of the disinfectant on embryos. Positive control consisted of ten 9-day-old ECE with each of four viral inoculum from $6,4\log_{10}$ $EID_{50}$/mL to $3,4\log_{10}$ $EID_{50}$/mL. The test was performed in duplicates resulting in 44 samples. The

experiment was repeated with the same protocol described above, 72 hours after dilution of the disinfectants have been performed. Therefore, a total of 88 samples were tested.

**Hemagglutination assays**. Hemagglutination assays were carried out using 0.5% chicken erythrocytes as previously described (Swenson et al., 2018).

**RT-PCR**. RNA was extracted from allantoic fluid using the Cador Pathogen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One Step-RT-qPCR analyses were run on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, US). The reaction was performed with the LSI VetMAX® Swine Influenza A kit (A/H1N1/2009) (Thermo Fisher Scientific, Waltham, MA, US) for a final volume of 20 µl/reaction.

**Data analysis**. The difference between the positive control and the test was recorded and Ct values were used to interpretate whether disinfectants reduced virus viability and infectivity. The results considered the Ct means of duplicate sets of control and test at time on hour 0 and hour 72 post disinfectant dilution.

## RESULTS

Influenza A [H1N1] virus titration and detection by RT-PCR are demonstrated on Table 2. The organic load, the neutralizer and the four disinfectants had no effect on embryos viability, demonstrating no toxicity.

**Table 2.** Influenza virus titration (IAV) on embryonated chicken eggs (ECE) and Cycle threshold (Ct) values from RT-PCR of viral inoculum.

| Viral titration ($\log_{10}$ $EID_{50}$) | IAV RT-PCR (Ct) | |
|---|---|---|
| | 0h | 72h |
| 6.2 | 21.7 | 22.6 |
| 5.2 | 24.3 | 25.2 |
| 4.2 | 28.8 | 28.1 |
| 3.2 | 31.0 | 31.6 |

Results of hemmaglutination and RT-PCR are demonstrated on Table 3. In the first evaluation using fresh disinfectant solution (Hour 0), oxidizing compost disinfectant and phenolic disinfectant inactivated influenza at all tested virus concentrations. The association of quaternary ammonium (QAC) and glutaraldehyde inactivated the virus up to a concentration of

5.4log$_{10}$ EID$_{50}$/mL, therefore the highest virus concentration (6.4log$_{10}$ EID$_{50}$/mL) tested was still viable.

**Table 3.** Results of influenza A virus (IAV) hemagglutination and RT-PCR at 0 hour and 72 hours after disinfectant dilution.

| Study group | Virus titer (log$_{10}$ EID$_{50}$) | Hemagglu-tination | IAV RT-PCR | Hemagglu-tination | IAV RT-PCR |
|---|---|---|---|---|---|
| | | Hour 0 | | Hour 72 | |
| Oxidizing agent | 6.4 | - | - | - | - |
| | 5.4 | - | - | - | - |
| | 4.4 | - | - | - | - |
| | 3.4 | - | - | - | - |
| | Neg. control | - | - | - | - |
| Phenol | 6.4 | - | - | + | + |
| | 5.4 | - | - | - | - |
| | 4.4 | - | - | - | - |
| | 3.4 | - | - | - | - |
| | Neg. control | - | - | - | - |
| Quaternary ammonium (QAC) and glutaraldehyde association | 6.4 | + | + | + | + |
| | 5.4 | - | - | - | - |
| | 4.4 | - | - | - | - |
| | 3.4 | - | - | - | - |
| | Neg. control | - | - | - | - |
| Quaternary ammonium compound (QAC) | 6.4 | + | + | + | + |
| | 5.4 | + | + | + | + |
| | 4.4 | + | + | + | + |
| | 3.4 | + | + | + | + |
| | Neg. control | - | - | - | - |
| Positive control | 6.4 | + | + | + | + |
| | 5.4 | + | + | + | + |
| | 4.4 | + | + | + | + |
| | 3.4 | + | + | + | + |

"+" positive, "-" negative.

