UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA E BIOCIÊNCIAS

Gleidson Biasi Carvalho Salles

Monitoramento de vírus respiratórios e a correlação com colibacilose em *Gallus Gallus domesticus* da avicultura de corte do Brasil

Florianópolis
2023

Gleidson Biasi Carvalho Salles

Monitoramento de vírus respiratórios e a correlação com colibacilose em *Gallus Gallus domesticus* da avicultura de corte do Brasil

Tese submetida ao Programa de Pós-Graduação em Biotecnologia e Biociências da Universidade Federal de Santa Catarina como requisito parcial para a obtenção do título de Doutor em Biotecnologia e Biociências

Orientador: Prof.ª Dr.ª Gislaine Fongaro
Coorientador: Prof. Dr. Eduardo Correa Muniz

Florianópolis
2023

Ficha de identificação da obra elaborada pelo autor,
através do Programa de Geração Automática da Biblioteca Universitária da UFSC.

Salles, Gleidson Biasi Carvalho
Monitoramento de vírus respiratórios e a correlação com colibacilose em Gallus Gallus domesticus da avicultura de corte do Brasil / Gleidson Biasi Carvalho Salles ; orientadora, Gislaine Fongaro, coorientador, Eduardo Correa Muniz, 2023.
125 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro de Ciências Biológicas, Programa de Pós Graduação em Biotecnologia e Biociências, Florianópolis, 2023.

Inclui referências.

1. Biotecnologia e Biociências. 2. Vigilância genômica de vírus de aves. 3. Bronquite Infecciosa das aves. 4. Metapneumovírus. 5. Coifecção colibacilose. I. Fongaro, Gislaine. II. Muniz, Eduardo Correa. III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Biotecnologia e Biociências. IV. Título.

Gleidson Biasi Carvalho Salles

Título: Monitoramento de vírus respiratórios e a correlação com colibacilose em *Gallus Gallus domesticus* da avicultura de corte do Brasil

O presente trabalho em nível de Doutorado foi avaliado e aprovado, em 15 de dezembro de 2023, pela banca examinadora composta pelos seguintes membros:

Dr. Mario Steindel
Universidade Federal de Santa Catarina

Dr. Paulo Augusto Esteves
Empresa Brasileira de Pesquisa Agropecuária

Dr. Sergio Moraes Aoki
Empresa Zoetis Indústria de Produtos Veterinários LTDA

Certificamos que esta é a versão original e final do trabalho de conclusão que foi julgado adequado para obtenção do título de Doutor em Biotecnologia e Biociências.

ICP-Edu
Documento assinado digitalmente
Aguinaldo Roberto Pinto
Data: 26/03/2024 17:00:37-0300
CPF: ***.874.718-**
Verifique as assinaturas em https://v.ufsc.br

Coordenação do Programa de Pós-Graduação

ICP-Edu
Documento assinado digitalmente
Gislaine Fongaro
Data: 26/03/2024 16:00:57-0300
CPF: ***.101.439-**
Verifique as assinaturas em https://v.ufsc.br

Dr.ª Gislaine Fongaro
Orientadora

Florianópolis, 2023

Dedico este trabalho a minha esposa Marina Jung, que esteve ao meu lado em todos os momentos dessa desafiadora e incrível jornada.

AGRADECIMENTOS

Agradeço a Zoetis por ter me apoiado imensamente nesse período, em especial aos meus colegas que contribuíram enormemente para conclusão dessa etapa: Antônio Neto, Antônio Kraieski, Carlos Silva, Dircélio Nascimento, Renato Verdi e principalmente, Eduardo Muniz, por ser paciente, parceiro, incentivador e inspirador.

Agradeço ao Laboratório de Virologia Aplicada, Bêa, Mari, Rafa, além deles, quero estender meus agradecimentos a Giulia Pilati, pois a sua dedicação, foco e parceria me ajudaram chegar até aqui.

Agradeço a UFSC por permitir sonhar e realizar esse grande sonho.

Agradeço a Prof.ª Dra. Gislaine Fongaro, sua transparência, foco, organização, parceria e lealdade, foram estímulos para conseguir concluir todas as etapas propostas, você é uma pessoa que inspira quem está a sua volta.

Agradeço a toda minha família, em especial, a minha mãe, que mesmo longe sempre me inspirou por sua determinação e garra.

Por fim, agradeço ao Supremo arquiteto do universo, por me dar condições físicas e emocionais para vencer todos os obstáculos.

RESUMO

O Brasil é o maior exportador e terceiro maior produtor de carne de frango do mundo, sendo o país destaque mundial na produção de proteína animal e a avicultura de corte assume o protagonismo nacional. As doenças respiratórias presentes na avicultura assumiram um protagonismo muito importante na economia brasileira, tendo em vista o aumento de condenas por aerossaculite nos abatedouros do Brasil, além dos casos de Influenza Aviária nos países do Hemisfério Norte e Sul. Nesse sentido, o monitoramento epidemiológico por meio de técnicas moleculares e sorológico devem ser ferramenta de uso cotidiano, para assim mensurar de maneira mais especifica quais agentes estão circulando nos planteis e avaliar os impactos relacionados às perdas zootécnicas. Dentre os patógenos avícolas destacam-se alguns vírus, sendo os Metapneumovírus (aMPV) e Vírus da Bronquite Infecciosa Aviária (IBV) comumente associados a quadros clínicos de aerossaculite que levam a prejuízos na avicultura de corte, sendo agravados os casos clínicos quando ocorre coinfecção bacteriana, especialmente com *Escherichia coli* aviária (APEC). O presente estudo, dividido em três capítulos, teve como objetivo principal monitorar a circulação de vírus de interesse respiratório (aMPV e IBV) e a coinfecção com *Escherichia coli* (APEC) na avicultura de corte brasileira. **O primeiro capítulo** apresenta uma revisão de literatura acerca do aMPV, sua prevalência e distribuição nos diferentes países do mundo, assim como aborda as características da partícula viral, classificação e técnicas de detecção e diagnóstico na avicultura. **O segundo capítulo** teve como objetivo monitorar aMPV em aves de corte da avicultura brasileira por meio de ensaio sorológico e de diagnóstico molecular, correlacionando com impactos gerados pela presença de APEC em casos de coinfecção pelos dois agentes. Para isso, carcaças de aves (*Gallus gallus domesticus)* já necropsiadas em campo para inspeção de rotina foram doadas para esse estudo, sendo coletados 100 lotes de aves dos estados do Paraná (n=30), Santa Catarina (n=15), Rio Grande do Sul (n=15), São Paulo (n=10), Minas Gerais (n=10) e Ceará (n=20). Para cada lote, foram coletadas amostras de swabs traqueais, fêmures, fígados e baços. Os lotes foram selecionados com base no histórico de problemas respiratórios, sorologia positiva para aMPV e sinais respiratórios clínicos, como espirros, roncos e secreção nasal. As aves necropsiadas tinham entre 13 e 32 dias de idade. Um total de 20% dos lotes (20/100) apresentaram respostas sorológicas compatíveis com a circulação de aMPV e desses 6,4% apresentaram coinfecção entre aMPV e APEC. Todas as amostras respiratórias foram submetidas ao ensaio de amplificação gênica usando transcrição reversa seguida da reação em cadeia da polimerase (RT-PCR), sendo possível detectar e identificar em 2 lotes avícolas o aMPV-B. **O terceiro capítulo** teve como objetivo realizar o monitoramento epidemiológico do IBV e suas variantes circulantes na avicultura de corte no Brasil, em aves imunizadas contra IBV. Um total de 1.000 swabs nasotraqueais foram avaliados, sendo essas amostras oriundas dos mesmos lotes avaliados para aMPV e amostrados nos estados do Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Minas Gerais e Ceará/Brasil. A triagem para a presença de IBV foi realizada usando a RT-qPCR da região hipervariável do gene S1 do IBV. Para compreender a filogenia e a evolução viral, 28 amostras de diferentes estados brasileiros foram selecionadas para sequenciamento de Sanger. Um total de 91% das amostras testadas foram positivas para o IBV, sendo classificadas em 7,14% pertencentes ao IVV-GI-1, 78,57% a IBV-GI-11 e 14,28% ao IBV- GI-23, ressaltando a detecção da nova variante do IBV-GI-23 no Brasil, em amostras do estado do Paraná. Os dados aqui gerados mostram um panorama nacional da epidemiologia de

aMPV e IBV, em especial os escapes vacinais frente ao IBV na avicultura Brasileira, bem como demostra que a coinfecção entre aMPV e APEC nas aves aumenta os agravos na saúde dos lotes avícolas. Em suma, cabe ressaltar a importância do monitoramento viral escalonado na avicultura de corte frente do Brasil nas regiões produtoras de aves de corte, impulsionando a necessidade por mais estudos que abordem a evolução viral para guiar estratégias e programas de enfrentamento às viroses no setor avícola.

Palavras-chave: Metapneumovirus, Monitoramento molecular, Bronquite Infecciosa das galinhas.

ABSTRACT

Brazil is the largest exporter and third largest producer of chicken meat in the world, the country stands out worldwide in the production of animal protein, and poultry farming takes on national prominence. Respiratory diseases present in poultry farming have assumed a very important role in the Brazilian economy, given the increase in convictions for aerosacculitis in slaughterhouses in Brazil, in addition to cases of Avian Influenza in countries in the Northern and Southern Hemisphere. In this sense, epidemiological monitoring by Using molecular techniques should be tools for everyday use, to more specifically measure which agents are circulating in plants and evaluate the impacts related to zootechnical losses. Viruses stand out among poultry pathogens, with Metapneumovirus (aMPV) and Avian Infectious Bronchitis Virus (IBV) commonly associated with clinical conditions that lead to losses in poultry farming, with clinical cases being aggravated when bacterial co-infection occurs, especially with avian Escherichia coli (APEC). The present study, divided into three chapters, had the main objective of monitoring the circulation of viruses of respiratory interest and co-infection with Escherichia coli (APEC) in Brazilian poultry farming. The first chapter presents a review of the literature on aMPV, its prevalence, and distribution in different countries around the world, as well as addressing the characteristics of the viral particle, classification, and detection and diagnosis techniques in poultry farming. The second chapter aimed to monitor MPV in meat birds in Brazilian poultry farming through serological and molecular diagnostic assays, correlating with the impacts generated by the presence of APEC in cases of co-infection by the two agents. For this, carcasses of birds (Gallus gallus domesticus) already necropsied in the field for routine inspection were carried out for this study, collecting 100 batches of birds from the states of Paraná (n=30), Santa Catarina (n=15), Rio Grande do Sul (n=15), São Paulo (n=10), Minas Gerais (n=10) and Ceará (n=20). For each batch, samples of tracheal swabs, femurs, livers and spleens were collected. The batches were selected based on the history of protection problems, positive serology for aMPV and clinical signs, such as sneezing, snoring and nasal obstruction. The necropsied birds were between 13 and 32 days old. A total of 20% of the batches (20/100) obtained serological responses compatible with the circulation of aMPV and these 6.4% corresponded to co-infection between aMPV and APEC. All respiratory samples were subjected to gene amplification assay using reverse transcription followed by polymerase chain reaction (RT-PCR), making it possible to detect and identify aMPV-B in 2 poultry batches. The third chapter aimed to carry out epidemiological monitoring of IBV and its variants circulating in poultry farming in Brazil, in birds immunized against IBV. A total of 1,000 nasotracheal swabs were evaluated, with these samples coming from the same batches evaluated for aMPV and sampled in the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Minas Gerais and Ceará/Brazil. Screening for the presence of IBV was performed using RT-qPCR of the hypervariable region of the IBV S1 gene. To understand viral phylogeny and evolution, 28 samples from different Brazilian states were selected for Sanger sequencing. A total of 91% of the samples tested were positive for IBV, with 7.14% belonging to IVV-GI-1, 78.57% to IBV-GI-11 and 14.28% to IBV-GI-23, highlighting the detection of the new variant of IBV-GI-23 in Brazil, in samples from the state of Paraná. The data generated here show a national overview of the epidemiology of aMPV and IBV, in particular, vaccine escapes against IBV in Brazilian poultry farming, as well as demonstrating that co-infection between aMPV and APEC in birds increases health problems in poultry

flocks. In short, it is worth emphasizing the importance of staggered viral monitoring in poultry farming across Brazil in meat bird-producing regions, driving the need for more studies that address viral evolution to guide strategies and programs to combat viruses in the poultry sector.Collectively, the data generated here will serve as a basis for viral epidemiological surveillance and its correlation with colibacillosis in poultry, as well as provide methodological alternatives for the prevention and control of pathogens before they cause significant losses to the sector.

Keywords: Metapneumovirus, Molecular Monitoring, Infectious Bronchitis in Chickens.

LISTA DE ABREVIATURAS E SIGLAS

ABPA	Brazilian Animal Protein Association
PIB	Gross Domestic Product
IA	Avian influenza
USDA	United States Department of Agriculture
DNC	New Castle disease
ILT	Infectious Laryngotracheitis
MG	*Mycoplasma Gallisepticum*
MS	*Mycoplasma Sinoviae*
aMPV	Avian Metapneumovirus
CI	Coriza Infecciosa
APEC	Avian Pathogenic *Escherichia coli*
RNA	Ribonucleic acid
IF	Impact factor
TRT	Turkin Rhionotracheitis
ART	Avian Rhionotracheitis
SHS	Swollen Head Syndrom
PCR	Polymerase chain reaction
RT -qPCR	Reverse transcription polymerase chain reaction
NGS	Next Generation Sequencing
DNA	Deoxyribonucleic acid
IBV	Infectious bronchitis virus
cDNA	Complementary deoxyribonucleic acid
MAPA	Ministry of Agriculture, Livestock and Supply
LVA	Laboratory of Applied Virology
UFSC	Federal University of Santa Catarina
ELISA	Enzyme Linked Immuno Sorbent Assay
OD	Optical Density
LMW	Low Molecular Weight
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2

SUMÁRIO

1. CONTEXTUALIZAÇÃO GERAL

O Brasil é destaque mundial na produção de proteína animal e a avicultura assume o protagonismo nacional nesse contexto, pois o país é o maior exportador e terceiro maior produtor de carne de frango do mundo (ABPA 2022). Só no setor de avicultura trabalham aproximadamente 2,6 milhões de pessoas, diretos ou indiretamente (ABPA 2022), esses dados demonstram a importância da atividade para o produto interno bruto (PIB), trazendo um impacto positivo importante na economia brasileira.

O ano de 2023 tem sido muito desafiador para todos os países que produzem frangos, pois o mundo todo ainda sofre com os impactos gerados pelo SARS-CoV-2, guerra entre Rússia e Ucrânia e principalmente pelos casos de Influenza Aviário (IA) nos continentes Europeu e Americano. Somente nos Estados Unidos, foram abatidas e descartadas aproximadamente 80 milhões de aves em virtude da presença do IA nos planteis avícolas (USDA 2022).

Nesse contexto, todo o continente Sul-americano está em alerta, pois a entrada de um vírus inédito em produções comerciais pode gerar impactos extraordinariamente grandes. Muitos agentes fazem parte do bloco de doenças respiratórias, podemos citar a própria IA, doença de New Castle (DNC), vírus da Bronquite Infecciosa (IBV), Laringotraqueíte (ILT), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), Coriza Infecciosa (CI) e o Metapneumovírus Aviário (aMPV). Esses patógenos levam a doenças que não possuem sinais patognomônicos, o que reforça a necessidade de identificação e caracterização molecular para compreender de maneira clara qual ou quais patógenos estão circulando no território nacional.

O aMPV é um vírus envelopado, pleomórfico, não hemaglutinante e que possui um genoma não segmentado de sentido negativo contendo 8 genes que codificam nove proteínas (kaboudi, Lachheb, 2021). O mais variável é o gene da glicoproteína G, que tem sido usado para subtipagem do aMPV, até o momento são conhecidos 6 subtipos, A, B, C, D e mais dois novos subtipos (Umar, 2019). O aMPV é membro da ordem Mononagavirales pertencendo a família Pnemoviridae e do gênero Metapneumovírus, sendo dois gêneros identificados pertencentes a essa família: *Orthopneumovirus* e o *Metapneumovirus* (Kaboudi, Lachheb, 2021).

Após o seu surgimento no final dos anos 70 na África do Sul (Buys and Prees, 1980), o aMPV se disseminou de maneira relativamente rápida para outras partes do mundo, sendo possível ser encontrado em praticamente todos os países que produzem aves. No Brasil, primeiro caso foi relato no início dos anos 90 e caraterizado com o aMPV subtipo A. Seus hospedeiros naturais são perus e galinhas, mas o vírus já foi identificado em outras aves, o que reforça a preocupação, pois a circulação e migração de aves silvestres pode auxiliar na transmissão de um subtipo ainda não identificado no país. Ao entrar em contato com ave saudável, o vírus se replica nas células ciliadas do trato respiratório superior, principalmente nos cornetos nasais, laringe e traqueia, levando a um aumento na produção de muco, perda progressiva dos cílios (deciliação) e da atividade ciliar (ciliostase), hiperplasia das células epiteliais e destruição do epitélio ciliado. Esse processo resulta na dificuldade de remoção do muco, que se acumula nas passagens e cavidades (Cook, 2000, Rautenschlein, 2020). Esse quadro clínico pode ser agravado por infecções secundarias, como é o caso da *Escherichia coli* patogênica aviária (APEC) levando a redução significativas no desempenho zootécnico das aves afetadas (Legnardi *et al.,* 2021).

Todos esses sinais clínicos são comuns para várias doenças já listadas, o que pode dificultar ainda mais o diagnostico, e o isolamento do agente, pois o aMPV pode ser detectado apenas no início dos sinais clínicos, persistindo entre 3 e 6 dias apenas (Cook e Cavanagh, 2002).

Além do aMPV, o Vírus da Bronquite Infecciosa também pertence ao bloco das doenças respiratórias, e tem trazido muitos problemas de desempenho, produtividade e impacto no bem-estar animal. Esse Coronavírus tem como porta de entrada o sistema respiratório superior e infecta primeiramente as células epiteliais da traqueia, gerando os primeiros sinais clínicos de natureza respiratória (Cavanagh, 2007).

Algumas estirpes podem desenvolver lesões em outras regiões e órgãos, principalmente reprodutivo e renal. Essas predileções por distintas células do hospedeiro estão ligadas a alta variabilidade das linhagens de IBV envolvido (Mendonza *et al*., 2022). Os coronavírus possuem vírus envelopado e pleomórficos, cujo diâmetro pode variar de 80 a 120 nm. A família *Coronaviridae* pertence a ordem *Nidovirales,* que está dividida em duas subfamílias: *Coronaviridae* e *Torovirinae* (Jackwood and De Wit, 2012).

Os coronavírus tem seu material genético composto por ácido ribonucleico (RNA) e seu genoma compreende aproximadamente 27 a 31 kilobases. O gene S codifica a principal proteína estrutural, a glicoproteína S da Espicula. Essa glicoproteína está associada com o envelope viral, posuindo a subunidade S2, que auxilia na fixação viral e a subunidade S1, responsável pela interação com os receptores específicos presentes nas células alvo da infecção (Cavanagh, 2007).

O IBV está em constante evolução, pois a taxa de mutação e recombinação gênica é alta, tornando-se onipresente na avicultura mundial (Guzman *et al*., 2020). Essa significativa variabilidade genética levou ao desenvolvimento de muitas vacinas em todo o mundo para prevenção e combate do IBV. De maneira geral, é muito comum a utilização de vacinas replicantes (vivas) e não replicantes (inativadas).

No Brasil o IBV circula desde a década de 1950, com muitos genótipos e linhagens distintas. A primeira identificação foi do sorotipo Mass (Fraga et al., 2018) e, com o advento da biologia molecular na década de 1990, tornou-se possível uma compreensão mais clara dos principais clados virais presentes no Brasil. Até 2021, apenas dois sorotipos vacinais estavam autorizados para uso, o sorotipo Mass (GI-I) e a Variante BR (GI-11).

Em 2022, uma nova linhagem, GI-23 (Variant-2), foi identificada no Brasil, e uma vacina homóloga foi introduzida (Ikuta et al., 2022; Trevisol et al., 2023). A Variante-2 do Vírus da Bronquite Infecciosa, foi descrita pela primeira vez em Israel em 1998 e foi responsável por lesões significativas nos sistemas respiratório e nefropatogênico em aves. Em 2016, foi relatada a identificação da mesma cepa na Europa (Lisowska et al., 2017; Valastro et al., 2016).

Nesse sentido o mapeamento, identificação e classificação por meio de técnicas moleculares pode contribuir para o desenvolvimento de novas alternativas para prevenção e controle do espalhamento viral, em especial ao se conhecer precocemente o agente patogênico. Técnicas sorológicas, por si só, não são capazes de prever eventos epidemiológicos e sim determinar uma infecção. Já os métodos moleculares como PCR ou RT-qPCR, sequenciamento de Sanger e mais recentemente sequenciamento de nova geração (NGS), demonstraram sua importância clínica e epidemiológica por meio da identificação e tipagem genética de patógenos (Yohe e Thyagarajan, 2017; Du et al., 2017).

Tendo em vista uma melhor compreensão das viroses respiratórioas na avicultura e agravos mediados por coinfecções virais e bacterianas, o presente estudo apresenta dados obtidos pelo monitoramento de aMPV e IBV nos estados de RS, SC, PR, SP, MG e CE entre o ano de 2021, bem como casos de coinfecção de aMPV com E. coli patogênica (APEC) na avicultura de corte brasileira.