The quaternary ammonium disinfectant did not eliminate virus viability after a 10-minute contact. Seventy-two hours after disinfectants were diluted, oxidizing compost disinfectant demonstrated the same result as the assessment with fresh disinfectant solution, inactivating influenza at all tested virus concentration. The phenolic disinfectant and the association of QAC and glutaraldehyde inactivated the virus up to 5,4log$_{10}$ EID$_{50}$/mL. Lastly,

QAC when was not associated with other active ingredient, had no effect on influenza virus inactivation (Table 3).

## DISCUSSION

The experiment evaluated four commercial disinfectants and their effectiveness on inactivating four concentrations of a pandemic H1N1 influenza virus. The tested disinfectants are widely used in swine production in order to decrease contamination on farms after cleaning facilities, equipment and vehicles. Our results demonstrated that oxidizing compost disinfectant and phenolic disinfectant were the most effective, even at high levels of influenza virus ($6.4\log_{10}$ EID$_{50}$/mL). The efficacy of these active ingredients has previously been reported using the high pathogenic avian influenza virus H7N2 (Suarez et al., 2003), H7N1 (Sonthipet et al., 2018) and H5N1 (Marzouk et al., 2014) when tested at the manufacturer's recommended dose. The QAC disinfectant did not inactivate the influenza virus under our in vitro test conditions, considering the initial virus concentration of $3.4\log_{10}$ EID$_{50}$/mL. QAC has previously been reported to be effective for enveloped virus (Jeffrey, 1995; Suarez et al., 2003) due to its hydrophobic activity (Gerba, 2015). It is important to note that there are different commercially available QAC-based disinfectants, regarding generation and active ingredients, which can lead to different results. Our results demonstrated that IAV is susceptible to QAC and glutaraldehyde association disinfectant, but the concentration may influence its efficacy. Marzouk et al. (2014) reported the inactivation of two strains of avian influenza virus ($7.15\log_{10}$ EID$_{50}$/mL and $8.13\log_{10}$ EID$_{50}$/mL) using 1% QAC and glutaraldehyde association with a contact time of 15 minutes.

Influenza viruses are described as relatively susceptible to chemical disinfection, particularly due to the presence of the viral envelope, which is composed of lipid bilayers (De Benedictis et al., 2007; Ivanova et al, 2015). Therefore, IAV is inactivated by most disinfectants when used properly. Organic matter has been described as an enhancer to increase viral persistence and tenacity (Hauck et al., 2017). Since organic load also decreases the efficacy of chemical disinfectants (Chandler-Bostock & Mellits, 2015; Marzouk et al., 2014), we considered the worst-case scenario and included autoclaved feces to simulate a real situation of an unsuccessful cleaning procedure, similar to other studies (Guan et al., 2014, Sonthipet et al., 2018).

The test was repeated 72 hours after disinfectants were diluted to assess effectiveness over time. All tested products demonstrated similar performance, except for phenolic

disinfectant that decreased effectiveness when compared to the fresh diluted test (hour 0). The effectiveness of oxidizing agent to inactivate the avian influenza virus was obtained after a 10-day-dilution and the virus was not eliminated, demonstrating the importance of the freshness of the disinfectant solution (Suarez et al., 2003). It is important to understand the stability of disinfectants, since some biosecurity procedures in a pig farm include disinfection arch or footbaths where the disinfectants are not necessarily diluted at the time of use, and could result in disinfection failure.

In vitro test to assess virus susceptibility to disinfectants may have some protocol variation, in addition to the class of disinfectants and virus strain. Susceptibility tests can be performed with carriers, using coupons with different surfaces or suspension model and can also be evaluated under different conditions, as temperature, pH, contact time between disinfectants and viruses, different concentrations of organic load and disinfectant neutralizing step. In our study, we standardized four different concentrations of the IAV and the disinfectants were diluted according to the manufacturer's recommendations. Viral recovery was performed using ECE inoculation in order to assess virus viability and infectivity. Allantoic fluid was harvested from the ECE 72hours post inoculation, RT-PCR and hemagglutination assays were run simultaneously and the agreement between assays was 100%, since all replicates had the same results using both methods. The limitation of our experiment is that our test was carried out with one virus strain. Hauck et al. (2017) reported different persistence time in manure when they tested two avian influenza viruses with different levels of pathogenicity. Moreover, viruses can tolerate extensive variations in the glycerophospholipids composition of their envelopes (Ivanova et al., 2015) and it is unknown how this characteristic may influence the virus persistence and susceptibility.

## CONCLUSION

Information on the effectiveness of disinfectants in inactivating pandemic H1N1 influenza virus isolated from pig is limited, therefore, we evaluated four disinfectants widely used on pig farms under the same controlled conditions. In conclusion, three of the four disinfectants tested were effective to inactivate pandemic H1N1 influenza virus in the presence of organic load. Test result performed 72 hours after disinfectant dilution suggest a decrease in the effectiveness of one disinfectant and further investigation is needed to understand the limit period of action of each active ingredient.

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

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## 6. Capítulo V – Quarto artigo científico

**Effect of pooling udder skin wipes on the detection of influenza A virus in pre-weaning pigs**

Artigo publicado na Journal of Veterinary Diagnostic Investigation (formato na norma da revista)

*Brief Report*

# Effect of pooling udder skin wipes on the detection of influenza A virus in preweaning pigs

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Running head: Pooling udder wipes to detect influenza in pigs.

**ABSTRACT**

Influenza A virus (IAV) active surveillance in pigs prior to weaning is commonly conducted by collecting individual samples, mostly nasal swabs. Recently, the use of udder skin wipes collected from lactating sows was identified as an effective sampling method to indicate IAV status of suckling piglets prior to weaning. However, there is limited information on the effect of pooling multiple udder wipes on the ability to detect IAV. We evaluated the effect of pooling 3, 5, or 10 udder wipes on the sensitivity of detecting IAV, and compared the results with testing the wipes individually. The likelihood of detecting positive udder wipes decreased with pooling when the initial positive cycle threshold value was $\geq 31.5$, and that pooling of up to 3 samples could be performed without affecting sensitivity significantly. Our results support pooling of udder skin wipes to conduct surveillance of IAV in pigs prior to weaning.

**Keywords:** Influenza A virus; pigs; pooling; surveillance; udder wipes.

**BRIEF REPORT**

Influenza A virus (IAV) is endemic in swine and causes respiratory disease that results in productivity and economic losses.[2] Pigs prior to weaning are one of the main subpopulations that help maintain infections endemic in breeding herds. Cost-effective surveillance programs directed at detecting IAV in piglets prior to weaning are needed to design effective IAV control and elimination programs, in particular if the desired outcome is to wean an IAV-negative pig. Traditionally, nasal swabs have been used as the reference sample to monitor pigs, and to not only to detect but also isolate IAV.[5] Recently, udder skin wipes collected from lactating sows have been identified as a suitable sample to monitor litters of pigs prior to weaning.[1] Udder skin wipes contain the nasal and oral secretions deposited on the skin of the lactating sows by suckling piglets and thus represent the litter status. Following the report of the use of udder wipes for IAV sampling, udder wipes have become a popular sample type to detect IAV in breeding herds in North America (Torremorell pers. comm. 2021). Pooling of samples prior to RT-PCR assays is common for some diseases,[6] given that it can help reduce diagnostic laboratory fees or increase the number of samples collected and tested at the same cost. Although studies have been performed using pooling of nasal swabs,[1,4] information on pooling udder skin wipes and its effect on sensitivity for IAV detection is missing. Therefore, the aim of our study was to assess the impact of pooling udder wipes collected from lactating sows on the sensitivity of detection of IAV by RT-PCR compared to individual wipes.