1.1 Hipóteses

Foram tomadas as seguintes hipóteses para esse estudo:

Hipótese I – aMPV circulam de forma não uniforme no Brasil, sendo prevalentemente em lotes de aves de corte com colibacilose e agravos respiratórios, justificando a introdução de ciclos vacinais contra tal vírus;

Hipótese II – O IBV está presente em diferentes regiões do país, com distintas variantes, independentemente dos programas vacinais utilizadas.

1.2 Referências da Contextualização Geral

ABPA (Brazilian Animal Protein Association) Annual Report ABPA 2022. Available at: < http://abpa-br.com.br/setores/avicultura/publicacoes/relatorios-anuais>. Accessed on: 10 Nov. 2022.

Buys, S. B. (1980). A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys*, *28*, 36.

Cavanagh, D. (2007). Coronavirus avian infectious bronchitis virus. *Veterinary Research*, *38*(2), 281–297.

Chacón JL, Mizuma M, Vejarano MP, Toquín D, Eterradossi N, Patnayak DP, Goyal SM, Ferreira AJ. Avian metapneumovirus subtypes circulating in Brazilian vaccinated and nonvaccinated chicken and turkey farms. Avian Dis. 2011.

Cook J.K. Cavanagh D., 2002. Detection and differentiation of avian pneumoviruses. Avian Pathol. 31 :117-132.

Cook, 2000 J.K.A. Cook Avian pneumovirus infections of turkeys and chickens Vet. J., 160 (2000), pp. 118-125.

Fraga, A. P. de, Gräf, T., Pereira, C. S., Ikuta, N., Fonseca, A. S. K., & Lunge, V. R. (2018). Phylodynamic analysis and molecular diversity of the avian infectious bronchitis virus of chickens in Brazil. *Infection, Genetics and Evolution*, *61*, 77–83. https://doi.org/10.1016/j.meegid.2018.03.014

Goodwin, S.; Mcpherson, J.D.; Mccombie, W.R. Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics, v. 17, n. 6, p. 333-351, 17 mai. 2016. Springer Science and Business Media LLC. http://dx.doi.org/10.1038/nrg.2016.49.

Guzmán, M., & Hidalgo, H. (2020). Live Attenuated Infectious Bronchitis Virus Vaccines in Poultry: Modifying Local Viral Populations Dynamics. *Animals*, *10*(11), 2058. https://doi.org/10.3390/ani10112058

Jackwood, M. W., & de Wit, S. (2013). Infectious Bronchitis. In *Diseases of Poultry* (pp. 139–159). Wiley. https://doi.org/10.1002/9781119421481.ch4

Kaboudi, K. (2019). Virus-induced immunosuppression in turkeys (Meleagris gallopavo): A review. *Open Veterinary Journal*, *9*(4), 349-360.

Legnardi, Matteo; ALLÉE, Chantal; Franzo, Giovanni; Cecchinato, Mattia; Brown, Paul. Research Note: detection of avian metapneumovirus subgroup c specific antibodies in a mallard flock in italy. Poultry Science, [S.L.], v. 100, n. 7, p. 101186, jul. 2021. Elsevier BV. http://dx.doi.org/10.1016/j.psj.2021.101186.

Lisowska, A., Sajewicz-Krukowska, J., Fusaro, A., Pikula, A., & Domanska-Blicharz, K. (2017). First characterization of a Middle-East GI-23 lineage (Var2-like) of infectious bronchitis virus in Europe. *Virus Research*, *242*, 43–48. https://doi.org/10.1016/j.virusres.2017.09.010

Mendoza-González, L., Marandino, A., Panzera, Y., Tomás, G., Williman, J., Techera, C., Gayosso-Vázquez, A., Ramírez-Andoney, V., Alonso-Morales, R., Realpe-Quintero, M., & Pérez, R. (2022). Research Note: High genetic diversity of infectious bronchitis virus from Mexico. *Poultry Science*, *101*(10), 102076. https://doi.org/10.1016/j.psj.2022.102076

Paim, W, Maggioli, M, Weber, M et al. Virome characterization in serum of healthy show pigs raised in Oklahoma demonstrated great diversity of ssDNA viruses. Virology

Rautenschlein, S. 2020. Avian metapneumovirus. Pages 135–143 in Diseases of Poultry. D. E. Swayne, ed. 14th ed. Wiley Blackwell, Hoboken, NJ.

Sophia Yohe, Bharat Thyagarajan; Review of Clinical Next-Generation Sequencing. *Arch Pathol Lab Med* 1 November 2017; 141 (11): 1544–1557. DOI: https://doi.org/10.5858/arpa.2016-0501-RA

Trevisol, I. M., Caron, L., Mores, M. A. Z., Voss-Rech, D., da Silva Zani, G., Back, A., Marchesi, J. A. P., & Esteves, P. A. (2023). Pathogenicity of GI-23 Avian Infectious Bronchitis Virus Strain Isolated in Brazil. *Viruses*, *15*(5), 1200. https://doi.org/10.3390/v15051200

Umar, S., Teillaud, A., Aslam, H. B., Guerin, J. L., & Ducatez, M. F. (2019). Molecular epidemiology of respiratory viruses in commercial chicken flocks in Pakistan from 2014 through to 2016. *BMC veterinary research*, *15*(1), 1-12.

USDA (United State Department of Agriculture) Monthly Report USDA. Available at: <https://www.aphis.usda.gov/animal_health/downloads/animal_diseases/ai/2022-23-wild-bird-ai-surveillance-implementation-plan.pdf>. Accessed on: 04 Nov. 2022

v. 556, p. 87-95, 2021 https://doi.org/10.1016/j.virol.2021.01.006.

Valastro, V., Holmes, E. C., Britton, P., Fusaro, A., Jackwood, M. W., Cattoli, G., & Monne, I. (2016). S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infection, Genetics and Evolution*, *39*, 349–364. https://doi.org/10.1016/j.meegid.2016.02.015

2. ORGANIZAÇÃO DA TESE DE DOUTORADO

A presente tese doutoral foi organizada em três capítulos, de acordo com os seus respectivos artigos científicos gerados e/ou redigidos no presente estudo (Figura 1).

O **primerio capítulo** foi composto pelo primeiro artigo intitulado *"Trends and challenges in the surveillance and control of Avian Metapneumovirus"*. Trata-se de uma revisão literária que buscou entender quais são os principais subtipos de aMPV presentes em diferentes países mundiais produtores de aves de corte, além de identificar quais estratégias são utilizadas para prevenir e controlar aMPV em aves. Esse manuscrito encontra-se publicado na revista *Viruses* (IF: 4.8 em 2023 – Qualis A CAPES).

O **segundo capítulo** refere-se ao manuscrito "*Epidemiological monitoring of avian metapneumovirus by molecular and serological methods in unvaccinated chicken suspeited for avian pathogenic Escherichia coli"*. Tal estudo teve como objetivo apresentar a soroprevalência e subtipos de aMPV presentes nos diferentes estados federados do Brasil e avaliar os impactos gerados pela *Escherichia coli* patogênica aviária (APEC) em casos de coinfecção pelos dois agentes. Este manuscrito encontra-se submetido na revista Microorganisms (IF: 4.9 em 2023 – Qualis A CAPES).

O **terceiro capítulo** refere-se ao manuscrito "*Monitoring Infectious Bronchitis Virus (IBV) in Vaccinated and Non-vaccinated Broiler Chickens in Brazil to surveillance of Vaccine Escapes and New viral Variants*". Tal estudo teve como objetivo monitorar IBV no Brasil, compreender potenciais escapes vacinais virais, identificar novas cepas variantes na população avícola e avaliar a persistência do vírus vacinal em frangos de corte vacinados. O artigo encontra-se submetido na revista Avian Pathology (IF: 2.7 em 2023 – Qualis A Capes).

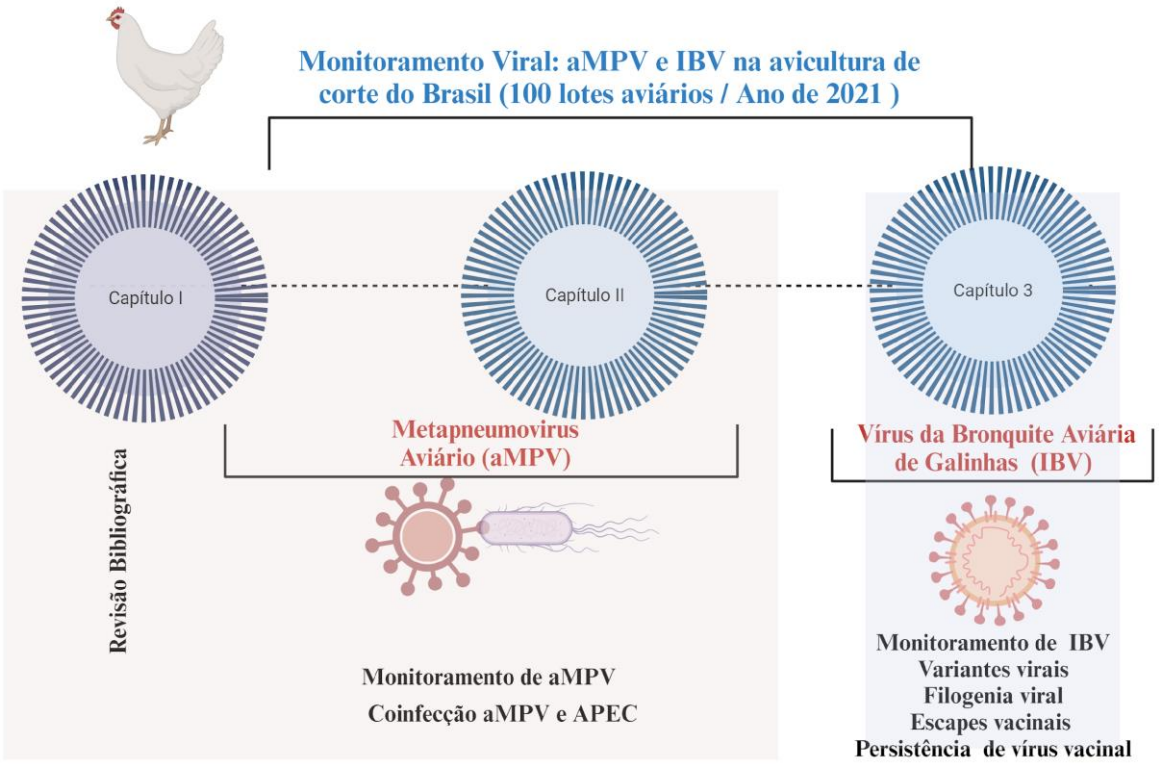


Figura 1. Esquema representativo da organização da presente tese doutoral, dividida em três capítulos, sendo: Capítulos I e II com foco em estudos de aMPV, obtendo-se dados que geraram uma revisão bibliográfica seguido de experimentos de diagnóstico para a detecção viral na avicultura de corte no Brasil, correlação com históricos sorológicos de aMPV e positividade para colibacilose, podendo-se avaliar os agravos clínicos. No terceiro capítulo o foco do estudo foi o estudo de IBV, estudando-se o monitoramento e a filogenia viral, bem como a persistência de vírus vacinais replicantes e escapes vacinais.

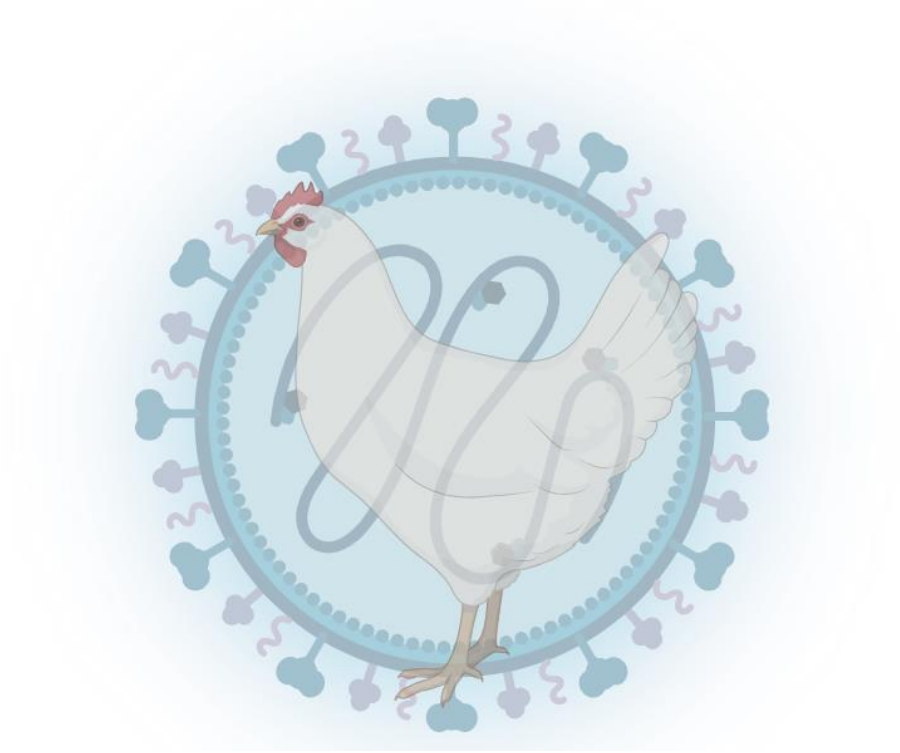
3. OBJETIVO GERAL

Monitorar a circulação de vírus de interesse respiratório (aMPV e IBV) e a coinfecção com *Escherichia coli* (APEC) em carcaças de *Gallus gallus domesticus* da avicultura de corte brasileira, bem como avaliar escapes vacinais em aves imunizadas contra IBV.

3.1 Objetivos Específicos

- Compor uma revisão bibliográfica abordando aspectos e desafios para o diagnóstico de aMPV;
- Avaliar a soroprevalência e a detecção gênica de aMPV subtipos A, B, C e D, em aves não vacinadas contra aMPV;
- Correlacionar agravos clínicos em caso de coinfecção entre aMPV e histórico de APEC nas aves avaliadas;
- Avaliar a ocorrência de IBV visando identificar novas variantes na população avícola, bem como compreender potenciais escapes vacinais virais;
- Avaliar a persistência de IBV vacinal em frangos de corte vacinados contra IBV.

CAPÍTULO I



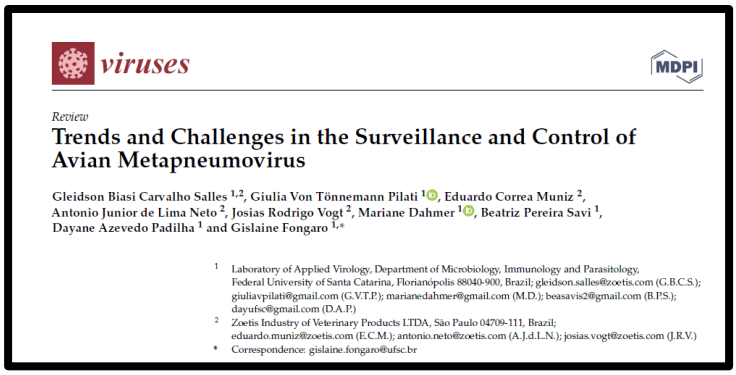
4. CAPÍTULO I:

Trends and challenges in the surveillance and control of Avian Metapneumovirus

Trends and challenges in the surveillance and control of Avian Metapneumovirus

Citation: Salles, G.B.C.; Pilati, G.V.T.; Muniz, E.C.; de Lima Neto, A.J.; Vogt, J.R.; Dahmer, M.; Savi, B.P.; Padilha, D.A.; Fongaro, G. Trends and Challenges in the Surveillance and Control of Avian Metapneumovirus. Viruses 2023, 15, 1960. https://doi.org/10.3390/v15091960

Artigo Publicado na Viruses (https://www.mdpi.com/1999-4915/15/9/1960).



viruses

MDPI

Review

Trends and Challenges in the Surveillance and Control of Avian Metapneumovirus

Gleidson Biasi Carvalho Salles [1,2], Giulia Von Tönnemann Pilati [1], Eduardo Correa Muniz [2], Antonio Junior de Lima Neto [2], Josias Rodrigo Vogt [2], Mariane Dahmer [1], Beatriz Pereira Savi [1], Dayane Azevedo Padilha [1] and Gislaine Fongaro [1,*]

[1] Laboratory of Applied Virology, Department of Microbiology, Immunology and Parasitology, Federal University of Santa Catarina, Florianópolis 88040-900, Brazil; gleidson.salles@zoetis.com (G.B.C.S.); giuliavpilati@gmail.com (G.V.T.P.); marianedahmer@gmail.com (M.D.); beasavis2@gmail.com (B.P.S.); dayufsc@gmail.com (D.A.P.)

[2] Zoetis Industry of Veterinary Products LTDA, São Paulo 04709-111, Brazil; eduardo.muniz@zoetis.com (E.C.M.); antonio.neto@zoetis.com (A.J.d.L.N.); josias.vogt@zoetis.com (J.R.V.)

* Correspondence: gislaine.fongaro@ufsc.br

Abstract: Among the respiratory pathogens of birds, the Avian Metapneumovirus (aMPV) is one of the most relevant, as it is responsible for causing infections of the upper respiratory tract and may induce respiratory syndromes. aMPV is capable of affecting the reproductive system of birds, directly impacting shell quality and decreasing egg production. Consequently, this infection can cause disorders related to animal welfare and zootechnical losses. The first cases of respiratory syndromes caused by aMPV were described in the 1970s, and today six subtypes (A, B, C, D, and two more new subtypes) have been identified and are widespread in all chicken and turkey-producing countries in the world, causing enormous economic losses for the poultry industry. Conventionally, immunological techniques are used to demonstrate aMPV infection in poultry, however, the identification of aMPV through molecular techniques helped in establishing the traceability of the virus. This review compiles data on the main aMPV subtypes present in different countries; aMPV and bacteria co-infection; vaccination against aMPV and viral selective pressure, highlighting the strategies used to prevent and control respiratory disease; and addresses tools for viral diagnosis and virus genome studies aiming at improving and streamlining pathogen detection and corroborating the development of new vaccines that can effectively protect herds, preventing viral escapes.

Keywords: Poutry virus; Virus variance; Diognóstic; Genome; Avian; Disease.

Introduction

Poultry farming has undergone great changes in its production systems; while the sheds where birds are housed and raised have better conditions for animal welfare and a high technological level, population densities have also increased proportionally [1], representing a large sanitary challenge. Although many measures are taken to control diseases, such as biosecurity, immunoprophylaxis, management and nutrition [2], this large population of animals in the same environment or shed becomes a risk factor for the health of the birds, which can lead to the emergence of diseases, most of which are respiratory. In this context, the flow of people, animals and migratory birds can pose a risk to the health of poultry batches [3], as this flow of people and animals can carry diseases into poultry facilities.

The blockade of respiratory diseases in birds presents a very large challenge for veterinarians, as these diseases usually do not show pathognomonic signs, that is, the clinical diagnosis is very complex [4]. Despite the presumptive diagnosis being difficult, the confirmatory diagnosis should be a common practice in the prevention of respiratory diseases. For this, a clear understanding of the means of transmission, viral incubation period, clinical signs and which material is most appropriate and when to collect it is necessary, in addition to choosing the best aMPV diagnostic method [5].

This category covers a spectrum of diseases, notably including Avian Influenza, New Castle Disease, Infectious Bronchitis of Chickens, Mycoplasmosis, Pasteurellosis, Infectious Laryngotracheitis and Avian Metapneumovirus [6].

The Avian Metapneumovirus (aMPV) is an important pathogen involved in diseases of the respiratory complex of birds, and although it is very neglected, its damage goes beyond respiratory symptoms and can affect the reproductive system [7,8,9,10,11], thus facilitating the development of other diseases, such as colibacillosis, and the association of these two diseases can cause serious damage to animals, where one disease can potentiate the effect of the other [12].

This review seeks to understand which the main subtypes are present in different countries, in addition to identifying which strategies are used to prevent and control this disease and the methods of prevention, control and viral ecology, based on the most elaborate studies to date. First, it discusses the main diagnostic tools that should be used to effectively assist in epidemiology and in the development of new tools that can protect poultry flocks, avoiding viral escapes and disease.

Characteristics of Viral Particles, Classification and Nomenclature

aMPV belongs to the Metapneumovirus genus of the Pneumoviridae family, it is an enveloped virus with non-segmented negative-stranded single-stranded RNA, pleomorphic spherical shape (diameters vary from 100 to 200 nm) and which can present long filaments, in addition to a helical nucleocapsid [13].

Early viral characterization through monoclonal serological assays showed some variability among aMPV strains [14,15,16,17] characterizing the molecular follow-up soon after, where genetic difference based on G protein variability was confirmed [18]. Later confirmed in several studies [19,20], this work used samples from the Central Veterinary Laboratory in Weybridge, United Kingdom. Two Subtypes, A and B, were identified, where subtype A samples came from the UK and subtype B samples from Italy and Hungary. These studies showed that the aMPV subtype A was the first to circulate in South Africa and later in the United Kingdom, and a few years later it was already possible to identify the subtype B present in Europe as well.

The aMPV genome is composed of eight viral genes (Figure 1), arranged in the order (3′-N-P-M-F-M2-SH-G-L-5′). These genes are identified as a nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix protein (M2), small hydrophobic protein (SH), surface glycoprotein (G) and a viral (L) RNA-dependent RNA polymerase, and these genes code for nine proteins [4,21,22].