Udder skin wipes collected from sows nursing piglets close to weaning (~ 21-d-old) were selected from a previous study.[3] Briefly, 45 lactating sows in a 3,000-sow farm located in the midwest United States were selected randomly. Sampling was performed as described previously,[1] using a $8 \times 8$ cm sterile gauze pads with 8 mL of Dulbecco modified Eagle medium (Gibco) supplemented with 4% of 7.5% bovine serum albumin, fraction V, and 1X antibiotics

Anti-Anti (100x) (Gibco), which is composed of 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B. Samples were placed in a cooler box containing ice packs and transported to the laboratory for processing and testing. Samples were processed for RNA extraction (Ambion MagMAXAM1835 viral RNA isolation kit; Applied Biosystems) and tested by RT-PCR to detect the IAV matrix gene.7 Samples with cycle threshold (Ct) <35 were considered positive. Of 45 samples, 19 samples tested positive for IAV with Ct values of 27.5-33.8. These samples were used as the starting material to evaluate the sensitivity of pooling. Negative samples were combined to serve as the IAV-negative control. In order to evaluate the effect of pooling, 0.1 mL of the positive samples were diluted in 0.2 mL sample solution for 1:2 ratio, in 0.4 mL for 1:4 ratio, and in 0.9 mL for 1:9 ratio, using the negative control udder wipe solution (Fig. 1) that had been confirmed IAV negative by RT-PCR. The 19 IAV-positive undiluted samples and their respective pools of 1:2, 1:4, and 1:9 were individually analyzed by RT-PCR, as described above.
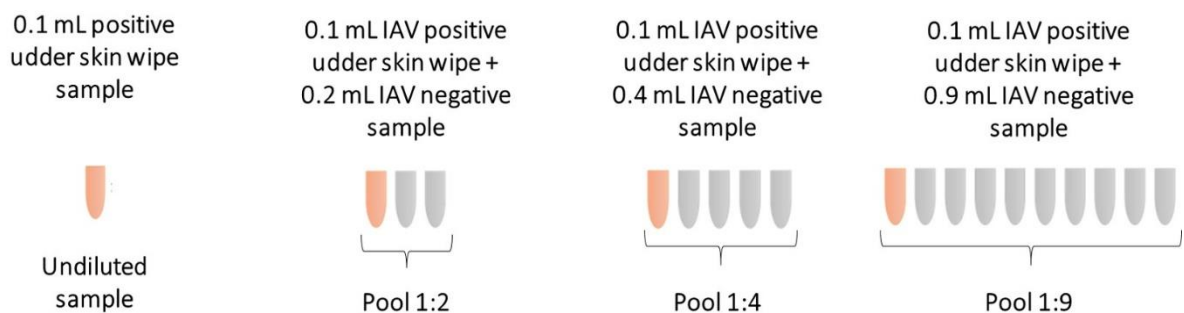


Figure 1. Summary of pooling procedures using positive (orange) and negative (gray) influenza A virus (IAV) udder skin wipes indicating the pooling ratio of no pooling (undiluted sample), 1:3, 1:5 and 1:10.

In order to calculate sensitivity, results from undiluted samples were considered reference values and sensitivity was calculated as the proportion of positive samples after pooling divided by the total number of positive samples. The 95% confidence interval (CI) was

estimated using the Jeffreys interval method. Briefly, samples were divided in 2 groups using the median of the undiluted samples, and sensitivity was calculated for the high and low Ct groups. A polynomial regression of a first-order model was used to estimate the increase in Ct values by each unit of dilution (equation 1). Prior to inclusion in the model, undiluted samples and dilutions 1:2, 1:4, and 1:9 were converted to the log10 scale; 0, 0.47, 0.70, 1.00, respectively. The number of dilutions that could be performed before reaching the positive cutoff (Ct <35) was calculated.

Equation 1: $Ct = B0 + initial\ Ct \times B1 + log10\ dilution \times B2$.

(B0 = intercept; B1, B2 = coefficients associated with predictors.)

The pooling of the positive udder wipes in pools of 3, 5, and 10 resulted in sensitivities of 84.2%, 68.0%, and 63.0%, respectively (Table 1). As expected, there was a slight increase in the Ct values of the pooled samples compared to the individual samples. The median Ct value of the undiluted positive samples was 31.5.