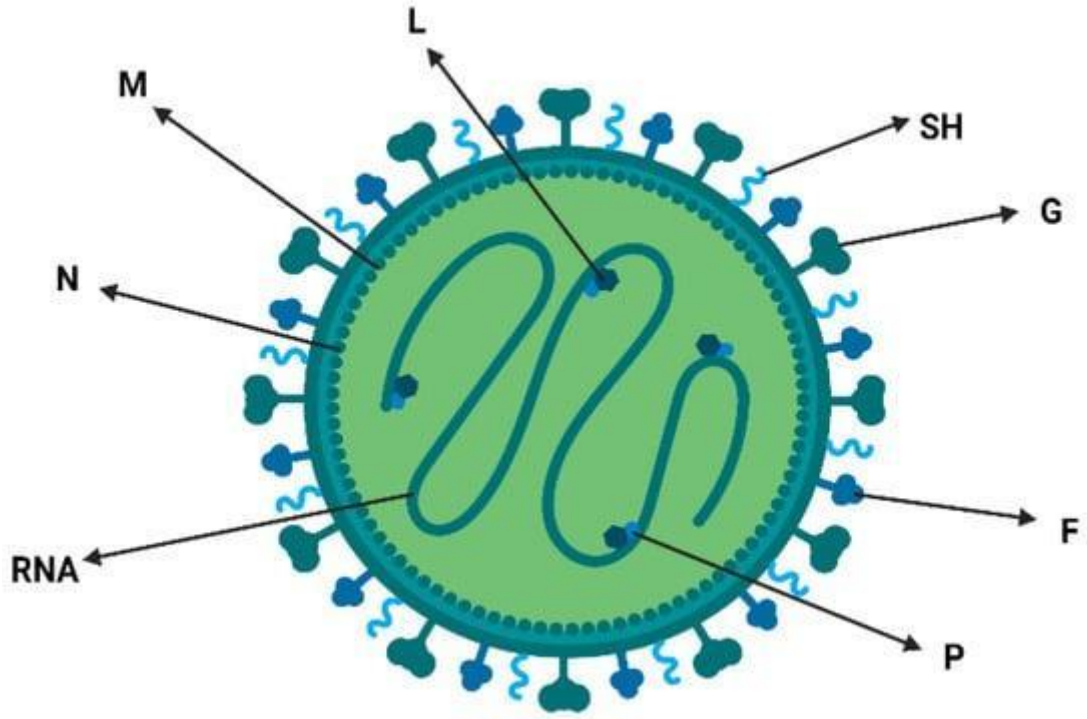


Figure 1. Schematic figure representing aMPV: (G) Glycoprotein, (F) Fusion protein, (SH) Small hydrophobic protein and other structural proteins, (M) Matrix protein, (N) Nucleocapsid protein, (P) Phosphoprotein, (L) RNA-dependent RNA polymerase and RNA strand.

Some genes, such as G, SH, M, N, P and F, present genetic heterogeneity and can be used to differentiate subtypes. However, the G protein, a highly glycosylated type II membrane protein known for its remarkable variation in length and sequence identity even within aMPV subtypes, can also show differences in size and nucleotide number between G gene sequences of the different subtypes. Due to this greater heterogeneity in relation to other genes, based on the analysis of the nucleotide sequences of the G gene, it is possible to perform the classification and characterization of the subtypes, as well as molecular epidemiological studies [4,22].

However, a study conducted in France described isolates of subtype D (aMPV-D) exhibiting a relatively low sequence identity in the G gene compared to subtypes aMPV-A, aMPV/B and aMPV-C [4]. While a study evaluating the use of quadriplex RT-qPCR reported that the G gene can be utilized for designing primers and probes for the detection of aMPV-A, aMPV-B and aMPV-D, the M gene was used for the detection of the aMPV-C subtype [23].

The classification of different aMPV into subtypes can be supported via the RT-ddPCR technique, which was developed using a region of low variability in the L

ORF, located between nucleotide positions 5′ 1980 and 5′ 2158, avoiding the limitations of previous tests. The result of this classification can be seen through the cutoff amplitude, set at ~6000 for CH1 and 3200 for CH2, the mean amplitude values for positive clusters in CH1 and CH2 were classified as aMPV samples in different subtypes. Thus, because it is based on a highly conserved region of the genome, there is less risk of the test being affected by the evolution of the viral genome and the impact it has on the specificity of such tests [24].

Protein N can be the target antigen in the development of serological assays, as it has the ability to induce a greater serological response in infected hosts. Comparison of the amino acid sequence of aMPVs indicated that within subtypes the N genes were 99–100% identical, even between viruses from different geographic regions. Amino acid identities between subtypes A and B were 90 to 91%, however, between subtypes C and A, B, or D, aa identities were only 70 to 71%, 70 to 72% and 73 to 74%, respectively [25].

Discovery and Distribution of Avian Metapneumovirus (aMPV)

The aMPV is a relatively new virus that affects turkeys and chickens and can also be found in guinea fowl [26], ducks [27] and pheasants [19]. The first report occurred in turkeys in South Africa in 1978 [28], a few years later the virus was found in chickens in England and classified as Swollen Head Syndrome (SHS) [29]. The aMPV rapidly spread across Europe, detected in the United Kingdom [30], France [31], Spain [30], Germany [32], Hungary [33], Italy [34,35], the United States [36] and Brazil [37]. Through epidemiological traceability, it was evident that the identification of subtype A was the first, however, within a few years the distribution of subtypes A and B was already identified in other countries, making control difficult [18].

In the early 1980s, the transmission between different species of birds was still unclear, but in 1987, through an experiment carried out by Picault et al., 1987, where they isolated a virus in chickens that became ill with aMPV; this virus was removed, homogenized from the respiratory tract tissue and was later inoculated into SPF turkeys and these showed characteristic clinical signs of avian Metapneumovirus, thus evidencing the transmission between chickens and turkeys [38].

The aMPV has a wide global distribution, essentially where there are poultry production or migratory bird routes the virus can be identified, but what changes is the

prevalence of subtypes A, B, C and D, in addition to the two new subtypes described: (a) subtypes A and B are likely to be found in Europe, Brazil and the African continent [39]; (b) the C subtype has been identified in the United States, Canada, China, France and recently in South Korea [40,41,42,43,44,45]; and (c) the D subtype was only reported in France [39] and the two new subtypes were found in the United States and Canada [45]. Today, the most prevalent subtype in the world is B [22,46].

Changes related to the G protein have an impact not only on subtyping characteristics, but also on virus replication in the target cell, thus changing its pathogenicity in the host [22].

Replication, Viral Persistence and Clinical Signs

The intense replication of the virus in the upper tract of birds (sinuses, larynx and trachea) causes the cessation of ciliary movements (ciliostasis), which can lead to the complete loss of these cilia (desciliation) [47]. This process results in difficulty in removing mucus, which accumulates in the passages and cavities, and gives rise to the main clinical sign of the disease, the swollen head, although this condition is not always present [22]. This primary infection favors the invasion of secondary agents, such as E. coli, which cause different clinical signs and whose intensity is linked to the pathogenicity of the agents involved. The aMPV is the cause of severe respiratory infection in turkeys, Turkey Rhinotracheitis (TRT), and usually occurs in young birds [48,49].

Common symptoms include sneezing, nasal and eye discharge, conjunctivitis, submandibular edema, infraorbital sinus swelling, cracking and rales [48,49,50]. In chickens, the virus has been associated with Swollen Head Syndrome (SHS), which is characterized by swelling of the periorbital and infraorbital sinuses, torticollis, disorientation and opisthotonos [50]. The clinical manifestation may progress to redness of the conjunctiva with edema of the lacrimal gland. After 12 to 24 h, the birds show a subcutaneous swelling on the head, which starts around the eyes, increases under the entire head and descends to the submandibular tissue and back of the neck. After three days, they may show neurological signs such as apathy and torticollis [48,49].

The permanence period of aMPV is extremely short in birds, not exceeding 4 to 7 days, which greatly impairs virus detection for molecular diagnosis [16,50].

Transmission and Economic Losses

The most common route of transmission of aMPV occurs horizontally through aerosol however, there are other paths taken by the virus until contact with birds, such as water, equipment, feed trucks that supply the farms and the transit of people [51].

So far, there is no clear evidence of vertical contamination through breeders to progeny [48,52,53]. In addition, migratory birds play an important role in the spread of the virus; there are reports of outbreaks of clinical cases of aMPV in birds in periods that coincide with the migration of wild birds [41]. The detection of antibodies in geese, house sparrows, gulls, parakeets, waterfowl and several other species suggests the circulation of this virus by wild birds [44,45,47,51], which reinforces the need for serological monitoring of these animals.

Economic losses in broilers due to respiratory complications related to aMPV alone or with secondary bacterial infections affect 1% to 3%, and 20% to 30% of cases, respectively [49]. In commercial batches, the first signs are mild respiratory failure, rhinitis and conjunctivitis, followed by neurological signs and swollen head. Reproductive alterations can be observed and alterations in the production or quality of the eggs can be common [7,50,54,55].

Morbidity and mortality are influenced by co-infections. When chickens show clinical signs, morbidity at all ages is often described as up to 100%, as mortality ranges from 0.4% to 50%, particularly in susceptible young birds [49].

Co-Infection of aMPV and Bacteria

Cases of aMPV often coincide with co-infection with Escherichia coli [7,48,49,50]. When such co-infection occurs, the clinical signs in birds tend to be more severe. This is because one agent can potentiate the action of the other, thereby increasing the overall pathogenicity of the clinical presentation [51,52,53,54,55,56,57].

Studies have shown high morbidity and exacerbation of the clinical picture in turkeys co-infected with aMPV and Mycoplasma gallisepticum [58], Ornithobacterium rhinotracheale [59] and lentogenic Newcastle disease virus [60]. Chickens

experimentally infected with aMPV and later infected with three different bacteria (Escherichia coli, Bordetella avium, Ornithobacterium rhinotracheale or a mixture of the three) were evaluated; animals infected with aMPV and the mixture of the three developed more severe clinical symptoms when compared to birds inoculated with aMPV or bacteria alone. The air sacs and lungs in this situation showed more severe alterations in birds inoculated with aMPV and Bordetella avium [61].

Infections by aMPV and Ornithobacterium rhinotracheale are one of the main problems related to the respiratory system in turkeys. Field cases and experimental studies have shown that the most common manifestations in cases of co-infection are airsacculitis and pneumonia [62,63,64].

Vaccination against aMPV and Viral Selective Pressure

The first vaccine used to control and prevent aMPV originated from a field strain called UK/3B/85 belonging to subtype A [65,66]. Until 1995 only vaccines belonging to subtype A were used in the UK, and the prevalence of this subtype appears to have declined, in contrast, what has been observed is an increase in the prevalence of aMPV subtype B [67].

This possible selection by vaccine pressure was demonstrated in a study in which eight aMPV strains (pre-1994) and six aMPV strains were evaluated between 2001 and 2007, with these samples coming from the Veneto region, Italy.

The strains, when compared, showed genetic mutations in specific amino acids of glycoprotein G, and the prevalence was of aMPV subtype B and the vaccines used also belonged to this same subtype [11]. These genetic mutations in glycoprotein G may favor viral escapes, as vaccines will present partial coverage, which would reduce their effectiveness when used.

Although vaccines played an excellent role in controlling aMPV with a homologous subtype, the pressure exerted by vaccines on the environment may have helped in the dominance of another subtype, in this case B. In addition to vaccine pressure, hosts and environment can help in this process. In studies [68,69,70,71], the heterologous protection capacity between vaccines of different subtypes is demonstrated, although it is susceptible to viral escapes.

There are two types of live vaccines available on the market, one subtype A and the other subtype B, information in the literature indicates that both products provide good cross-protection [68,69,70,71].

Other very important measures, such as a rigorous biosecurity program and proper management, including bird density, litter conditions, sanitary intervals, cleaning and disinfection, multiple ages and environmental conditions (ventilation, temperature variations) are also of great importance for the successful control of this disease [72]. Thus, in high-risk regions, vaccination together with a biosecurity program is an indispensable part of the strategic control of aMPV. The introduction of vaccines into immunoprophylaxis programs is not a simple activity, as in addition to the costs involved, there is an enormous limitation of labor for the application of vaccines, whether through mass (spray, drinking water) or individual (ocular or intramuscular) application, which require greater attention, as in these cases vaccines are applied bird by bird, which often limits the use of this method. [73].

Vaccination programs can be used through different strategies, it is important to know how they work and what the final objective is of protection for aMPV. It is possible to use replicating (live) and inactivated (non-replicating) vaccines, and it is important to respect the purpose of each tool. Replicating vaccines end up stimulating both a cellular and humoral response. When necessary, these vaccines can be used in broiler chickens from the first day of life. Non-replicating vaccines are widely used in long-lived birds (broiler-breeders). Non-replicating vaccines induce greater production of circulating antibodies (IgY) mainly to protect the reproductive tract of birds in addition to reducing viral excretion [74]. In long-lived birds, the ideal would be to associate the two technologies, where the replicating vaccine would serve as a primer for the non-replicating vaccine, providing broader and more uniform protection for birds [75].

aMPV has been considered a relatively slow-evolving virus when compared to other avian RNA viruses, however, other studies estimate that this rate of viral evolution is within the normal range [76,77,78]. Viral evolution is based both on the pressure exerted by vaccine programs and on the type of host and the environment, since different strains belonging to the same subtype circulate phenotypically in different regions of the world [78].

Thus, the vaccines used for the prevention and control of aMPV, although capable of reducing clinical conditions and viral dissemination, are not equally effective

in preventing aMPV infection and circulation, contributing to viral persistence within a given region or country [78].

It is very important to monitor the animals, this includes indirect methods such as serology, but molecular diagnosis is essential to identify the prevalence of the subtype, which can help in making more assertive decisions [79,80].

Methodological Trends for the Discovery of New Viral Strains in Poultry

Molecular techniques such as PCR or RT-qPCR, Sanger sequencing and next generation sequencing (NGS), have demonstrated their clinical and epidemiological importance through the identification and genetic typing of pathogens [80,81,82,83]. The NGS and Sanger sequencing techniques are distinguished by their ability to identify mutations during the sequencing process, where Sanger is used to identify short DNA sequences (500 to 900 base pairs), while NGS is capable of sequencing 50 to 900 base pairs 300 nucleotides in length, in addition to its ability to read billions of genetic fragments at the same time [84]. While the Sanger sequencing technique is confined, in clinical practice, to the detection of point mutations, NGS has introduced significant innovations. Although NGS does have some limitations related to the potential for errors in genomic regions with repetitive nucleotide bases, it stands out for its enhanced capability to analyze mutations during the same process, as well as its greater efficiency and speed in generating and identifying results [81,82,83].

NGS techniques seem more accurate in identifying not only point mutations, but also already circulating or new variants (whole genomes) of infectious and contagious pathogens, such as aMPV [83,84]. NGS is characterized via DNA or cDNA sequencing, which can generate short or long reading fragments, depending on the methodology used. The most used NGS platforms for respiratory virus detection are: Illumina sequencers, Life Technologies sequencers, Oxford Nanopore sequencers and Roche (Metapneumovirus) sequencers [84,85].

Considering that aMPV is one of the most important respiratory agents in birds and associated with economic losses in production, and because it is an RNA virus, where mutations and recombinations occur at higher rates, genomic surveillance is an

important tool for tracking the dissemination of variants and monitoring of genetic alterations [84,85,86,87,88,89].

Currently, serological methods are widely used for screening and field monitoring of batches where serum antibody titers are detected. However, molecular techniques, mainly RT-qPCR, are widely used for aMPV detection, where primers are usually designed to amplify the G gene region both for viral detection and for the identification of subtypes, as it is a region of substantial heterogeneity [88].

In Brazil, there are studies describing the circulation of the two main subtypes of aMPV, A and B. However, it is important to highlight that the epidemiological survey of the pathogen is outdated, as there is no genomic surveillance system implemented in the country. Therefore, whole-genome sequencing techniques, such as next-generation sequencing (NGS), are essential for the identification of small mutations in the aMPV genome, which may lead to vaccine inefficiency, as well as for the development of vaccines [87,88,89,90].

Kariithi et al. (2022) used the Illumina MiSeq platform to sequence complete genomes of aMPV subtype A in broilers from Mexico [90]. Based on the recent impact caused by aMPV at the clinical and economic levels in Europe, the platform reconstructed the phylogeny and viral dispersion based on sequences deposited in GenBank from 1985 to 2019 and identified the heterogeneity of circulating strains among the countries analyzed and, although the authors did not report any significant host adaptation, there was a shift in bloodlines between turkeys, guinea fowls and chickens; this heterogeneity can lead to low coverage and vaccine failures [22].

Unfortunately, despite the visible need for more accurate genomic surveillance, genetic analysis of aMPV is still very scarce. Currently, studies with SNG are more focused on human Metapneumovirus, with birds being the majority analyzed via RT-PCR (Table 1).

Table 1. Epidemiological analysis studies based on aMPV genomic analysis.

Target	Sample	Methodology	Country	aMPV Subtype	Publication Year	Author
Gene—G	Cloacal/throat double swabs	RT-PCR	China		2022	[91]
Gene—G and Protein G	Choanal cleft swab	ELISA	North Vietnam		2021	[92]
		RT-PCR		aMPV B	2021	
Genes—G, N e M	Choanal cleft swab	RT-PCR	Iran	aMPV B	2017	[93]
Gene—G	Respiratory tract swabs	RT-PCR	Northern Italy	aMPV B	2018	[86]
Gene—M	Oropharyngeal and cloacal swabs	RT-PCR	Canada	aMPV C	2018	[45]
Gene—G	Tracheal swabs	RT-PCR and Sanger sequencing	Greece	aMPV B	2019	[94]
Gene—G	Throat swabs	-	China	aMPV B	2019	[95]
Gene—N, M, F, L, M2, SH e G	Tissues swabbed (choana, lung)	Illumina sequencing	Mexico	aMPV A	2022	[96]
Genome	Uninformed	NGS (Illumina MiSeq)	Hungary	aMPV B	2020	[97]
Gene—G	Tracheal and cloacal swabs	RT-PCR and Sanger sequencing	Brazil	aMPV A and B	2011	[72]

The studies presented here affirm the importance of effective genomic surveillance, including for aMPV, which can influence prevention, clinical and economic improvement in animals for producers, as well as improvements in animal diagnosis and therapy and the development of more effective vaccines that have greater protection coverage compared to current aMPV vaccines.

Conclusions

The evolution of molecular techniques for viral diagnosis, mainly of aMPV, has played an important role in fast and accurate viral detection, understanding the epidemiology and helping to inform the best control strategies over the years. Although there are important tools to support and assist in accurate diagnosis, it is essential to evolve further in the detection and control of this disease. In this sense, in addition to traditional serological and molecular methods, we emphasize genomic sequencing which has allowed the broad characterization of the genetic variability of the virus, the detection of mutations, and the identification of new variants, as well as allowing the simultaneous evaluation of pathogens present in the same sample in cases of co-infections. This approach has allowed us to understand the evolution of viruses over time and can be used to evaluate the efficiency of vaccination programs in poultry.

It is evident that the control of aMPV requires a holistic view, focused on knowledge of the agent, epidemiological surveillance, effective diagnosis, adequate immunoprophylactic programs and constant discussing of the precepts of biosafety,

thus, a new approach must be used for control and disease prevention, mainly respiratory, in birds.

References

1. Goossens, E.; Dehau, T.; Ducatelle, R.; Van Immerseel, F. Omics technologies in poultry health and productivity—Part 2: Future applications in the poultry industry. Avian Pathol. 2022, 51, 418–423. [Google Scholar] [CrossRef] [PubMed]
2. Dehau, T.; Ducatelle, R.; Van Immerseel, F.; Goossens, E. Omics technologies in poultry health and productivity—Part 1: Current use in poultry research. Avian Pathol. 2022, 51, 407–417. [Google Scholar] [CrossRef] [PubMed]
3. Graziosi, G.; Mescolini, G.; Silveira, F.; Lupini, C.; Tucciarone, C.M.; Franzo, G.; Cecchinato, M.; Legnardi, M.; Gobbo, F.; Terregino, C.; et al. First detection of avian metapneumovirus subtype C Eurasian lineage in a Eurasian wigeon (Mareca penelope) wintering in Northeastern Italy: An additional hint on the role of migrating birds in the viral epidemiology. Avian Pathol. 2022, 51, 283–290. [Google Scholar] [CrossRef] [PubMed]
4. Kaboudi, K.; Lachheb, J. Avian metapneumovirus infection in turkeys: A review on turkey rhinotracheitis. J. Appl. Poult. Res. 2021, 30, 100211. [Google Scholar] [CrossRef]
5. Zaffuto, K.M.; Estevez, C.N.; Afonso, C.L. Primary chicken tracheal cell culture system for the study of infection with avian respiratory viruses. Avian Pathol. 2008, 37, 25–31. [Google Scholar]
6. Croville, G.; Foret, C.; Heuillard, P.; Senet, A.; Delpont, M.; Mouahid, M.; Ducatez, M.F.; Kichou, F.; Guerin, J.-L. Disclosing respiratory co-infections: A broad-range panel assay for avian respiratory pathogens on a nanofluidic PCR platform. Avian Pathol. 2018, 47, 253–260. [Google Scholar] [CrossRef]
7. Cook, J.K. Avian rhinotracheitis. Rev. Sci. Tech. OIE 2000, 19, 602–613. [Google Scholar]
8. Cook, J.K.; Cavanagh, D. Detection and differentiation of avian pneumoviruses. Avian Pathol. 2002, 31, 117–132. [Google Scholar]
9. Sugiyama, M.; Koimaru, H.; Shiba, M.; Ono, E.; Nagata, T.; Ito, T. Drop of egg production in chickens by experimental infection with an avian metapneumovirus

strain PLE8T1 derived from swollen head syndrome and the application to evaluate vaccine. J. Vet. Med. Sci. 2006, 68, 783–787. [Google Scholar]

10. Alexander, D.J.; Jones, R.C. Paramyxoviridae. In Poultry Diseases; Pattison, M., McMullin, P., Bradbury, J.M., Alexander, D., Eds.; Saunders Elsevier Publ.: London, UK, 2008; pp. 294–316. [Google Scholar]
11. Cecchinato, M.; Catelli, E.; Lupini, C.; Ricchizzi, E.; Clubbe, J.; Battilai, M.; Naylor, C. Avian metapneumovirus (AMPV) attachment protein involvement in probable virus evolution concurrent with mass live vaccine introduction. Veter.-Microbiol. 2010, 146, 24–34. [Google Scholar] [CrossRef]
12. Giovanardi, D.; Lupini, C.; Pesente, P.; Rossi, G.; Ortali, G.; Catelli, E. Longitudinal field studies of Avian Metapneumovirus and Turkey Hemorrhagic Enteritis Virus in turkeys suffering from colibacillosis associated mortality. Vet. Res. Commun. 2014, 38, 129–137. [Google Scholar] [CrossRef] [PubMed]
13. Afonso, C.L.; Amarasinghe, G.K.; Bányai, K.; Bào, Y.; Basler, C.F.; Bavari, S.; Bejerman, N.; Blasdell, K.R.; Briand, F.-X.; Briese, T.; et al. Taxonomy of the order Mononegavirales: Update 2016. Arch. Virol. 2016, 161, 2351–2360. [Google Scholar] [CrossRef] [PubMed]
14. Hafez, H.M. Comparative investigation on different turkey rhinotracheitis (TRT) virus isolates from different countries. Dtsch. Tierarztl. Wochenschr. 1992, 99, 486–488. [Google Scholar] [PubMed]
15. Jing, L.; Cook, J.K.A.; David, T.; Brown, K.; Shaw, K.; Cavanagh, D. Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. Avian Pathol. 1993, 22, 771–783. [Google Scholar] [CrossRef]
16. Cook, J.K.A.; Kinloch, S.; Ellis, M.M. In vitro and in vivo studies in chickens and turkeys on strains of turkey rhinotracheitis virus isolated from the two species. Avian Pathol. 1993, 22, 157–170. [Google Scholar] [CrossRef]
17. Collins, M.S.; Gough, R.E.; Alexander, D.J. Antigenic differentiation of Avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. Avian Pathol. 1993, 22, 469–479. [Google Scholar]
18. Juhasz, K.; Easton, A.J. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: Evidence for two distinct subgroups. J. Gen. Virol. 1994, 75, 2873–2880. [Google Scholar] [CrossRef]

19. Milwright, R.D.P.; Toquin, D.; Bayon-Auboyer, M.H.; Jestin, V.; Morin, H. Isolation of a pneumovirus from a Muscovy duck. Vet. Rec. 1999, 145, 680. [Google Scholar]
20. Seal, B.S.; Sellers, H.S.; Meinersmann, R.J. Fusion protein predicted amino acid sequence of the first US Avian pneumovirus isolate and lack of heterogeneity among other US isolates. Virus Res. 2000, 66, 139–147. [Google Scholar]
21. Easton, A.J.; Domachowske, J.B.; Rosenberg, H.F. Animal Pneumoviruses: Molecular genetics and pathogenesis. Clin. Microbiol. Rev. 2004, 17, 390–412. [Google Scholar] [CrossRef]
22. Franzo, G.; Legnardi, M.; Mescolini, G.; Tucciarone, C.M.; Lupini, C.; Quaglia, G.; Catelli, E.; Cecchinato, M. Avian Metapneumovirus subtype B around Europe: A phylodynamic reconstruction. Vet. Res. 2020, 51, 88. [Google Scholar] [CrossRef] [PubMed]
23. Lemaitre, E.; Bougeard, S.; Allée, C.; Eterradossi, N.; Courtillon, C.; Brown, P.A. Establishment and application of a quadruple real-time RT-PCR for detecting avian metapneumovirus. PLoS ONE 2022, 17, e0270708. [Google Scholar] [CrossRef]
24. Lemaitre, E.; Bougeard, S.; Allée, C.; Eterradossi, N.; Courtillon, C.; Brown, P.A. Avian metapneumovirus: A five-plex digital droplet RT-PCR method for identification of subgroups A, B, C, and D. Front. Veter. Sci. 2022, 9, 1058294. [Google Scholar] [CrossRef] [PubMed]
25. Xu, W.; Suderman, M.; Koziuk, J.; Ojkic, D.; Berhane, Y. Development of A recombinant nucleocapsid based indirect ELISA for the detection of antibodies to avian metapneumovirus subtypes, A, B, and C. Veter.-Immunol. Immunopathol. 2020, 231, 110151. [Google Scholar] [CrossRef] [PubMed]
26. Cecchinato, M.; Lupini, C.; Silveira, F.; Listorti, V.; Mescolini, G.; Morandini, E.; Franzo, G.; Catelli, E. Molecular characterization of avian metapneumovirus from Guinea fowls (Numida meleagridis). Pak. Vet. J. 2018, 38, 419–423. [Google Scholar] [CrossRef]
27. Catelli, E.; De Marco, M.A.; Delogu, M.; Terregino, C.; Guberti, V. Serological evidence of Avian pneumovirus infection in reared and free-living pheasants. Vet. Rec. 2001, 149, 56–58. [Google Scholar] [CrossRef]

28. Buys, S.B. A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. Turkeys 1980, 28, 36. [Google Scholar]
29. Morley, A.J.; Thomson, D.K. Swollen-head syndrome in broiler chickens. Avian Dis. 1984, 28, 238–243. [Google Scholar] [CrossRef]
30. Anon, K.R. Turkey rhinotracheitis of unknown aetiology in England and Wales. Vet. Rec. 1985, 117, 653–654. [Google Scholar]
31. Giraud, P.; Bennejean, G.; Guittet, M.; Toquin, D. Turkey rhinotracheitis in France: Preliminary investigations on a ciliostatic virus. Vet. Rec. 1986, 119, 606–607. [Google Scholar]
32. Hafez, H.M.; Woernle, H. Turkey rhinotracheitis, serological results in Baden-Wurttemnberg. Tierarztl. Umsch. 1989, 44, 476–480. [Google Scholar]
33. Lantos, C. Actual problems of poultry hygenes. Bfiteny. Es Feldogozas 1990, 37, 54–58. [Google Scholar]
34. Fabris, G.; D'aprile, P.N. Rinotracheite infettiva del tacchino: Osservazioni sul campo ed indagini di laboratorio. Zootec. Int. 1990, 6, 36–40. [Google Scholar]
35. Catelli, E.; Cecchinato, M.; Delogu, M.; De Matteo, P.; Ortali, G.; Franciosi, C.; De Marco, M.A.; Naylor, C.J. Avian pneumovirus infection in turkey and broiler farms in Italy: A virological, molecular and serological field survey. Ital. J. Anim. Sci. 2004, 3, 287–292. [Google Scholar] [CrossRef]
36. Senne, D.; Edson, J.C.; Pederson, B.P. Avian pneumovirus update. In Proceedings of the American Veterinary Medical Association, 134th Annual Congress, Reno, NV, USA, 19–24 July 1997; p. 190. [Google Scholar]
37. Dani, M.A.; Arns, C.W.; Durigon, E.L. Molecular characterization of Brazilian Avian pneumovirus isolates using reverse transcription-polymerase chain reaction, restriction endonuclease analysis and sequencing of a G gene fragment. Avian Pathol. 2016, 28, 473–476. [Google Scholar] [CrossRef]
38. Picault, J.P.; Giraud, P.; Drouin, P.; Gulttet, M.; Bennejean, G.; Lamande, J.; Toquin, D.; Gueguen, C. Isolation of a TRTV-like virus from chickens with swollen head syndrome. Vet. Rec. 1987, 121, 135. [Google Scholar] [CrossRef]
39. Umar, S.; Teillaud, A.; Aslam, H.B.; Guerin, J.L.; Ducatez, M.F. Molecular epidemiology of respiratory viruses in commercial chicken flocks in Pakistan from 2014 through to 2016. BMC Vet. Res. 2019, 15, 351. [Google Scholar] [CrossRef]

40. Seal, B.S. Avian pneumoviruses and emergence of a new type in the United States of America. Anim. Health Res. Rev. 2000, 1, 67–72. [Google Scholar] [CrossRef]
41. Shin, H.J.; Cameron, K.T.; Jacobs, J.A.; Turpin, E.A.; Halvorson, D.A.; Goyal, S.M.; Nagaraja, K.V.; Kumar, M.C.; Lauer, D.C.; Seal, B.S.; et al. Molecular epidemiology of subgroup C avian pneumoviruses isolated in the United States and comparison with subgroup a and B viruses. J. Clin. Microbiol. 2002, 40, 1687–1693. [Google Scholar] [CrossRef]
42. Lee, E.H.; Song, M.-S.; Shin, J.-Y.; Lee, Y.-M.; Kim, C.-J.; Lee, Y.S.; Kim, H.; Choi, Y.K. Genetic characterization of avian metapneumovirus subtype C isolated from pheasants in a live bird market. Virus Res. 2007, 128, 18–25. [Google Scholar] [CrossRef]
43. Sun, S.; Chen, F.; Cao, S.; Liu, J.; Lei, W.; Li, G.; Song, Y.; Lu, J.; Liu, C.; Qin, J.; et al. Isolation and characterization of a subtype C avian metapneumovirus circulating in Muscovy ducks in China. Vet. Res. 2014, 45, 74. [Google Scholar] [CrossRef] [PubMed]
44. Jardine, C.M.; Parmley, E.J.; Buchanan, T.; Nituch, L.; Ojkic, D. Avian metapneumovirus subtype C in Wild Waterfowl in Ontario, Canada. Transbound. Emerg. Dis. 2018, 65, 1098–1102. [Google Scholar] [CrossRef] [PubMed]
45. Retallack, H.; Clubb, S.; DeRisi, J.L. Genome sequence of a divergent avian metapneumovirus from a Monk Parakeet (Myiopsitta monachus) Microbiol. Resour. Announc. 2019, 8, e00284-19. [Google Scholar]
46. Kaboudi, K. Virus-induced immunosuppression in turkeys (Meleagris gallopavo): A review. Open Vet. J. 2019, 9, 349–360. [Google Scholar] [CrossRef]
47. Canuti, M.; Kroyer, A.N.; Ojkic, D.; Whitney, H.G.; Robertson, G.J.; Lang, A.S. Discovery and characterization of novel RNA viruses in aquatic North American wild birds. Viruses 2019, 11, 768. [Google Scholar] [CrossRef]
48. Cook, J.K.A. Cook Avian pneumovirus infections of turkeys and chickens. Vet. J. 2000, 160, 118–125. [Google Scholar] [CrossRef]
49. Rautenschlein, S. Avian metapneumovirus. In Diseases of Poultry, 14th ed; Swayne, D.E., Ed.; Wiley Blackwell: Hoboken, NJ, USA, 2020; pp. 135–143. [Google Scholar]
50. Cook, J.K.; Huggins, M.B.; Orbell, S.J.; Mawditt, K.; Cavanagh, D. Infectious bronchitis virus vaccine interferes with the replication of Avian pneumovirus

vaccine in domestic fowl. Avian Pathol. 2001, 30, 233–242. [Google Scholar] [CrossRef]

51. Jones, R.C.; Rautenschlein, S. Avian metapneumovirus. In Diseases of Poultry; Wiley-Blackwell: Ames, IA, USA, 2013; pp. 125–138. [Google Scholar]
52. Jones, R.C.; Williams, R.A.; Baxter-Jones, C.; Savage, C.E.; Wilding, G.P. Experimental infection of laying turkeys with rhinotracheitis virus: Distribution of virus in the tissues and serological response. Avian Pathol. 1988, 17, 841–850. [Google Scholar] [CrossRef]
53. Shin, H.J.; Rajashekara, G.; Jirjis, F.F.; Shaw, D.P.; Goyal, S.M.; Halvorson, D.A.; Nagaraja, K.V. Specific detection of Avian pneumovirus (APV) US isolates by RT-PCR. Arch. Virol. 2000, 145, 1239–1246. [Google Scholar] [CrossRef]
54. Arns, C.W.; Zuanaze, M.; Berchieri, J.A.; Silva, E.N.; Di Fabio, J.; Sesti, L.; Zuanaze, M.A.F. Doenças das Aves. Metapneumovírus Aviário, 2nd ed.; FACTA: Campinas, Brazil, 2009; pp. 777–783. [Google Scholar]
55. Gough, R.E.; Jones, R.C. Diseases of Poultry, 12th ed.; Avian Metapneumovirus; Iowa State Press: Ames, IA, USA, 2008; pp. 101–110. [Google Scholar]
56. Samy, A.; Naguib, M. Avian Respiratory Coinfection and Impact on Avian Influenza Pathogenicity in Domestic Poultry: Field and experimental findings. Vet. Sci. 2018, 5, 23. [Google Scholar] [CrossRef]
57. Mary, J.; Jackwood, P.; Erica, S. Multicausal Respiratory Diseases In Diseases of Poultry, 14th ed.; Swayne David, E., Ed.; John Wiley & Sons: Hoboken, NJ, USA, 2019. [Google Scholar]
58. Naylor, C.J.; Al-Ankari, A.R.; Al-Afaleq, A.I.; Bradbury, J.M.; Jones, R.C. Exacerbation of Mycoplasma gallisepticum infection in Turkeys by rhinotracheitis virus. Avian Pathol. 1992, 21, 295–305. [Google Scholar] [CrossRef] [PubMed]
59. Marien, M.; Decostere, A.; Martel, A.; Chiers, K.; Froyman, R.; Nauwynck, H. Synergy between Avian pneumovirus and Ornithobacterium rhinotrachealein turkeys. Avian Pathol. 2005, 34, 204–211. [Google Scholar] [CrossRef] [PubMed]
60. Turpin, E.A.; Perkins, L.E.L.; Swayne, D.E. Experimental Infection of Turkeys with Avian Pneumovirus and Either Newcastle Disease Virus or Escherichia coli. Avian Dis. 2002, 46, 412–422. [Google Scholar] [CrossRef] [PubMed]
61. Jirjis, F.F.; Noll, S.L.; Halvorson, D.A.; Nagaraja, K.V.; Martin, F.; Shaw, D.P. Effects of Bacterial Coinfection on the Pathogenesis of Avian Pneumovirus Infection in Turkeys. Avian Dis. 2004, 48, 34–49. [Google Scholar] [CrossRef]

62. Hafez, M.; Awad, A.; Ger, S. Turkey production and health: Current challenges Authors. J. Vet. Res. 2021, 1, 3–14. [Google Scholar] [CrossRef]
63. van Empel, P.C.M.; HafezH, M. Ornithobacterium rhinotracheale: A review. Avian Pathol. 1999, 28, 217–227. [Google Scholar] [CrossRef]
64. Paudel, S.; Easwaran, M.; Jang, H.; Jung, H.-K.; Kim, J.-H.; Shin, H.-J. Immunization with avian metapneumovirus harboring chicken Fc induces higher immune responses. Virus Res. 2016, 220, 129–135. [Google Scholar] [CrossRef]
65. Cook, J.K.; Ellis, M.M.; Dolby, C.A.; Holmes, H.C.; Finney, P.M.; Huggins, M.B. A live attenuated turkey rhinotracheitis virus vaccine. 1. Stability of the attenuated strain. Avian Pathol. 1989, 18, 511–522. [Google Scholar] [CrossRef]
66. Cook, J.K.A.; Marjorie, M.E. Attenuation of turkey rhinotracheitis virus by alternative passage in embryonated chicken eggs and tracheal organ cultures. Avian Pathol. 1990, 19, 181–185. [Google Scholar] [CrossRef]
67. Naylor, C.; Shaw, K.; Britton, P.; Cavanagh, D. Appearance of type B Avian pneumovirus in great Britain. Avian Pathol. 1997, 26, 327–338. [Google Scholar] [CrossRef]
68. Cook, J.K.; Huggins, M.B.; Woods, M.A.; Orbell, S.J.; Mockett, A.P. Protection provided by a commercially available vaccine against different strains of turkey rhinotracheitis virus. Vet. Rec. 1995, 136, 392–393. [Google Scholar] [CrossRef] [PubMed]
69. Cook, J.K.; Huggins, M.B.; Orbell, S.J.; Senne, D.A. Preliminary antigenic characterization of an Avian pneumovirus isolated from commercial turkeys in Colorado, USA. Avian Pathol. 1999, 28, 607–617. [Google Scholar] [CrossRef] [PubMed]
70. Toquin, D.; Bäyon-Auboyer, M.; Jestin, V.; Eterradossi, N. Résponse Sérologique et Protection Croisée Visa vis de L'infection par Une Souche Non-A Non-B du Virus de La Rhinotrachéite Infectieuse De La Dinde; Comptes Rendus des 3emes Journées de la Recherche Avicole: St Malo, France, 1999; pp. 223–224. [Google Scholar]
71. Christopher, B.; Basim, M.; Andreas, H.; Stephane, L.; Kannan, G. Vaccination against Avian metapneumovirus subtype B in commercial broiler chicks: Heterologous protection and selected host transcriptional responses to subtype A or B challenge. Avian Pathol. 2022, 51, 181–196. [Google Scholar] [CrossRef]

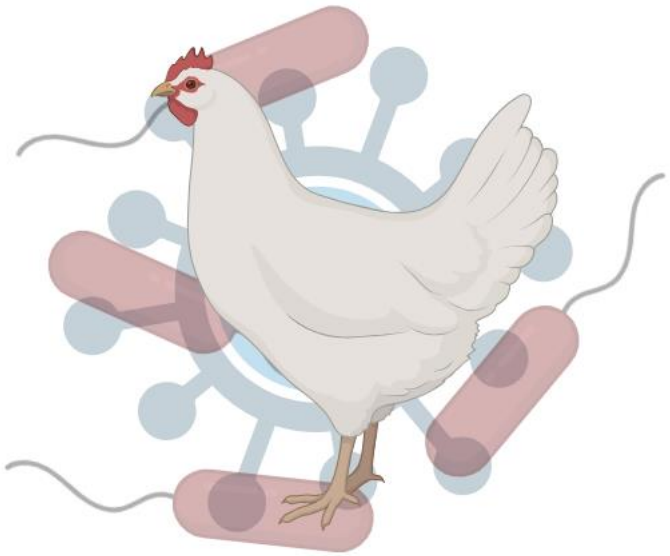
72. Felippe, P.A.; Silva, L.H.A.; Santos, M.B.; Sakata, S.T.; Arns, C.W. Detection of and phylogenetic studies with avian metapneumovirus recovered from feral pigeons and wild birds in Brazil. Avian Pathol. 2011, 40, 445–452. [Google Scholar] [CrossRef]
73. Ganapathy, K.; Bufton, A.; Pearson, A.; Lemiere, S.; Jones, R.C. Vaccination of commercial broiler chicks against avian metapneumovirus infection: A comparison of drinking-water, spray and oculo-oral delivery methods. Vaccine 2010, 28, 3944–3948. [Google Scholar] [CrossRef]
74. Tamam, S.M.; Hussein, A.S.; Arafa, A.M.; Madbouly, H.M. Preparation and evaluation of inactivated avian Metapneumovirus vaccine from recently isolated Egyptian strain. J. Appl. Poult. Res. 2015, 24, 168–176. [Google Scholar] [CrossRef]
75. Cook, J.K.A.; Orthel, F.; Orbell, S.; Woods, M.A.; Huggins, M.B. An experimental turkey rhinotracheitis (TRT) infection in breeding turkeys and the prevention of its clinical effects using live-attenuated and inactivated TRT vaccines. Avian Pathol. 1996, 25, 231–243. [Google Scholar] [CrossRef]
76. Duffy, S.; Shackelton, L.; Holmes, E. Rates of evolutionary change in viruses: Patterns and determinants. Nat. Rev. Genet. 2008, 9, 267–276. [Google Scholar] [CrossRef]
77. Franzo, G.; Massi, P.; Tucciarone, C.M.; Barbieri, I.; Tosi, G.; Fiorentini, L.; Ciccozzi, M.; Lavazza, A.; Cecchinato, M.; Moreno, A. Think globally, act locally: Phylodynamic reconstruction of infectious bronchitis virus (IBV) QX genotype (GI-19 lineage) reveals different population dynamics and spreading patterns when evaluated on different epidemiological scales. PLoS ONE 2017, 12, e0184401. [Google Scholar] [CrossRef]
78. Franzo, G.; Cecchinato, M.; Tosi, G.; Fiorentini, L.; Faccin, F.; Tucciarone, C.M.; Trogu, T.; Barbieri, I.; Massi, P.; Moreno, A. GI-16 lineage (624/I or Q1), there and back again: The history of one of the major threats for poultry farming of our era. PLoS ONE 2018, 13, e0203513. [Google Scholar] [CrossRef]
79. Rubbenstroth, D.; Rautenschlein, S. Investigations on the protective role of passively transferred antibodies against avian metapneumovirus infection in turkeys. Avian Pathol. 2009, 38, 427–436. [Google Scholar] [CrossRef] [PubMed]
80. Yohe, S.; Thyagarajan, B. Review of Clinical Next-Generation Sequencing. Arch. Pathol. Lab. Med. 2017, 141, 1544–1557. [Google Scholar] [CrossRef] [PubMed]