Table 1. Sensitivity (95% confidence interval, CI), mean and median cycle threshold (Ct) value of undiluted samples, and samples pooled in a final ratio of 1:2, 1:4, and 1:9.

| | Undiluted | Pooled | | |
|---|---|---|---|---|
| | | 1:2 | 1:4 | 1:9 |
| Sensitivity (95% CI) | Reference | 84.2% (67.8-100) | 68.0% (47.5-89.3) | 63.0% (41.5-84.9) |
| Median Ct | 31.5 | 32.7 | 33.7 | 34.0 |
| Mean Ct ± 1SD | 31.0 ± 1.9 | 32.8 ± 2.1 | 34.0 ± 3.3 | 34.5 ± 3.0 |

There was no difference in sensitivity in the group of samples with low ($\leq$ 31.5) Ct values (Fig. 2). In the group of samples with high (>31.5) Ct values, sensitivity ranged between 30% (95% CI 42-98%) and 100% (95% CI 2-58%), depending on the pooling ratio (Fig. 2).
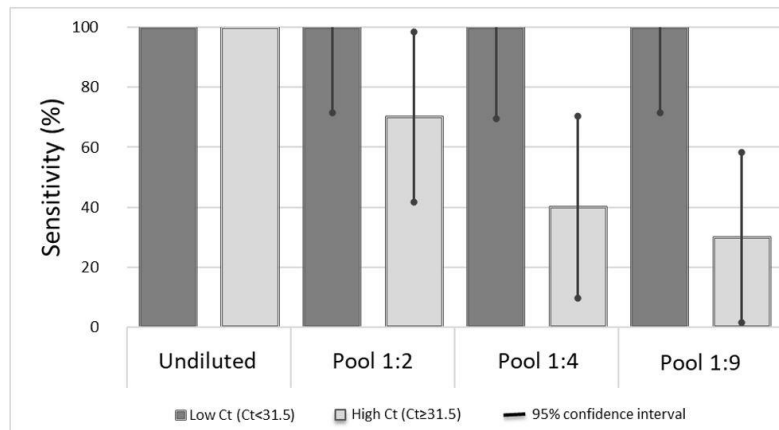


Figure 2. Sensitivity (%) and 95% confidence interval in groups with low (Ct<31.5) and high (Ct$\geq$31.5) initial cycle threshold (Ct) values, based on Ct values from undiluted samples (reference values).

Estimated Ct values after pooling samples varied based on the initial samples Ct (Table 2). For samples with a Ct value of 34, only 2 dilutions were needed to reach the negative cutoff value of 35. In contrast, 36 dilutions were needed when the starting Ct value was 30.). The intercept from polynomial regression was 3.53. The coefficient associated with initial Ct was 0.89 and the dilution ratio was 3.04.

We conclude that pooling of udder skin wipes can be used to test for IAV by RT-PCR. However, pooling resulted in a slight decrease in sensitivity when pooling ratios were 1:3, and a moderate decrease when pooling ratios were 5 or higher. As expected, our study demonstrated that the likelihood of detecting a positive udder skin wipe is correlated with the initial Ct value, which reflects viral load. In the samples used in our study, sensitivity was not compromised when samples with Ct <31.5 were pooled for up to 10 samples. Instead, sensitivity decreased

Table 2. Estimated cycle threshold (Ct) values from pooled samples based on the initial Ct value and the estimated maximum number of dilutions necessary to reach the Ct cutoff of 35 based on the RT-PCR.

| Initial Ct | Pooling ($\log_{10}$) | | | Maximum dilution to reach Ct>35 |
|:---:|:---:|:---:|:---:|:---:|
| | 1:2 (0.47) | 1:4 (0.70) | 1:9 (1) | |
| 30 | 31.7 | 32.4 | 33.3 | 36 |
| 31 | 32.6 | 33.3 | 34.2 | 19 |
| 32 | 33.5 | 34.2 | 35.1 | 9 |
| 33 | 34.4 | 35.1 | 36.0 | 5 |
| 34 | 35.2 | 35.9 | 36.9 | 2 |