81. Du, S.H.; Li, D.R.; Wang, H.J.; Wang, Q. Application of RT-qPCR in the Study of Forensic Pathology. Fa Yi Xue Za Zhi 2017, 33, 526–531. (In Chinese) [Google Scholar] [CrossRef] [PubMed]
82. Behjati, S.; Tarpey, P.S. What is next generation sequencing? Arch. Dis. Child. Educ. Pract. Ed. 2013, 98, 236–238. [Google Scholar] [CrossRef]
83. Goodwin, S.; Mcpherson, J.D.; Mccombie, W.R. Coming of age: Ten years of next-generation sequencing technologies. Nat. Rev. Genet. 2016, 17, 333–351. [Google Scholar] [CrossRef]
84. Schuster, J.E.; Williams, J.V. Human metapneumovirus. Pediatr. Rev. 2013, 34, 558–565. [Google Scholar] [CrossRef]
85. Hodinka, R.L. Respiratory RNA Viruses. Microbiol. Spectr. 2016, 4, 233–271. [Google Scholar] [CrossRef]
86. Chacón, J.L.; Mizuma, M.; Vejarano, M.P.; Toquín, D.; Eterradossi, N.; Patnayak, D.P.; Goyal, S.M.; Ferreira, A.J. Avian metapneumovirus subtypes circulating in Brazilian vaccinated and nonvaccinated chicken and turkey farms. Avian Dis. 2011, 55, 82–89. [Google Scholar] [CrossRef]
87. Andreopoulou, M.; Franzo, G.; Tucciarone, C.M.; Prentza, Z.; Koutoulis, K.C.; Cecchinato, M.; Chaligianni, I. Molecular epidemiology of infectious bronchitis virus and avian metapneumovirus in Greece. Poult. Sci. 2019, 98, 5374–5384. [Google Scholar] [CrossRef]
88. Rizotto, L.S.; Scagion, G.P.; Cardoso, T.C.; Simão, R.M.; Caserta, L.C.; Benassi, J.C.; Keid, L.B.; Oliveira, T.M.F.S.; Soares, R.M.; Arns, C.W.; et al. Complete Genome Sequence of an Avian Metapneumovirus Subtype A Strain Isolated from Chicken (Gallus gallus) in Brazil. Genome Announc. Genome Announc. 2017, 5, e00688-17. [Google Scholar] [CrossRef]
89. Aly, S.M.; Sabri, D.M. Next generation sequencing (NGS): A golden tool in forensic toolkit. Arch. Forensic Med. Criminol. 2015, 4, 260–271. [Google Scholar] [CrossRef] [PubMed]
90. Kariithi, H.M.; Christy, N.; Decanini, E.L.; Lemiere, S.; Volkening, J.D.; Afonso, C.L.; Suarez, D.L. Detection and Genome Sequence Analysis of Avian Metapneumovirus Subtype A Viruses Circulating in Commercial Chicken Flocks in Mexico. Vet. Sci. 2022, 9, 579. [Google Scholar] [CrossRef] [PubMed]
91. Wang, X.; Stelzer-Braid, S.; Scotch, M.; Rawlinson, W.D. Detection of respiratory viruses directly from clinical samples using next-generation sequencing: A

literature review of recent advances and potential for routine clinical use. Rev. Med. Virol. 2022, 32, e2375. [Google Scholar] [CrossRef] [PubMed]

92. Nguyen, V.G.; Chung, H.C.; Do, H.Q.; Nguyen, T.T.; Cao, T.B.; Truong, H.T.; Mai, T.N.; Le, T.T.; Nguyen, T.H.; Le, T.L.; et al. Serological and Molecular Characterization of Avian Metapneumovirus in Chickens in Northern Vietnam. Vet. Sci. Sep. 2021, 8, 206. [Google Scholar] [CrossRef] [PubMed]
93. Mayahi, M.; Momtaz, H.; Jafari, R.A.; Zamani, P. Detection and subtyping avian metapneumovirus from turkeys in Iran. Vet. Res. Forum 2017, 8, 105–108. [Google Scholar] [PubMed]
94. Tucciarone, C.M.; Franzo, G.; Lupini, C.; Alejo, C.T.; Listorti, V.; Mescolini, G.; Brandão, P.E.; Martini, M.; Catelli, E.; Cecchinato, M. Avian Metapneumovirus circulation in Italian broiler farms. Poult. Sci. 2018, 97, 503–509. [Google Scholar] [CrossRef]
95. Yu, M.; Xing, L.; Chang, F.; Bao, Y.; Wang, S.; He, X.; Wang, J.; Wang, S.; Liu, Y.; Farooque, M.; et al. Genomic sequence and pathogenicity of the first avian metapneumovirus subtype B isolated from chicken in China. Vet. Microbiol. 2019, 228, 32–38. [Google Scholar] [CrossRef]
96. Goraichuk, I.V.; Kapczynski, D.R.; Seal, B.S.; Suarez, D.L. Complete Genome Sequence of an avian metapneumovirus Subtype B Strain from Hungary. Microbiol. Resour. Announc. Genome Announc. 2020, 9, e00177-20. [Google Scholar] [CrossRef]
97. Sugiyama, M.; Ito, H.; Hata, Y.; Ono, E.; Ito, T. Complete nucleotide sequences of avian metapneumovirus subtype B genome. Virus Genes. 2010, 41, 389–395. [Google Scholar] [CrossRef]

CAPÍTULO II



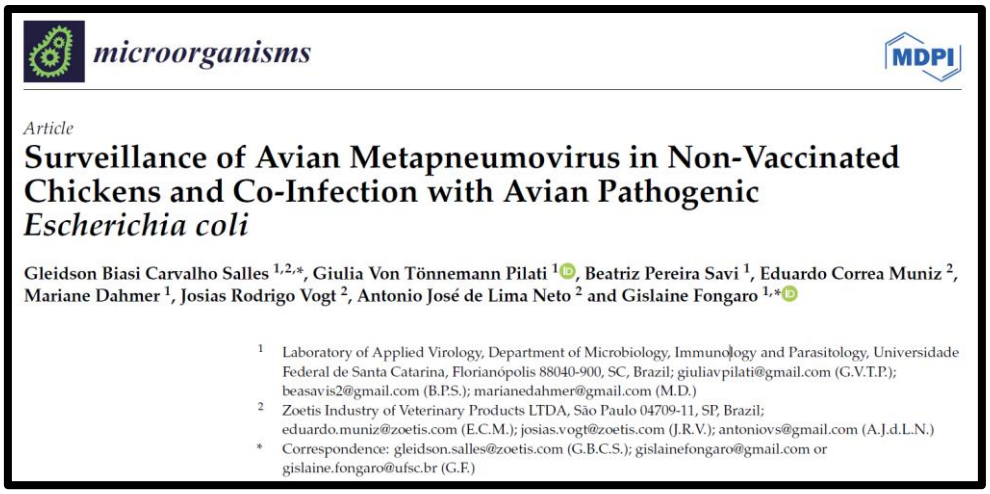
5. CAPÍTULO II

Surveillance of Avian Metapneumovirus in non-vaccinated chickens and co-infection with Avian Pathogenic *Escherichia coli*

Surveillance of Avian Metapneumovirus in non-vaccinated chickens and co-infection with Avian Pathogenic *Escherichia coli*

Citation: Salles GBC, Pilati GVT, Savi BP, Muniz EC, Dahmer M, Vogt JR, de Lima Neto AJ, Fongaro G. Surveillance of Avian Metapneumovirus in Non-Vaccinated Chickens and Co-Infection with Avian Pathogenic *Escherichia coli*. Microorganisms. 2023 Dec 28;12(1):56: 10.3390/microorganisms12010056

Artigo Publicado em (https://www.mdpi.com/2076-2607/12/1/56)



microorganisms MDPI

Article

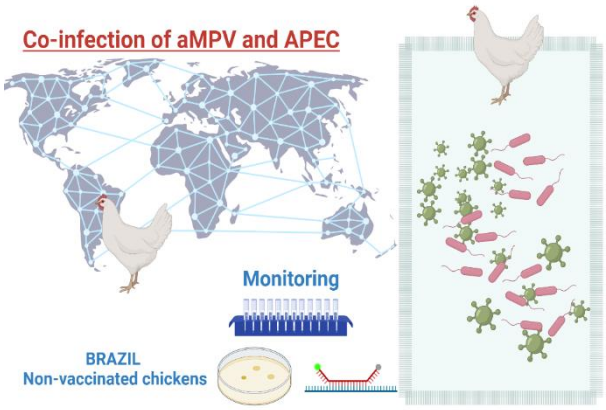
Surveillance of Avian Metapneumovirus in Non-Vaccinated Chickens and Co-Infection with Avian Pathogenic *Escherichia coli*

Gleidson Biasi Carvalho Salles [1,2,*], Giulia Von Tönnemann Pilati [1], Beatriz Pereira Savi [1], Eduardo Correa Muniz [2], Mariane Dahmer [1], Josias Rodrigo Vogt [2], Antonio José de Lima Neto [2] and Gislaine Fongaro [1,*]

[1] Laboratory of Applied Virology, Department of Microbiology, Immunology and Parasitology, Universidade Federal de Santa Catarina, Florianópolis 88040-900, SC, Brazil; giuliavpilati@gmail.com (G.V.T.P.); beasavis2@gmail.com (B.P.S.); marianedahmer@gmail.com (M.D.)

[2] Zoetis Industry of Veterinary Products LTDA, São Paulo 04709-11, SP, Brazil; eduardo.muniz@zoetis.com (E.C.M.); josias.vogt@zoetis.com (J.R.V.); antoniovs@gmail.com (A.J.d.L.N.)

* Correspondence: gleidson.salles@zoetis.com (G.B.C.S.); gislainefongaro@gmail.com or gislaine.fongaro@ufsc.br (G.F.)



Graphical Abstract

Abstract: Brazil is the second largest producer of broiler chicken in the world, and the surveillance of avian pathogens is of great importance for the global economy and nutrition. Avian metapneumovirus (aMPV) infection results in high rates of animal carcass losses due to aerosacculitis and these impacts can be worsened through co-

infection with pathogenic bacteria, particularly Escherichia coli (APEC). The present study evaluated the seroprevalence of the main aMPV subtypes in unvaccinated broiler chickens from poultry farms in Brazil, as well as the clinical effects of co-infection with APEC. Blood samples, respiratory swabs, femurs, liver, and spleen of post-mortem broiler chickens were collected from 100 poultry production batches, totaling 1000 samples. The selection of the production batch was based on the history of systemic and respiratory clinical signs. The results indicated that 20% of the lots showed serological evidence of the presence of aMPV, with two lots being positive for aMPV-B. A total of 45% of batches demonstrated co-infection between aMPV and APEC. The results point to the need for viral surveillance, targeted vaccination, and vaccination programs, which could reduce clinical problems and consequently reduce the use of antibiotics to treat bacterial co-infections.

Keywords: clinical signs; slaughter convictions; virus–bacteria co-infection; colibacillosis

Introduction

The transmission of respiratory agents in poultry farming generates constant chal-lenges for the global activity, losses are related to the decrease in zootechnical perfor-mance and direct impacts on the quality of life of affected animals [1]. The presump-tive diagnosis of these diseases is difficult to perform, as there are no pathognomonic signs for viral respiratory diseases, such as Influenza (IA), New Castle Disease (NCD), Infectious Bronchitis Virus (IVB) and Avian Metapneumovirus (aMPV) [2].

The aMPV is a virus belonging to the Pneumoviridae family, Metapneumovirus genus, which mainly affects the respiratory and reproductive systems of birds when infected [3]. The classification of aMPV can be carried out based on its envelope glycoproteins (G, F and SH), the main of which is the G glycoprotein, which is responsible for bind-ing to the host cell receptor [4]. The distinction in some amino acids present in the ge-netic material can alter the subtypes of aMPV, only 4 subtypes are described based on their antigenicity: A, B, C and D [5], 2 intermediate subtypes have also been described [6]. Subtypes A and B are more similar to each other than subtype C, for example [5]. In Brazil, the first reported case of aMPV occurred in the mid-1990s [7], although this dis-ease is relatively new in the country and few epidemiological studies have been de-veloped. aMPV has already been identified on most continents and its first description occurred in South Africa in turkeys, as TRT (Turkish Rhinotracheitis) [8]. In just a few years, it has been possible to identify aMPV in several different regions since its first appearance. A factor that can significantly contribute to this spread, in addition to mi-gratory birds, is the intercontinental movement of people [9].

The aMPV infection in turkeys and chickens continues to be a serious problem for producers worldwide. Subtypes A and B are responsible for causing the greatest pro-duction losses, mainly associated with swollen head syndrome, which produces signs such as swelling of the periorbital and infraorbital sinuses, in addition to the produc-tion of mucus and nasal secretion [10, 1]. The problems are not restricted to the respir-atory system, the two main subtypes can affect egg production and quality due to their predilection for replication in tissues of the respiratory and genitourinary tract [11]. Although there are vaccines available to prevent aMPV in Brazil, the

immunoprophy-laxis strategy to prevent this agent is not commonly used, especially in broiler chick-ens.

Upper respiratory tract infections caused by aMPV can be isolated or, in many cases, associated with bacteria such as Escherichia coli [1]. Coinfection-related viral damage and persistence may be altered compared to viral monoinfections [12]. Infections caused by avian pathogenic Escherichia coli (APEC) can be primary or secondary [13,14]. The development of secondary infections by APECs is conditioned by predis-posing factors that can disturb the host's organic balance, such as compromised integ-rity of the skin or mucous membranes, poor hygiene practices, influence of immuno-suppressive factors, inadequate ventilation and the presence of viral diseases [13, 15, 14].

In this context, a primary viral infection of the airways can lead to a secondary bacteri-al infection. The increase in bacterial binding factors induced by the virus favors the clinical manifestation caused by Escherichia coli [16], and the damage generated by vi-ral replication in mucociliary tissues favors bacterial maintenance in the respiratory tract [17, 18].

Regarding the economy and poultry production, Brazil is the second largest producer and largest exporter of chicken meat in the world, being in evidence in global health and nutrition. In relation to the slaughter of broiler chickens, the states of Paraná, San-ta Catarina and Rio Grande do Sul, São Paulo, Goiás, Minas Gerais stand out (together they represent 88.33% of the total birds slaughtered in Brazil and exported). The state of Ceará allocates its production for Brazilian domestic consumption [19]. This high percentage of birds housed in a geographic region can pose health risks to

the animals' health, mainly through the transmission of respiratory infectious agents [20].

In view of the above, the present study aimed to evaluate the seroprevalence of aMPV in unvaccinated broiler chickens, perform molecular detection by RT-PCR and identify the subtypes present in Brazil, in addition to evaluating the impacts caused in batches of broiler chickens that presented co-infection between aMPV and APEC.

Material and Methods

Sample collections

A total of 100 batches of broiler chickens were evaluated (Gallus gallus domesticus) distributed throughout Brazil. The definition of the states where the samples were collected respected the proportionality of broiler chicken production, 10 chickens were sampled per batch, totaling one thousand chickens, coming from the South Region (states of Paraná (n = 30 lots), Santa Catarina (n = 15 lots), Rio Grande do Sul (n = 15 lots)), Southeast Region (states of São Paulo (n = 10 lots) and Minas Gerais (n = 10 lots)) and Northeast Region (state of: Ceará (n = 20 lots)), which represent 80% of chicken meat production in Brazil [19]. Figure 1 shows the regions

and collection areas. Zootechnical data were not available for this study.

Figure 1. Map of Brazil, highlighting the South, Southeast and Northeast Regions and the Brazilian states sampled in the present study, being Rio Grande do Sul (RS), Santa Catarina (SC), Paraná (PR), São Paulo (SP), Minas Gerais (MG) and Ceará (CE).

The batches were selected based on the history of respiratory problems and animals that presented some respiratory disorder, such as sneezing, rales, snoring, nasal secre-tions and swollen head and suspected colibacillosis. Furthermore, the birds were not vaccinated for aMPV.

The liver and spleen organs were collected and evaluated (taking into consideration, the presence of macroscopic lesions), femurs and 10 nasotracheal

swabs were collect-ed. The samples were stored at a temperature of 2° to 8 °C for the purposes of diag-nosing aMPV and identifying APEC.

For the purpose of serological evaluation for aMPV, blood samples were collected 15 to 21 days after the first collection. Collection was carried out in pools of 20 animals and the sera were stored individually by batches and organs of animals undergoing post-mortem inspection aged between 13 and 32 days. The liver and spleen organs were collected and evaluated (taking into consideration, the presence of macroscopic lesions), femurs and 10 nasotracheal swabs were collected. The samples were stored at a temperature of 2° to 8 °C for the purposes of diagnosing aMPV and identifying APEC.

All biological samples evaluated here were donated by farms that carry out routine inspections, eliminating the need for an ethics committee as they are leftover biological samples collected by routine health surveillance services - Consultation with the Ethics Committee on the Use of Animals (CEUA nº 4434190521 / Federal University of Santa Catarina).

Clinical signs in batches

To survey the clinical signs of the sampled batches, anamnesis was carried out and the individual sanitary control sheets of the batches were evaluated, where information such as medications used, clinical signs, average weight and feed consumption were recorded.

Serological detection of aMPV

To detect antibodies against aMPV, the ELISA (Enzyme Linked Immuno Sorbent As-say) method was used, using the BioChek commercial kit (Netherlands),

following the manufacturer's instructions. The results were analyzed according to an optical density (OD) using the BioChek ii software (version 2015) with sample/positive ratios (SP) > 0.5 (titer ≥ 0.501), indicating the average titer of the 20 birds evaluated per batch against possible natural exposure to aMPV, since they are not birds vaccinated against the pathogenic agent.

Molecular detection of aMPV by RT-PCR

Total RNA from the samples was extracted using the RNeasy® Mini kit (QIAGEN) following the manufacturer's instructions. The M-MLV Reverse Transcriptase kit (Promega) was used to perform reverse transcription, following the manufacturer's in-structions. For the polymerase chain reaction (PCR), the G protein gene was used for the detection of subtypes A and B (Table 1), using the following reagents and concen-trations 2 mM magnesium chloride, 0.25 mM deoxyribonucleotide phosphates, 0.3 µM of each primer, 1 U of Taq DNA polymerase GoTaq® DNA Polymerase (Promega, Madison, WI, USA), 1x Green GoTaq® Reaction Buffer (Promega, Madison, WI, USA), 3 µL of sample and sterile ultrapure water to make 25 µL. The reactions were carried out in a thermocycler, using the following cycling parameters: 94 °C for 2 minutes; 35 cycles of 94 °C for 30 seconds, 63 °C for 30 seconds, 68 °C for 3 minutes; and final cycle of 72 °C for 10 minutes [21].

The samples were subjected to horizontal electrophoresis in 1% agarose gel, using GelRed as a DNA intercalating agent. Amplicon sizes were determined by comparison with the low molecular weight (LMW) marker.

Table 1. Primers, gene target and size of gene fragments used in molecular detection of aMPVA A and B.

Virus	Target gene	Primer sequence	Amplicon Size	Reference.
aMPV/A	G protein	F 5'-GGACATCGGGAGGAGGTACA -3' R 5'-CACTCCTCTAACACTGACTGTTCAACT -3'	116 bp	[21]
aMPV/B	G protein	F 5'-TCATCCCGGAAGCCTCCCTCACTAT-3' R 5'-TAGCGTTTGCTGCACTGGCTTCTGATAC -3'	135 bp	[21]

Assessment of APEC co-infection

For the isolation of Escherichia coli, femur swabs were inoculated on MacConkey agar and incubated at 37 °C for 24 hours. Typical Escherichia coli colonies were confirmed as APEC using qualitative PCR, as described by [22, 23] using the genes: iroN, ompT, hlyF, iss e iutA as the predictors of APEC virulence (Table 2).

Table 2. Primers, gene and size of gene fragments used in APEC detection.

Target gene	Primer sequence	Amplicon size	Reference
iroN	F 5'-AAGTCAAAGCAGGGGTTGCCCG -3' R 5'-GATCGCCGACATTAAGACGCAG -3'	667 bp	[23]
ompT	F 5'- TCATCCCGGAAGCCTCCCTCACTACTAT -3' R 5'- TAGCGTTTGCTGCACTGGCTTCTGATAC -3'	496 bp	[22]
hlyF	F 5'- GGCCACAGTCGTTTAGGGTGCTTACC -3' R 5'- GGCGGTTTAGGCATTCCGATACTCAG -3'	450 bp	[22]
Iss	F 5'- CAGCAACCCGAACCACTTGATG -3' R 5'- AGCATTGCCAGAGCGGCAGAA -3'	323 bp	[23]
iutA	F 5'- GGCTGGACATCATGGGAACTGG -3' R 5'- CGTCGGGAACGGGTAGAATCG -3'	302 bp	[22]

Results

Clinical signs in batches

A total of 43 lots showed no clinical signs (attribute “0”), 29 lots showed only 1 clinical sign (attribute “+”) and 28 lots showed the presence of more than 2 clinical signs (attribute “++”). The ranking of the batches evaluated according to the clinical signs obtained from the batch health control sheets, containing information on the clinical signs observed and medications used (Table 3).

Table 3. Classification of batches according to clinical signs, injuries, medication used and origin of samples.

Ranking Scores	Clinical Signs and Injuries	Batches	Total Batches Medicated	Medications
0		*n* = 29 (13, 40, 46, 48, 49, 50, 51, 52, 53, 54, 61, 62, 63, 64, 65, 66, 67, 68, 69,70, 71, 73, 74, 75, 76, 77, 78, 79, 80)	0	Unmedicated
+	sneezing, crackles, nasal discharge, aerosacculitis, nasal discharge,	*n* = 42 (1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 19, 20, 21, 27, 28, 34, 35, 37, 38, 39, 47, 55, 56, 81, 82, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 97, 98, 99, 100)	3	Sultfa + Trimethoprim, Ciprofloxacin, Norfloxacin, Florfenicol,
++	sneezing and mucopurulent nasal discharge, sneezing and rales, rales and swelling in the periocular region, swollen head and rales, sneezing and presence of airsacculitis, sneezing, rales and nasal discharge, sneezing and tracheitis, airsacculitis and colibacillosis, septicemia, suspected colibacillosis.	*n* = 29 (9, 11, 14, 15, 17, 18, 22, 23, 24, 25, 26, 29, 30, 31, 32, 33, 36, 41, 42, 43, 44, 45, 57, 58, 59, 60, 72, 83, 96)	21	Ciprofloxacin, Sulfachlorpyridazine + Trimethopim, Norfloxacin, Florfenicol,

Classification of clinical conditions in birds based on clinical signs and lesions observed, where: “0” No clinical signs (n=29), “+” Only 1 clinical sign observed (n=42) and “++” 2 or more signs observed (n=29). The origin of the samples by state is identified as per the following description: 1 – 15 batches from the state of Santa Catarina. 16 – 30 batches in the state of Rio Grande do Sul. 31 – 60 batches in the state of Paraná. 61, 62, 65, 66, 67, 69, 75, 76, 77, 78, 80 batches in the state of Minas Gerais. 62, 63, 64, 68, 70, 71, 72, 73, 74, 79 batches in the state of São Paulo. 81 –

100 batches in the state of Ceará. It is also possible to check the total number of medicated batches and the medications used by the group.

During sample collection, it was observed that 71% of the batches showed clinical respiratory signs, including rales, sneezing, nasal discharge and discharge, enlargement of the infraorbital sinus and swollen head. Among the batches from the southern states (Santa Catarina and Paraná), 13.3% used antibiotics during the birds' housing phase, among the drugs used were ciprofloxacin, sulfachlorpyridazine+trimethopim and florfenicol. In the Southeast region (São Paulo and Minas Gerais), only one batch (5%) of the batches showed clinical signs and this was medicated with ciprofloxacin on the day of collection. The clinical signs observed were different between the batches, when the batch control sheets were checked.

The states of Rio Grande do Sul, Paraná and Santa Catarina, which make up the southern region of Brazil, stand out with 83.3% of the batches showing various respiratory clinical signs. Additionally, 36.6% of these batches used antibiotic therapy at some point in the production cycle. On the other hand, in the states of São Paulo and Minas Gerais, representing the Southeast region, only 5% of the batches showed clinical signs of respiratory diseases, coinciding with the use of antibiotics in one of these batches. In the state of Ceará, representing the Northeast region, all batches collected exhibited clinical signs at the time of sampling, and 20% of them were under drug treatment. This diversity of scenarios highlights the variation in the prevalence of symptoms and the use of antibiotics in different regions of the country.

Seropositivity and molecular detection of aMPV

As a result, 20% of the samples showed the presence of antibodies against aMPV. The positive samples were concentrated in the southern region of Brazil, as

70% came from Paraná and the remaining 30% from Santa Catarina. In the State of Paraná, the results indicate positivity in 14 of the 30 batches sampled, revealing a seroprevalence for aMPV of 46.6% of the batches evaluated, with an average titratable weight of 6,881.4 IU. In Santa Catarina, the results indicate positivity in 6 of the 15 batches sampled, with aMPV seroprevalence of 40% of the batches evaluated with a titratable average of 780 IU (Figure 2).

Of the 100 batches evaluated for detection and molecular typing of aMPV by RT-qPCR, 2 batches were positive for the aMPV-B subtype, while no batches were positive for aMPV-A. Both positive samples came from the state of Paraná, which had positive serology with serological titers of 3.909 and 4.821 IU.

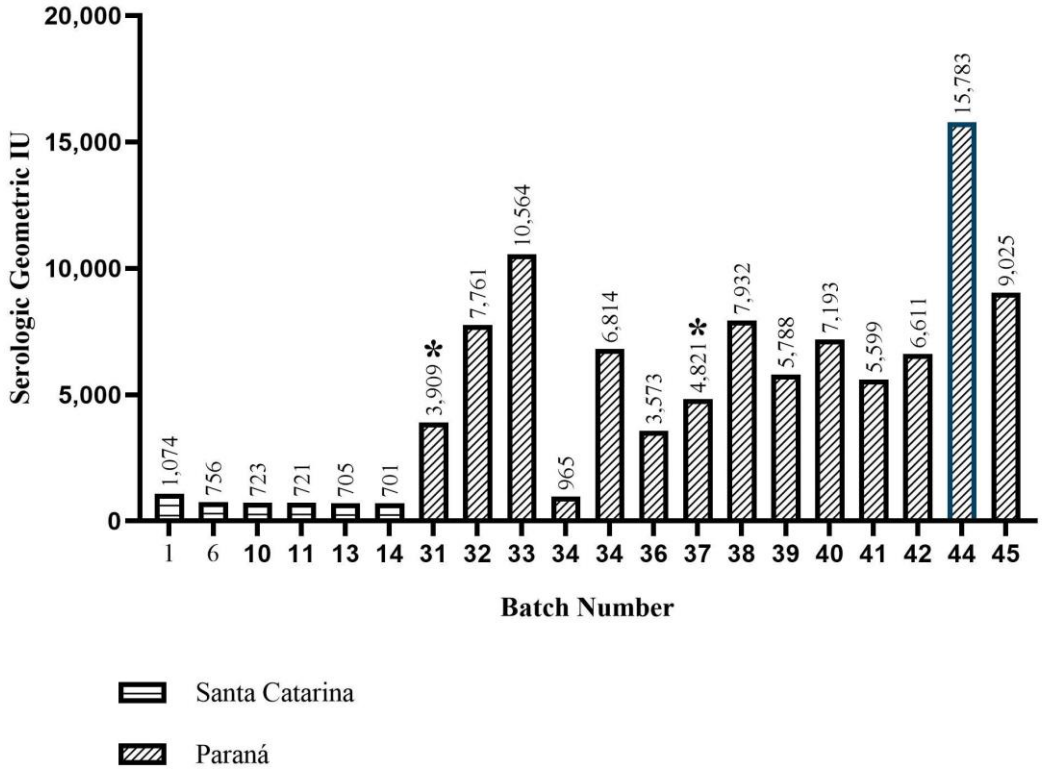


Figure 2. Serology for aMPV from different Brazilian states batches.

(*) Batches with RT-qPCR positivity for aMPV-B.

Co-infection between aMPV and APEC

All batches with serology compatible with aMPV were tested for the presence of APEC. Of the batches in which Escherichia coli was isolated from femurs, 20% (13/65) presented antibodies against aMPV in the ELISA assay, and in 1 batch the genetic material of type B aMPV was detected.

Clinical signs in these animals were generally more severe compared to either disease alone. The batches that showed co-infection between aMPV and APEC came from the states of Paraná and Santa Catarina (Table 4).

Table 4. Co-infection between aMPV and APEC and classification of different levels of clinical signs/lot lesions: (0) no clinical signs, (+) only one clinical sign and (++) 2 or more clinical signs.

Batches	Brazilian State	Serology aMPV (UI/mL)	APEC	Injury Scores by Clinical Signs
1	Santa Catarina	1074	Yes	+
6	Santa Catarina	756	Yes	+
31	Paraná	3909	Yes	++
32	Paraná	7761	Yes	++
33	Paraná	10,564	Yes	++
38	Paraná	7932	Yes	++
39	Paraná	5788	Yes	+
40	Paraná	7194	Yes	0
45	Paraná	9025	Yes	++

Discussion

The present study demonstrated seroconversion to aMPV in batches of broiler chickens not vaccinated against aMPV in the southern region of Brazil, specifically in the states of Paraná and Santa Catarina, which are the main poultry producers in the country, occupying the first and second positions, respectively [19]. In this study, 20/100 batches demonstrated seroconversion to aMPV and 2/100 of these were characterized as aMPV-B using RT-PCR. The detection of the viral genome and isolation of aMPV rep-resents a considerable challenge, since the virus has a relatively short period of resi-dence in the host, and is often detected in the early stages of infection, without demonstrating characteristic clinical signs [24].

The high density of poultry farms in certain regions and the frequent nonuse of vaccines to prevent aMPV, allows viral spread in poultry flocks. It is also noteworthy that in the southern region of Brazil there is intense production of turkeys, which may also contribute to the spread and maintenance of the virus in the region, considering that subtypes A and B can be found mainly in turkeys and chickens [25].

The aMPV is widely distributed worldwide [26, 2, 27]. In Latin America, the first report occurred in 1995 [28], using field samples of aMPV and cells derived from chicken em-bryos, they identified subtype A. At the first appearance, an increase in cases of aMPV, mainly in long-lived turkeys and chickens. In 2011 [29] characterized the first appear-ance of aMPV-B in Brazil.

Despite being present in poultry flocks and often neglected in broiler chickens, aMPV causes significant damage in poultry farming revealed that aMPV, after viral infection, causes thickening of the tracheal mucosa [30]. This occurs due to congestion, edema and infiltration of mononuclear cells in the lamina propria of the

trachea, generally appearing between three and seven days after infection. Furthermore, flattening of ep-ithelial cells and focal disciliation were observed, which may facilitate the emergence of secondary infections, worsening clinical signs.

The detection of aMPV-B in Brazilian poultry flocks may be related to the massive use of vaccines against aMPV-A for many years, which may have exerted vaccine pressure generating alternation of aMPV-B. It is worth mentioning that aMPV-A has more lim-ited transmission, as it is via the oral-fecal route, while MPV-B is respiratory, making it more easily disseminated [24].

Although replicating subtype A and subtype B (live) vaccines are available for use in immunoprevention, both are cross-protective [31, 32, 33], however, are not used in all states in Brazil. Some regions use replicating vaccines to prevent aMPV in broiler chickens, such as the Southeast (São Paulo and Minas Gerais) and Northeast (Ceará) regions, although the batches were collected from farms that do not use them, but this practice is relatively common in these regions. This factor may explain the low circulation of aMPV in these locations, since vaccination generates selection and control pressure, reducing clinical signs and viral excretion when used, although in the state of Ceará 100% of batches showed clinical respiratory signs, possibly another viral agent must be present at that time.

In the states of the Southern region (Paraná, Santa Catarina and Rio Grande do Sul), of the 20 batches in which the presence of antibodies to aMPV was detected, only two did not show clinical signs at the time of collection, which may be linked to the character-istic of infection and viral replication in early stages [34, 35, 36].

Regarding the use of medicines during production cycles, there is a significant concentration in the southern region of Brazil, which represented 88% of all batches

medicated in Brazil. These treatments were primarily aimed at controlling opportunistic bacteria or those naturally present in birds. Isolates confirmed as APEC were obtained in 45% of the batches in which there was seroconversion to aMPV, demonstrating the condition of co-infection. This was linked to the clinical condition of the birds, which leads to production losses during the life of the flock, as well as the loss of slaughtered birds.

The identification and characterization of APEC in aMPV-positive batches in Brazilian states demonstrates the importance of this agent, regardless of its primary or secondary role, especially in batches that were medicated to reduce impacts related to co-infection with aMPV associated with APEC [37].

Conclusions

The study presents the seroprevalence of aMPV in 20% (20/100) of the batches evaluated in Brazil, with the presence of subtype B detected, with 45% (9/20) demonstrating greater clinical problems in the presence of APEC co-infection.

This study points to the need to design constant monitoring strategies aimed at combating aMPV in the poultry sector, as well as reducing viral circulation and bacterial co-infections. This, together, will certainly have a positive impact on production, with a view to protecting livestock, improving animal health, and consequently reducing the use of antimicrobials.

References

1. Yehia, N., Salem, H. M., Mahmmod, Y., Said, D., Mahmoud S., Mawgod, S. A., Sorour, H. K., AbdelRahman, M. A. A., Selim, S., Saad, A. M., El-Saadony, M.

T., El-Meihy, R. M., Abd El-Hack, M. E., El-Tarabily, K. A., & Zanaty, A. M. (2023). Common viral and bacterial avian respiratory infections: An updated review. *Poultry Science*, *102*(5), 102553. https://doi.org/10.1016/j.psj.2023.102553

2. Kaboudi, K., & Lachheb, J. (2021). Avian metapneumovirus infection in turkeys: A review on turkey rhinotracheitis. *Journal of Applied Poultry Research*, *30*(4), 100211. https://doi.org/10.1016/j.japr.2021.100211
3. Afonso, C. L., Amarasinghe, G. K., Bányai, K., Bào, Y., Basler, C. F., Bavari, S., Bejerman, N., Blasdell, K. R., Briand, F.-X., Briese, T., Bukreyev, A., Calisher, C. H., Chandran, K., Chéng, J., Clawson, A. N., Collins, P. L., Dietzgen, R. G., Dolnik, O., Domier, L. L., … Kuhn, J. H. (2016). Taxonomy of the order Mononegavirales: Update 2016. *Archives of Virology*, *161*(8), 2351-2360. https://doi.org/10.1007/s00705-016-2880-1
4. Gough, R. E., & Jones, R. C. (2008). Avian Metapneumovirus. In *Diseases of Poultry* (p. 75-117). Blackwell Publishing.
5. Brown, P. A., Lemaitre, E., Briand, F.-X., Courtillon, C., Guionie, O., Allée, C., Toquin, D., Bayon-Auboyer, M.-H., Jestin, V., & Eterradossi, N. (2014). Molecular comparisons of full length metapneumovirus (MPV) genomes, including newly determined French AMPV-C and –D isolates, further supports possible subclassification within the MPV genus. *PLOS ONE*, *9*(7), e102740. https://doi.org/10.1371/journal.pone.0102740
6. Canuti, M., Kroyer, A. N. K., Ojkic, D., Whitney, H. G., Robertson, G. J., & Lang, A. S. (2019). Discovery and characterization of novel RNA viruses in aquatic North American wild birds. *Viruses*, *11*(9), 768. https://doi.org/10.3390/v11090768

7. Chacón, J. L., Brandão, P. E., Buim, M., Villarreal, L., & Piantino Ferreira, A. J. (2007). Detection by reverse transcriptase-polymerase chain reaction and molecular characterization of subtype B avian metapneumovirus isolated in Brazil. *Avian Pathology*, *36*(5), 383-387. https://doi.org/10.1080/03079450701589142
8. Buys, B. S. (1980). A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys*, *28*, 36.
9. Franzo, G., Cecchinato, M., Tosi, G., Fiorentini, L., Faccin, F., Tucciarone, C. M., Trogu, T., Barbieri, I., Massi, P., & Moreno, A. (2018). GI-16 lineage (624/I or Q1), there and back again: The history of one of the major threats for poultry farming of our era. *PLOS ONE*, *13*(12), e0203513. https://doi.org/10.1371/journal.pone.0203513
10. Cook, J. K. A. (2000). Avian pneumovirus infections of turkeys and chickens. *The Veterinary Journal*, *160*(2), 118-125. https://doi.org/10.1053/tvjl.2000.0486
11. Hess, M., B. Huggins, M., Mudzamiri, R., & Heincz, U. (2004). Avian metapneumovirus excretion in vaccinated and non-vaccinated specified pathogen free laying chickens. *Avian Pathology*, *33*(1), 35-40. https://doi.org/10.1080/0307945031000163273
12. Weerts, E. A. W. S., Matthijs, M. G. R., Bonhof, J., van Haarlem, D. A., Dwars, R. M., Gröne, A., Verheije, M. H., & Jansen, C. A. (2021). The contribution of the immune response to enhanced colibacillosis upon preceding viral respiratory infection in broiler chicken in a dual infection model. *Veterinary Immunology and Immunopathology*, *238*, 110276. https://doi.org/10.1016/j.vetimm.2021.110276

13. Dho-Moulin, M., & Fairbrother, J. M. (1999). Avian pathogenic Escherichia coli (APEC). *Veterinary Research, 30*(2-3), 299-316.

14. Nolan, L. K., Vaillancourt, J., Barbieri, N. L., & Logue, C. M. (2020). Colibacillosis. *Diseases of Poultry*, 770-830. https://doi.org/10.1002/9781119371199.ch18

15. La Ragione, R. M., & Woodward, M. J. (2002). Virulence factors of Escherichia coli serotypes associated with avian colisepticaemia. *Research in Veterinary Science*, *73*(1), 27-35. https://doi.org/10.1016/s0034-5288(02)00075-9

16. McCullers, J. A., & Rehg, J. E. (2002). Lethal Synergism between Influenza Virus and Streptococcus pneumoniae: Characterization Of a Mouse Model and the Role of Platelet-Activating Factor Receptor. *The Journal of Infectious Diseases*, *186*(3), 341-350. https://doi.org/10.1086/341462

17. Kotani, H., Ishizaki, Y., Hiraoka, N., & Obayashi, A. (1987). Nucleotide sequence and expression of the cloned gene of bacteriphage SP6 RNA polymerase. *Nucleic Acids Research*, *15*(6), 2653-2664. https://doi.org/10.1093/nar/15.6.2653

18. Christensen, H., Bachmeier, J., & Bisgaard, M. (2021). New strategies to prevent and control avian pathogenic *Escherichia coli* (APEC). *Avian Pathology*, *50*(5), 370-381. https://doi.org/10.1080/03079457.2020.1845300

19. Brazilian Association of Animal Protein. (2023). *Annual Report*. https://abpa-br.org/wp-content/uploads/2023/04/Relatorio-Anual-2023.pdf

20. Graziosi, G., Mescolini, G., Silveira, F., Lupini, C., Tucciarone, C. M., Franzo, G., Cecchinato, M., Legnardi, M., Gobbo, F., Terregino, C., & Catelli, E. (2022). First detection of avian metapneumovirus subtype C Eurasian lineage in a Eurasian wigeon (Mareca penelope) wintering in Northeastern Italy: An

additional hint on the role of migrating birds in the viral epidemiology. *Avian Pathology*, *51*(3), 283-290. https://doi.org/10.1080/03079457.2022.2051429

21. Guionie, O., Toquin, D., Sellal, E., Bouley, S., Zwingelstein, F., Allée, C., Bougeard, S., Lemière, S., & Eterradossi, N. (2007). Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. *Journal of Virological Methods*, *139*(2), 150-158. https://doi.org/10.1016/j.jviromet.2006.09.022
22. Johnson, T. J., Wannemuehler, Y., Doetkott, C., Johnson, S. J., Rosenberger, S. C., & Nolan, L. K. (2008). Identification of Minimal Predictors of Avian Pathogenic Escherichia coli. Virulence for Use as a Rapid Diagnostic Tool. *Journal of Clinical Microbiology*, *46*(12), 3987-3996. https://doi.org/10.1128/jcm.00816-08
23. Rodriguez-Siek, K. E., Giddings, C. W., Doetkott, C., Johnson, T. J., Fakhr, M. K., & Nolan, L. K. (2005). Comparison of Escherichia coli isolates implicated in human urinary tract infection and avian colibacillosis. *Microbiology*, *151*(6), 2097-2110. https://doi.org/10.1099/mic.0.27499-0
24. Brown, P. A., Allée, C., Courtillon, C., Szerman, N., Lemaitre, E., Toquin, D., Mangart, J.-M., Amelot, M., & Eterradossi, N. (2019). Host specificity of avian metapneumoviruses. *Avian Pathology*, *48*(4), 311-318. https://doi.org/10.1080/03079457.2019.1584390
25. Wei, L., Zhu, S., Yan, X., Wang, J., Zhang, C., Liu, S., She, R., Hu, F., Quan, R., & Liu, J. (2013). Avian metapneumovirus subgroup C infection in Chickens, China. *Emerging Infectious Diseases*, *19*(7), 1092-1094. https://doi.org/10.3201/eid1907.121126

26. Franzo, G., Legnardi, M., Mescolini, G., Tucciarone, C. M., Lupini, C., Quaglia, G., Catelli, E., & Cecchinato, M. (2020). Avian Metapneumovirus subtype B around Europe: A phylodynamic reconstruction. *Veterinary Research*, *51*(1), 1-10. https://doi.org/10.1186/s13567-020-00817-6

27. Rizotto, L. S., Simão, R. M., Scagion, G. P., Simasaki, A. A., Caserta, L. C., Benassi, J. C., Arns, C. W., & Ferreira, H. L. (2019). Detection of avian metapneumovirus subtype A from wild birds in the State of São Paulo, Brazil. *Pesquisa Veterinária Brasileira*, *39*(3), 209-213. https://doi.org/10.1590/1678-5150-pvb-6021

28. D'Arce, R. C. F., Coswig, L. T., Almeida, R. S., Trevisol, I. M., Monteiro, M. C. B., Rossini, L. I., Di Fabio, J., Hafez, H. M., & Arns*, C. W. (2005). Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. *Avian Pathology*, *34*(2), 133-136. https://doi.org/10.1080/03079450500059180