with pooling of samples with initial Ct values $\geq 31.5$. We understand that as a cost mitigation strategy, pooling of samples at the laboratory prior to testing them by RT-PCR, is a very attractive proposition. However, pooling of more than 3 samples would significantly reduce the probability of detection, and could lead to false-negative results and the inability to detect litters infected with IAV. The decision on the number of samples in the pool should be made in conjunction with the sampling strategy at the farm level. A decrease in sensitivity caused by increased pooling may be compensated by increasing the number of litters sampled at the farm at the same cost. When increasing sampling, there is a greater likelihood of detecting at least one positive litter in the population, in particular in scenarios of low IAV prevalence when the aim is to detect IAV at the herd level. It is important to note that our study was limited to investigating the effect of pooling on the sensitivity to detect IAV by RT-PCR in udder wipes. It is likely that pooling may affect the sensitivity of virus isolation or other test methods and as such, this should be further investigated.

To our knowledge, the effect of pooling udder skin wipes to detect IAV by RT-PCR has not been reported previously. The approach described here can help guide sampling strategies and surveillance approaches to detect IAV in litters of pigs prior to weaning.

Declaration of conflicting interests

Funding

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## 7.    CONSIDERAÇÕES FINAIS

Influenza é um agente patogênico de grande relevância na produção de suínos. Tendo em vista a necessidade em controlar esse vírus nas diferentes fases de produção é necessário entender o comportamento do vírus, bem como as principais medidas de controle assertivas de redução da transmissão do agente. Sabendo disso, esse projeto teve o objetivo de avaliar a dinâmica de infecção do vírus e também desenvolver medidas de controle, acompanhado de adequada monitoria das ações.

O primeiro projeto demonstrou a circulação dos vírus nas granjas de sítio 1, creches e terminações do Brasil e que é alto o percentual de leitões positivos ao desmame. Nas análises longitudinais, detectamos o vírus da Influenza até quatro vezes no mesmo animal ao longo de sua vida produtiva, demonstrando a suscetibilidade dos animais a esse vírus. Concluímos com esse estudo que o vírus infecta os animais nos diferentes sítios de produção e que a alta variabilidade genética pode prejudicar a resposta imune dos animais.

O segundo projeto avaliou a persistência do vírus da Influenza no aparelho mamário de fêmeas ao desmame e o desenvolvimento de um protocolo de intervenção para reduzir essa contaminação. O projeto concluiu que o vírus permanece ao menos 3 horas após a retirada dos leitões no desmame, e que procolos de desinfecção com uso de produto tópico podem contribuir na redução dos níveis de influenza no aparelho mamário, reduzindo o risco de disseminação deste agente, principalmente quando utilizadas práticas de manejo com transferência de fêmeas como é o caso da utilização de mães de leite.

Os resultados do terceiro trabalho podem contribuir para o controle do vírus considerando a disseminação através do ambiente e por fômites. Conclui-se com este estudo que há diferença entre os princípios ativos de desinfetantes na eficiência para reduzir o vírus da influenza. Ainda, o tempo entre o preparo da diluição do produto e a utilização da solução pode comprometer a eficiência, mostrando a importância do procedimento adequado no momento da desinfecção. A utilização da matéria orgânica no teste *in vitro* é um outro ponto importante, considerando a real situação das granjas.

O quarto projeto avaliou o efeito de utilizar *pool* de lenços de aparelho mamário de fêmeas na sensibilidade de detecção do vírus da Influenza por RT-PCR. Concluiu-se que a utilização de *pool* com até três amostras não impacta na sensibilidade analítica, podendo otimizar análises, sem perder qualidade de detecção. Resultados desse estudo fornece

informações a produtores e veterinários para que seja possível implementar e cumprir planos de amostragem mesmo em situações de orçamento limitado.

Os resultados destes estudos contribuem para o controle sistemático do vírus da influenza, uma vez que informações sobre a dinâmica de infecção em granjas brasileiras podem nortear as ações para reduzir sua transmissão. Protocolos de intervenção são importantes para que em momentos críticos, a exemplo do desmame, os produtores e veterinários possam utilizar ferramentas simples e aplicáveis para auxiliar na redução da contaminação viral. E finalmente, informações sobre otimização de amostragem contribuem para o atendimento de monitorias, especialmente em situações onde há restrição de orçamento.

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