29. Chacón, J. L., Mizuma, M., Vejarano, M. P., Toquín, D., Eterradossi, N., Patnayak, D. P., Goyal, S. M., & Piantino Ferreira, A. J. (2011). Avian metapneumovirus subtypes circulating in Brazilian vaccinated and nonvaccinated chicken and turkey farms. *Avian Diseases*, *55*(1), 82-89. https://doi.org/10.1637/9501-081310-reg.1

30. Hartmann, S., Sid, H., & Rautenschlein, S. (2015). Avian metapneumovirus infection of chicken and turkey tracheal organ cultures: Comparison of virus–host interactions. *Avian Pathology*, *44*(6), 480-489. https://doi.org/10.1080/03079457.2015.1086974

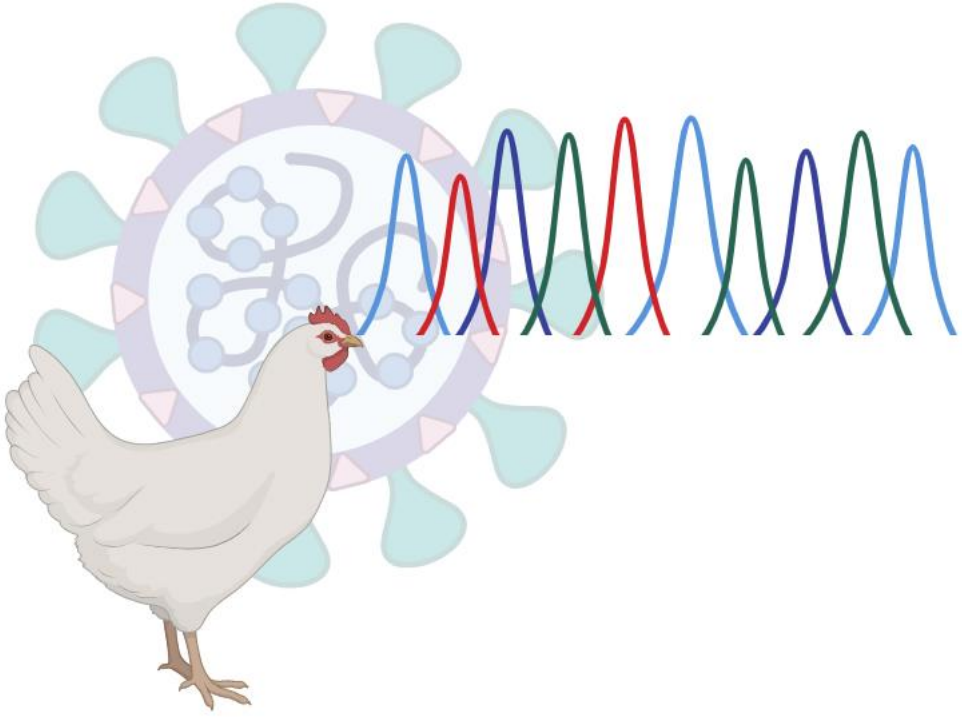
31. Cook, J. K. A., Huggins, M., Woods, M., Orbell, S., & Mockett, A. (1995). Protection provided by a commercially available vaccine against different strains

of turkey rhinotracheitis virus. *Veterinary Record*, *136*(15), 392-393. https://doi.org/10.1136/vr.136.15.392

32. Cook, J. K. A., Huggins, M. B., Orbell, S. J., & Senne, D. A. (1999). Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA. *Avian Pathology*, *28*(6), 607-617. https://doi.org/10.1080/03079459994407
33. Toquin, D., Bäyon-Auboyer, M., Jestin, V., Eterradossi, N. Résponse Sérologique et Protection Croisée Visa vis de L'infection par Une Souche Non-A Non-B du Virus de La Rhinotrachéite Infectieuse De La Dinde. *Comptes Rendus des 3emes Journées de la Recherche Avicole*: St Malo, France, 1999, p. 223-224.
34. Cook, J. K. A., Kinloch, S., & Ellis, M. M. (1993). In vitro and in vivo studies in chickens and turkeys on strains of turkey rhinotracheitis virus isolated from the two species. *Avian Pathology*, *22*(1), 157-170. https://doi.org/10.1080/03079459308418907
35. Cook, J. K.A., Huggins, M. B., Orbell, S. J., Mawditt, K., & Cavanagh, D. (2001). Infectious bronchitis virus vaccine interferes with the replication of avian pneumovirus vaccine in domestic fowl. *Avian Pathology*, *30*(3), 233-242. https://doi.org/10.1080/03079450120054640
36. Loock, M., Loots, K., Zande, S., Heerden, M., Nauwynck, H., Goddeeris, B., & Vanrompay, D. (2006). Pathogenic interactions between Chlamydophila psittaci and avian pneumovirus infections in turkeys. *Veterinary Microbiology*, *112*(1), 53-63. https://doi.org/10.1016/j.vetmic.2005.10.013
37. Sid, H., Benachour, K., & Rautenschlein, S. (2015). Co-infection with multiple respiratory pathogens contributes to increased mortality rates in Algerian poultry

flocks. *Avian Diseases*, *59*(3), 440-446. https://doi.org/10.1637/11063-031615-

CAPÍTULO III

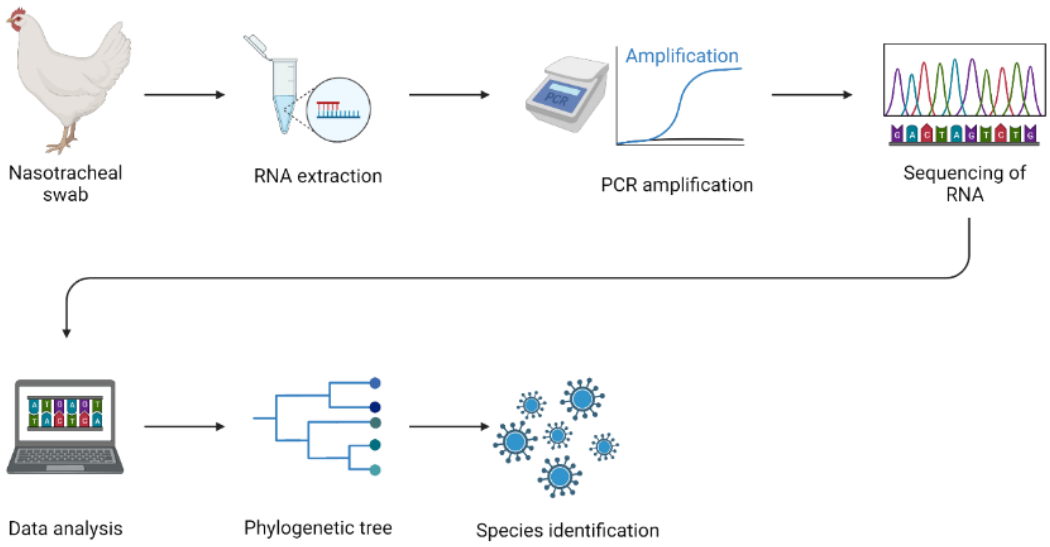


6. CAPÍTULO III

Monitoring Infectious Bronchitis Virus (IBV) in Vaccinated and Non-vaccinated Broiler Chickens in Brazil to surveillance of Vaccine Escapes and New viral Variants

Monitoring Infectious Bronchitis Virus (IBV) in Vaccinated and Non-vaccinated Broiler Chickens in Brazil to surveillance of Vaccine Escapes and New viral Variants

Artigo submetido na Revista Avian Pathology / Status atual: “Em revisão” na data de 30/11/2023)



Graphical Abstract:

Abstract: The infectious bronchitis virus (IBV) is ubiquitous, posing a threat to poultry batches worldwide. Biosecurity measures and vaccination are common practices to mitigate productivity losses. The objective of this study was to conduct IBV surveillance in Brazil, understand potential viral escapes, identify new variant strains in the poultry population, and assess the persistence of vaccine virus in vaccinated broilers. A total of 1,000 nasotracheal swabs from 100 batches were collected from poultry carcasses in six Brazilian states (Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Minas

Gerais, and Ceará). Screening for IBV presence was performed using Reverse Transcription Polymerase Chain Reaction (RT-PCR), resulting in the amplification of 392 base pair (bp) fragments within the hypervariable region of the IBV S1 gene. Remarkably, 91% of the samples tested positive for IBV. Our analysis revealed the presence of IBV from three distinct lineages, with numerous samples exhibiting the co-occurrence of more than one lineage. For understanding viral phylogeny and evolution, 28 samples from different Brazilian states were selected from the total of 100 batches for Sanger sequencing. Phylogenetic analysis identified the lineage origin, classifying them as 7.14% belonging to GI-1, 78.57% to GI-11, and 14.28% to GI-23. The results of this research can assist Brazilian poultry companies in designing more effective vaccination programs. This study also identifies a new IBV variant (GI-23) in Brazil.

Keywords: Infectious Bronchitis Virus, GI-23 Strain, Viral Escapes, Vaccination, Emerging Variant Strains

Introduction

The historical transformation in global poultry farming has brought about numerous challenges, including the control of diseases, especially respiratory diseases in birds (Yehia et al., 2023). The concentration of poultry in specific regions has significantly increased sanitary risks, leading to the introduction of many diseases in various parts of the world. In this context, Infectious Bronchitis Virus (IBV) was first reported in the 1930s in the United States, with the identification of the Mass serotype (Sjaak de Wit et al., 2011). Within a few years, it was identified in various regions across the globe, characterizing the virus as omnipresent in the worldwide poultry industry (Molenaar et al., 2020).

IBV is a highly contagious viral disease that affects chickens (*Gallus gallus domesticus*) as well as other domestic and wild birds, including partridges, geese, pigeons, guinea fowls, teals, ducks, and peafowl (Cavanagh, 2007). The virus has an envelope and a single-stranded positive-sense RNA with a length of 27 Kb. It belongs to the Coronaviridae family (Cavanagh & Gelb, 2008), *Gammacoronavírus* genus, and encodes four structural proteins (spike, envelope, matrix, and nucleocapsid) (Jordan, 2017). The spike protein (S) is post-translationally cleaved into S1 at the amino-terminal and S2 at the carboxy-terminal subunits (Cavanagh et al., 1986). The spike protein is responsible for virus-host contact and binding, playing a crucial role in host cell specificity and containing epitopes that induce the production of neutralizing antibodies. Based on complete sequences obtained from the S1 gene, it is possible to define 6 genotypes comprising 32 distinct lineages (GI-1 - GI-32) (Jackwood et al., 2020; Valastro et al., 2016).

The disease is primarily characterized by upper respiratory symptoms in birds, but it is also common to affect other organs such as the kidneys and the reproductive

tract. This can lead to issues related to airsacculitis, proventriculitis, nephritis, zootechnical losses, poor egg quality, high morbidity, and mortality (Moreno et al., 2017; Sjaak de Wit et al., 2011).

One of the main ways to control IBV is through a combination of good production practices, along with biosecurity measures on poultry farms (Sjaak de Wit et al., 2011). Additionally, the use of live (replicating) or inactivated (non-replicating) vaccines can be highly effective in controlling this disease. However, due to the extensive genetic diversity and high mutation rate, this results in many distinct viral lineages, thereby reducing the protective coverage of vaccines (Sjaak de Wit et al., 2011).

There are two mechanisms involved in the genetic change of IBV. The first mechanism is the generation of mutations that accumulate over time through simple evolution or as a result of pressures exerted by vaccines, resulting in genetic differentiation (Britton et al., 2012). This occurs because the polymerase enzyme makes "mistakes" when replicating the viral RNA genome to create a new particle. Since these errors occur at a high speed, and the ability to go back and correct them is limited, the result is the rapid evolution of the virus (Wang & Khan, 2000). When errors introduced into the genome provide a selective advantage to the virus, this "genetic variant" becomes the emerging new virus that can cause diseases in birds (Jia et al., 1995). The second mechanism that leads to changes in the IBV genome is called recombination, which can occur when two types of IBV infect the same cell (Jia et al., 1995). During virus replication, the polymerase enzyme starts copying the genome of one virus and then switches to the other, creating a genetic mutation that contains portions of the genomes of both parent viruses. This process results in a relatively rapid change in the viral genome composition (Chacón et al., 2023). Although genetic changes can lead to rapid and significant alterations, they are often

incompatible, for example, when two vaccine viruses recombine. Thus, viral recombination only occurs when specific genes involved in the pathogenicity and/or immunogenicity of a new virus can emerge to cause disease, and it is a relatively rare event.

This constant Darwinian evolution of IBV results in the emergence of new virus types (Fraga et al., 2013). Some types of IBV vaccines are particularly good at inducing antibodies that will cross-react with more distant virus types, providing broad protection, but each case is different and should be experimentally tested in chickens (Mendoza-González et al., 2022).

The significant antigenic variability of IBV has led to the development of many vaccines worldwide to combat this disease. Additionally, the use of two genetically distinct vaccines can provide broad coverage, although not complete, against heterologous viruses (Abdel-Sabour et al., 2021; Sjaak de Wit et al., 2011). Epidemiological surveillance can help characterize which genotype or lineage is present in a particular region, evaluate possible viral escapes, and identify new wild strains of IBV (Valastro et al., 2016).

In Brazil, IBV has been circulating since the 1950s, with many distinct genotypes and lineages identified. The first identification was of the Mass serotype (Fraga et al., 2018), and with the advent of molecular biology in the 1990s, a clearer understanding of the main viral clades present in Brazil became possible. Until 2021, only two vaccine serotypes were authorized for use in Brazil, the Mass serotype and the Variant BR.

In 2022, a new lineage, GI-23 (Variant-2), was identified in Brazil, and a matching vaccine was introduced (Ikuta et al., 2022; Trevisol et al., 2023). The Variant-2 of the Infectious Bronchitis Virus, belonging to the GI-23 lineage, was first described

in Israel in 1998 and was responsible for significant lesions in the respiratory and nephropathogenic systems in birds. In 2016, the identification of the same strain in Europe was reported (Lisowska et al., 2017; Valastro et al., 2016).

Therefore, the primary objective of this study was to monitor IBV in both vaccinated and non-vaccinated birds, assess vaccine escapes and viral persistence in hosts, evaluate the prevalence of different lineages, and investigate the possible introduction of new IBV lineages in Brazilian territory.

Material and Methods

Sampling

A total of 100 broiler chicken batchs (*Gallus gallus domesticus*) were collected and evaluated. The samples were obtained from different states in Brazil and were collected from August to December 2021. Zootechnical data were not available for this study.

The total number of samples collected per state followed the proportional poultry production of each region or location. Ten chickens were sampled per batches, totaling 1,000 chickens from the following regions: South (states of Paraná (n = 30 batches), Santa Catarina (n = 15 batches), Rio Grande do Sul (n = 15 batches, Southeast (states of São Paulo (n = 10 batches) and Minas Gerais (n = 10 batches), and Northeast (state of Ceará (n = 20 batches). These regions collectively represent 80% of the broiler chicken production in Brazil (ABPA, 2023). Sterilized swabs were used for collecting material from the nasal sinuses of the birds to detect IBV in the samples. All collection was carried out from dead animals, donated by the producing

farms and submitted to inspection aged between 13 and 32 days. Figure 1 illustrates the regions and collection areas.

All biological samples evaluated here were donated by farms that carry out routine inspections, eliminating the need for an ethics committee as they are leftover biological samples collected by routine health surveillance services - Consultation with the Ethics Committee on the Use of Animals (CEUA nº 4434190521 / Federal University of Santa Catarina).

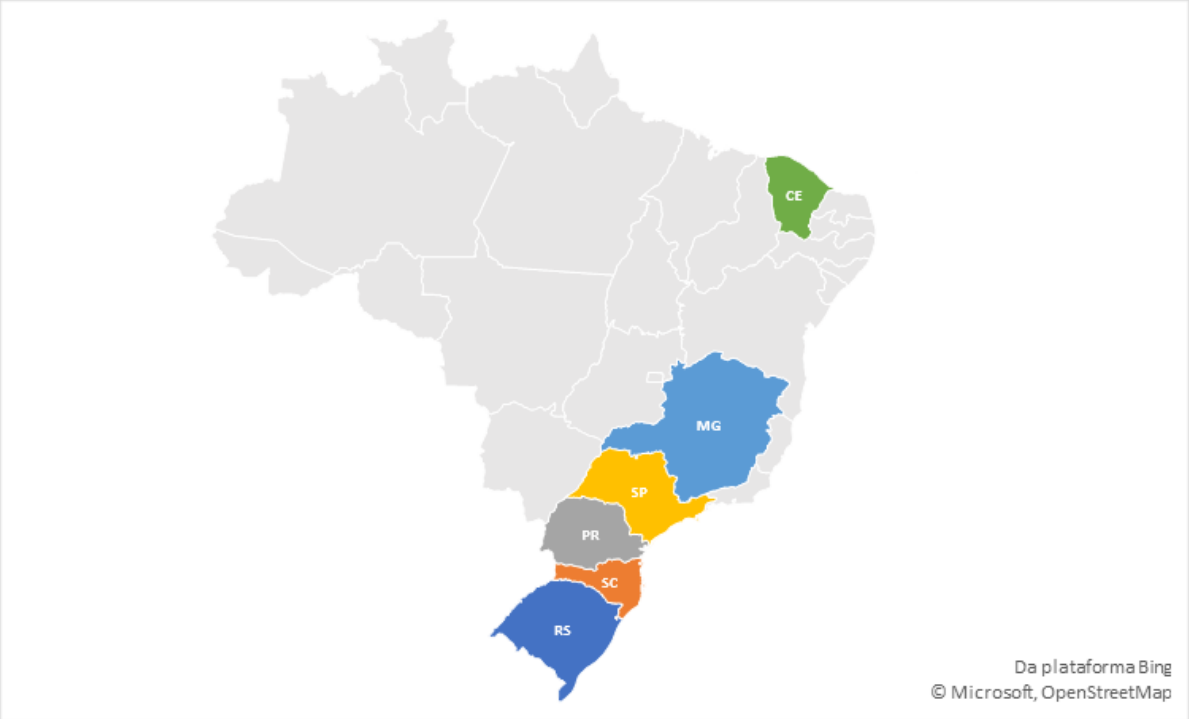


Figure 1. Map of Brazil, highlighting the South, Southeast and Northeast Regions and the Brazilian states sampled in the present study, being Rio Grande do Sul (RS), Santa Catarina (SC), Paraná (PR), São Paulo (SP), Minas Gerais (MG) and Ceará (CE).

Batches were selected based on a history of respiratory problems, and the vaccination programs used are described in Table 1.

Table 1: Demonstrate the different vaccination programs in Brazilian states. Each region utilizes programs based on its local challenges, which may vary between the use of one or two strains in the same immunoprevention program, where the day and method of application are the same for all companies.

State	Vaccination program	Vaccination date	Location and vaccination route
Santa Catarina	Massachusetts + BR-1	1st day of life	Spray in the hatchery
Rio Grande do Sul	Massachusetts + BR-1	1st day of life	Spray in the hatchery
Paraná	Massachusetts + BR-1	1st day of life	Spray in the hatchery
Minas Gerais	Unvaccinated birds for IBV	1st day of life	Spray in the hatchery
São Paulo	Massachusetts	1st day of life	Spray in the hatchery
Ceará	Massachusetts	1st day of life	Spray in the hatchery

Extraction of genetic material and molecular detection of IBV by RT-PCR

Nasotracheal swabs were eluted in PBS (1X), and total RNA extraction from the samples was performed using the RNeasy® Mini kit (QIAGEN) following the manufacturer's instructions. Reverse Transcription followed by Polymerase Chain Reaction (RT-PCR) was carried out to determine the presence of IBV, generating amplicons of 392 base pairs (bp) corresponding to the hyper-variable target region of the S1 gene of Infectious Bronchitis Virus (IBV) (Jones et al., 2005). Reagents used included the High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM) and PlatinumTM Taq DNA Polymerase (Thermo Fisher Scientific).

Samples in which IBV identification was performed with a CT value below 35 were subjected to typing, based on the analysis of the hypervariable region of the S1 Spike gene, to identify samples belonging to the BR-I, BR-II, Massachusetts, and GI-23 lineages of the infectious bronchitis virus (IBV).

Genetic characterization of the IBV S1 gene

Of the samples in which virus typing was performed, 28 were selected for sequencing of the S1 gene to carry out phylogenetic classification. To this end, the amplicons obtained were sequenced using the Sanger method to obtain phylogenetic information and confirm the viral lineage and strain.

For phylogenetic analysis, the MEGA-X program was employed (Kumar et al., 2018). The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model. The tree with the highest log likelihood (-2056.43) is shown. The percentage of trees in which the associated taxa cluster together is shown

next to the branches. The initial tree for the heuristic search was automatically obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with the highest log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 71 nucleotide sequences. The codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 308 positions in the final dataset (Kumar *et al*., 2018).

Results and Discussion

Prevalence of IBV in vaccine and non-vaccine samples based on RT-PCR

Out of the 100 batches assessed for IBV detection, 91% of the samples tested positive. The positive samples were further subtyped, except for five samples that exhibited a cycle threshold (CT) greater than 35 and were consequently categorized as untyped. In Table 2, the results of IBV positivity in different regions are presented.

Table 2: Prevalence of IBV in the different states that originated from the samples, showing the total number of batches that exhibited viral persistence, whether with the presence of one or more IBV strains. Additionally, it is possible to verify the age of the evaluated animals.

State	Age of collection (days old)	Total Samples	Number of Positive samples - IBV	IBV strains identified through RT-PCR

Rio Grande do Sul	16 - 26	15	15	(07) BR-I/BR-II (01) BR-I/BR-II, GI-23 (05) BR-I/BR-II, Massachusetts (01) BR-I/BR-II, Massachusetts, GI-23
Santa Catarina	17 -25	15	15	(02) Massachusetts (01) Not detected (02) BR-I/BR-II (10) BR-I/BR-II, Massachusetts
Paraná	20 - 32	30	30	(14) BR-I/BR-II (06) BR-I/BR-II, GI-23 (03) BR-I/BR-II, Massachusetts, GI-23 (03) Massachusetts, GI-23

				(05) GI-23
São Paulo	21 - 27	10	10	(05) Not detected (01) Untyped (01) BR-I/BR-II (02) BR-I/BR-II, Massachusetts (01) Massachusetts
Minas Gerais	13 - 20	10	07	(03) Not detected (07) BR-I/BR-II
Ceará	24 - 28	20	14	(06) Not detected (04) Untyped (01) BR-I/BR-II (09) Massahusets

The subtyping revealed that two (2.19%) samples were characterized as the Massachusetts strain and 28 (30.76%) were classified as the BR-I/BR-II strain. Eight

(8.79%) of them were categorized as positive for both BR-I/BR-II and Massachusetts strains, and seven (7.69%) were classified as BR-I/BR-II and Variant-2 strains. Additionally, three (3.29%) samples were classified as BR-I/BR-II, Massachusetts, and Variant-2 strains. Three (3.29%) samples were identified as Massachusetts and Variant-2 strains. Finally, six (6.59%) batches were classified as Variant-2 strains.

The first case of IBV in Brazil was linked to the Mass serotype, belonging to genogroup 1, lineage 1, which occurred in the 1950s. Since then, the virus has evolved significantly, and new lineages have been identified over time (Sjaak de Wit et al., 2011). Additionally, the introduction of vaccines was a crucial step in reducing productivity losses, whether in short-cycle or long-cycle poultry. Until the year 2016, only the Mass serotype was allowed for vaccination in birds (Valastro et al., 2016). However, after some respiratory outbreaks in birds and the epidemiological identification of genogroup GI-11, strain BR, the Ministry of Agriculture and Supply approved the use of a new vaccine for IBV prevention, this time with a strain homologous to the Brazilian variant, although this lineage had been previously described (Chacón et al., 2019; Jackwood, 2012). As the vaccine was introduced into Brazilian batches, the outbreaks decreased, to some extent, helping to control the damage caused by this lineage (Fraga et al., 2018). In this context, the six states that comprised the epidemiological research in this study represent approximately 80% of the country's chicken production (ABPA, 2023).

In total, 90% of the birds received vaccines containing the Massachusetts strain, either individually (30%) or in combination with vaccines containing the BR-I strain (60%), as demonstrated in Table 1. Additionally, 10% of the birds were not vaccinated, and this choice may pose risks to the batch. In the region where no vaccination occurred, 70% (07 samples) tested positive for IBV (BR-I/BR-II). This underscores the

sanitary risk associated with not using vaccines in immunoprophylaxis. In this regard, vaccination programs can vary significantly depending on the country, region, biosecurity measures, and findings from molecular diagnostics. These pillars must be respected when introducing a vaccine, as regional production characteristics vary. Therefore, companies can make more informed decisions when composing their vaccination programs.

From the results obtained, it is worth noting that 19% of the samples detected Variant-2, which belongs to Genogroup I, Lineage 23. At the end of 2020, a new IBV outbreak was reported by many companies in the country, and this study can contribute to the elucidation of the problems that occurred during that period. It identified and characterized a new lineage, belonging to genotype 1, lineage 23, Variant-2, originally described in Israel (Houta et al., 2021).

Two studies, also evaluating samples from the year 2021, mainly from the Southern region, particularly from the state of Paraná, identified the presence of the IBV GI-23 lineage circulating in broilers. Since its introduction, there has been a rapid spread of this lineage among farms in the major producing regions of the country, with birds exhibiting severe clinical symptoms in the upper respiratory tract and renal lesions (Ikuta et al., 2022, 2023; Trevisol et al., 2023).

S1 sequencing and phylogenetic analysis

Following the initial screening through RT-PCR, the sequencing of the S1 glycoprotein was carried out for potential differentiation between vaccine strains and field challenge strains, even to identify new strains in circulation within the national territory. These analyses were conducted on batches representing the production areas where strains potentially non-vaccine-related were detected (all regions were

evaluated here, except for Ceará, as the IBV-positive samples, when typed, were positive for the Massachusetts/Vaccine strain). In this regard, 28 (32.9%) of the positive batches were found to contain strains related to field challenges.

According to the phylogenetic relationship depicted in Figure 2 (GenBank: OR972341-OR972368). two batches (3 and 4) from the state of Santa Catarina were grouped and classified as belonging to the GI-1 strain, with the nucleotide sequences showing high similarity to the USP-13 samples (GenBank: FJ791254.1) and the H120 strain (GenBank: M21970.1).

The batches correspond to the states of Santa Catarina (11 and 13), Rio Grande do Sul (20, 21, 27, and 28,), and Paraná (46, 48, 49, 51, 52, 53, 55, and 58), were clustered and categorized as members of the GI-11 strain (GenBank: OR972341-OR972368).

The nucleotide sequences of batches exhibited high similarity to the BR-I strain (GenBank: KY626044.1). The nucleotide sequence of batch 12 (from Santa Catarina state) showed high similarity to the USP-16 sample (GenBank: FJ791757.1).

The nucleotide sequence of batch 62 (from the São Paulo state), showed more than 99.98% similarity to the USP-24 sample (GenBank: FJ91265.1). From the state of Minas Gerais (state that does not use IBV vaccines), the nucleotide sequence of six batches (65, 66, 67, 69, 77, and 78) from the southeast region (GenBank: OR972341-OR972368), exhibited high similarity with the samples USP-27 (GenBank: FJ791268.1), USP-29 (GenBank: FJ791270.1), and USP-30 (GenBank: FJ791271.1).

According to the phylogenetic relationship depicted in Figure 2, four batches (31, 33, 35, 43) were grouped within the GI-23 strain, all of them from the state of Paraná. The nucleotide sequence exhibited a similarity exceeding 99.99% with the IBV Israel Variant-2 strain (GenBank: JX027070.1). As in the present study, the strains

identified by Trevisol *et al*. (2023), who conducted a phylogenetic analysis and grouped them together with strains circulating in Poland and Israel the strains identified by Ikuta *et al*. (2022) were clustered with the parental GI23 strain from Israel (Variant-2), forming a distinct clade separated from all other IBV lineages.

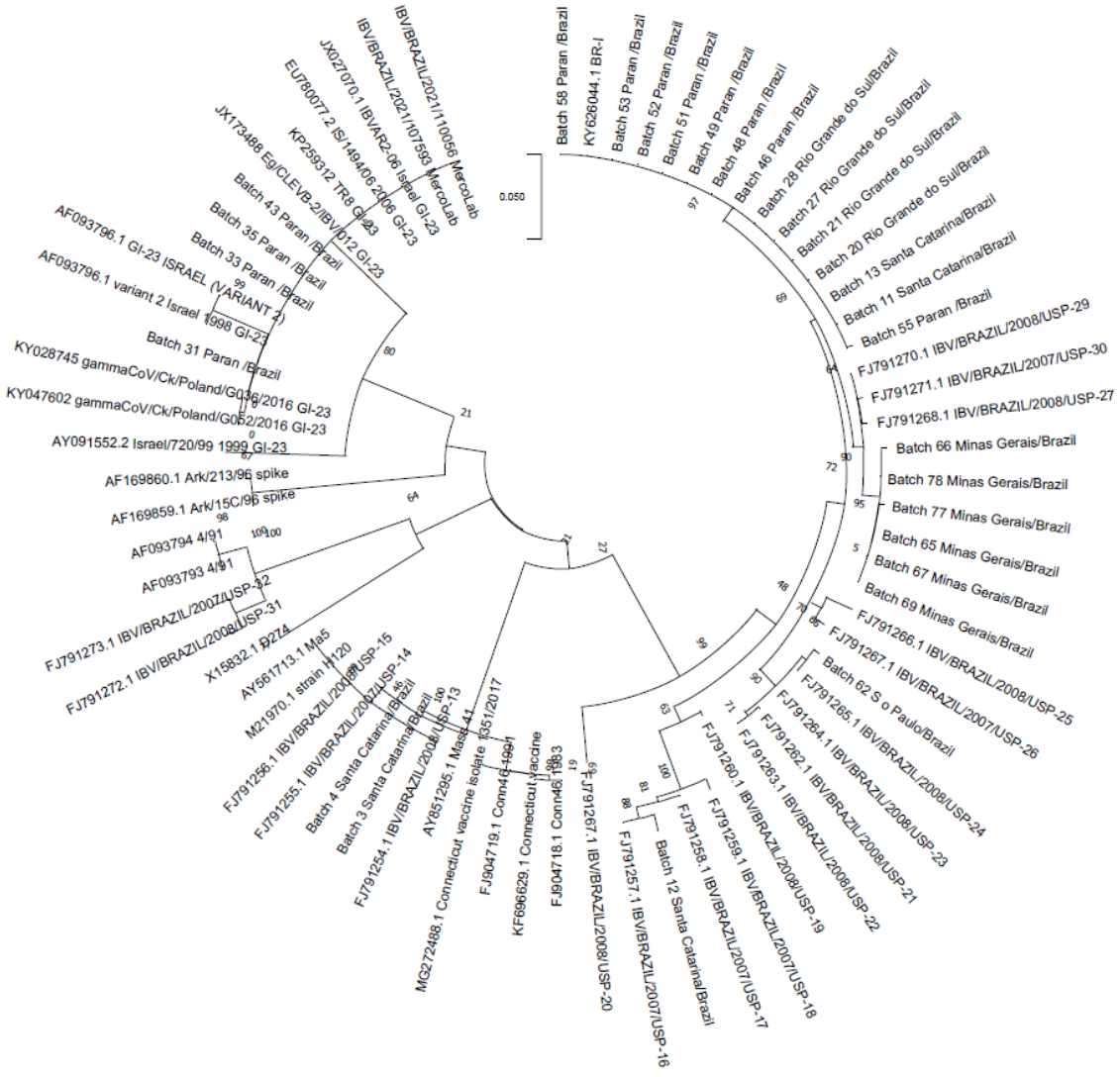


Figure 2 - Phylogenetic analysis of partial S1 sequences of Brazilian and international IBV strains. The tree was generated by the Maximum Likelihood method and the Tamura-Nei model. The tree with the highest log likelihood (-2056.43) is shown. The percentage of trees in which the associated taxa cluster together is shown next to the branches. The initial tree for the heuristic search was automatically obtained by

applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with the highest log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The fact that coronaviruses have RNA genomes, and these genomes are more extensive, ranging from 27 kb to 32 kb in length, facilitating the occurrence of errors during replication, resulting in a high mutation rate (Masters, 2006; Woo et al., 2009). New variants of IBV, capable of evading vaccine-induced immunity, continuously emerge due to mutations resulting from replication errors and/or recombination events in the S1 sequences (Kariithi et al., 2022).

The comprehensive analysis of the S1 glycoprotein sequences demonstrates the diversity and distribution of IBV strains in different regions of Brazil. Identifying strains related to field challenges, particularly those not linked to vaccine strains, highlights the dynamic nature of IBV and the continuous evolution of the virus. The phylogenetic analysis, as depicted in Figure 2, provides a visual representation of the genetic relationships among Brazilian and international IBV strains.

Vaccine persistence in the samples

There is a wide variety of vaccines, differing in strains and vaccine types according to local requirements. In Brazil, different regions or companies implemented distinct vaccination programs for the Infectious Bronchitis Virus (IBV), customized to address local challenges (Saraiva et al., 2018). In the case of live vaccines, the aim is to simulate the natural infection process by the field virus, but in an attenuated manner,

so that the virus replication induces an immune response without causing the complete disease (Guzmán & Hidalgo, 2020).

Table 3 presents the state to which the batches belonged, the age of the birds, the vaccination program employed, and the typing results for batches where the persistence of the vaccine virus may have occurred.

Table 3 - Information on the state affiliation of the batches, the age of the birds, the employed vaccination program, and the IBV genotype results for batches where the persistence of the vaccine virus is potentially observed.

State	Batch number	Age (days)	Vaccine	IBV genotyope
Santa Catarina	1	17	Infectious Bronchitis - Massachusetts (live) + BR-I (live)	Positive for strain BR-I/BR-II, Massachusetts
	2	17		Positive for strain BR-I/BR-II, Massachusetts
	3	19		Massachusetts
	4	25		Massachusetts
	5	25		Positive for strain BR-I/BR-II, Massachusetts
	6	20		Positive for strain BR-I/BR-II, Massachusetts

	7	18		Positive for strain BR-I/BR-II, Massachusetts
	8	18		Positive for strain BR-I/BR-II, Massachusetts
	9	17		Positive for strain BR-I/BR-II, Massachusetts
	10	21		Positive for strain BR-I/BR-II, Massachusetts
	11	22		Positive for strain BR-I/BR-II
	13	19		Positive for strain BR-I/BR-II
	14	18		Positive for strain BR-I/BR-II, Massachusetts
	15	17		Positive for strain BR-I/BR-II, Massachusetts
	16	24		Positive for strain BR-I/BR-II and strain Variant-2

Rio Grande do Sul	17	23	Infectious Bronchitis - Massachusetts (live) + BR-I (live)	Positive for strain BR-I/BR-II
	18	26		Positive for strain BR-I/BR-II, Massachusetts
	19	27		Positive for strain BR-I/BR-II, strain Massachusetts and strain Variant-2
	20	21		Positive for strain BR-I/BR-II
	21	20/21		Positive for strain BR-I/BR-II
	22	24		Positive for strain BR-I/BR-II and Massachusetts
	23	24		Positive for strain BR-I/BR-II and Massachusetts

	24	16		Positive for strain BR-I/BR-II and Massachusetts
	25	22		Positive for strain BR-I/BR-II
	26	22		Positive for strain BR-I/BR-II and Massachusetts
	27	21		Positive for strain BR-I/BR-II
	28	21		Positive for strain BR-I/BR-II
	29	22		Positive for strain BR-I/BR-III
Paraná	32	32	Infectious Bronchitis - Massachusetts (live) + BR-I (live)	Positive for strain Massachusetts and strain Variant-2
	34	25		Positive for strain Massachusetts and strain Variant-2

Paraná	36	21	Infectious Bronchitis - Massachusetts (live) + BR-I (live)	Positive for strain BR-I/BR-II and strain Variant-2
	37	24		Positive for strain BR-I/BR-II and strain Variant-2
	38	24		Positive for strain BR-I/BR-II and strain Variant-2
	39	22		Positive for strain BR-I/BR-II, strain Massachusetts and strain Variant-2
	41	31		Positive for strain BR-I/BR-II, strain Massachusetts and strain Variant-2
	42	28		Positive for strain Massachusetts and strain Variant 2

	44	32		Positive for strain BR-I/BR-II and strain Variant-2
	45	27		Positive for strain BR-I/BR-II and strain Variant-2
	46	22		Positive for strain BR-I/BR-II
	47	23		Positive for strain BR-I/BR-II
	48	23		Positive for strain BR-I/BR-II
	49	25		Positive for strain BR-I/BR-II
	50	25		Positive for strain BR-I/BR-II and Massachusetts
	51	25		Positive for strain BR-I/BR-II

	52	25		Positive for strain BR-I/BR-II
	53	25		Positive for strain BR-I/BR-II
	54	20		Positive for strain BR-I/BR-II and Massachusetts
	55	24		Positive for strain BR-I/BR-II
	56	20		Positive for strain BR-I/BR-II and strain Variant-2
	57	25		Positive for strain BR-I/BR-II
	58	25		Positive for strain BR-I/BR-II
	59	24		Positive for strain BR-I/BR-II and Massachusetts

	60	24		Positive for strain BR-I/BR-II
São Paulo	62	26	Infectious Bronchitis - Massachusetts (live)	Positive for strain BR-I/BR-II
	71	23		Positive for strain BR-I/BR-II and Massachusetts
	72	27		Positive for strain BR-I/BR-II and Massachusetts
	73	23		Positive for strain Massachusetts
Ceará	81	24	Infectious Bronchitis - Massachusetts (live)	Positive for strain Massachusetts
	84	24		Positive for strain BR-I/BR-II and Massachusetts
	85	24		Positive for strain Massachusetts

	87	24		Positive for strain Massachusetts
	89	24		Positive for strain Massachusetts
	91	24		Positive for strain Massachusetts
	92	24		Positive for strain Massachusetts
	94	24		Positive for strain Massachusetts
	95	24		Positive for strain Massachusetts
	98	24		Positive for strain Massachusetts

In total, 67 samples exhibited vaccine persistence, regardless of the vaccination programs used by different companies. At the time of vaccine virus detection, the batches were between 16 and 32 days old. Between days 16 and 21, 14 batches showed vaccine persistence, batches from 22 to 27 days, 38 batchess exhibited vaccine persistence, and from 28 to 32 days, 6 batches displayed vaccine persistence.

There is a very clear predominance of strains present in Brazil, with a high percentage of GI-11 BR strains identified in the biological samples of this study. This data may be related to the high pressure exerted by vaccines, as over time, the Mass lineage was widely used by companies (Carranza et al., 2017; Legnardi et al., 2019).

In the state of Paraná, out of 30 biological samples, 18 samples revealed a new IBV variant in Brazil, belonging to GI-23, Variant-2, accounting for 60% of the samples from that state. This discovery justifies the issues faced in that state, where many companies reported IBV outbreaks associated with high condemnations in slaughterhouses between 2020 and 2021. Outbreaks linked to this new variant may be related to the partial coverage of vaccination programs used during that period.

The state of Santa Catarina was the only one that presented a challenge with the Mass strain, which may be related to vaccine application failures in hatcheries (Jackwood & de Wit, 2013). Outbreaks linked to this new variant may be related to partial coverage of the vaccination programs used during that period (Lisowska et al., 2021).

In Rio Grande do Sul, 3 biological samples tested positive for Variant-2 strain, which accounts for 20% of the samples from that state. Although the company employs a vaccination program that provides partial protection against this variant, the program does not completely block the infection and replication of this virus (Jackwood & de Wit, 2013).

In the state of São Paulo, the vaccination program consisted only of the Mass serotype, providing partial protection for other serotypes, justifying the presence of 1 sample belonging to GI-11, strain BR-I. In Minas Gerais, the company did not use vaccines for IBV prevention, creating an opportunity for infection and replication of field viruses. In this state, out of the 10 samples, 7 were positive for IBV, with 6 of them

being classified as GI-11, strain BR-I, and 1 sample was related to the Mass vaccine virus. In this case, there may have been vaccine virus transmission from a neighboring company, thus, indicating the circulation of the BR-I strain (Legnardi et al., 2019).

In the state of Ceará, all the typed samples were related to vaccine strains. Many studies report these situations where vaccinated batches show field strains when evaluated. These results, after the use of molecular diagnostic technologies, are relatively common (Ikuta et al., 2023).

One of the main concerns regarding its continued use is how live vaccines can alter the pathogenic populations in the field through recombination across the entire genome, reversion to virulence, or enabling strain replacement. This concern is heightened when introducing a new vaccine strain in countries where the pathogenic variant has not yet been detected, as, after introduction, the new vaccine strain may evolve in unpredictable ways, presenting additional challenges for disease control strategies (Guzmán & Hidalgo, 2020).

It is not clear how poultry can respond to the combination of two or more IBV vaccines, which could be a limiting factor in protecting the birds against challenges (Jackwood et al., 2020). The high percentage of vaccine virus found in the birds may be related to the high excretion of the virus in the facilities, leading to viral transmission. This also raises the hypothesis of a high rate of virus persistence in the host, which is similar to what has been demonstrated (Legnardi et al., 2019). The concentration of vaccine viruses is highest after vaccine administration on the first day of life, and it decreases as the birds' immunity is established (Lisowska et al., 2021).

In general, the results emphasize the need for continued surveillance and monitoring of IBV in poultry batches in Brazil. Biosecurity measures and vaccination strategies should consider the diversity of IBV strains to provide effective protection.

The genetic information obtained in this study can serve as a foundation for the development of more targeted and efficient vaccines. Tailoring vaccines to specific lineages and variants can enhance their efficacy in mitigating IBV outbreaks.

Conclusion

The present study identified the circulation of the GI-23 lineage in nasotracheal swabs from batches collected in 2021, suggesting that the virus was already in circulation in the states of Paraná and Rio Grande do Sul during that year. Further research is needed to explore the properties and assess the potential impact of this variant on bird health. Further research is needed to investigate the properties and potential impact of this variant on poultry health.

Considering the large number of vaccine-derived strains identified, questions arise about the ability of live vaccines to spread and persist. This issue raises important points related to the effectiveness, safety, and potential environmental impact of live vaccines used in avian immunization contexts.

Overall, this study underscores the importance of genomics and molecular epidemiology in understanding and managing infectious diseases in poultry. The knowledge gained from this research can contribute to more effective control and prevention strategies for Infectious Bronchitis Virus in the poultry industry.

Collaboration between researchers, poultry companies, and regulatory authorities is essential to ensure the implementation of informed and effective biosecurity and vaccination practices, reducing the economic impact of IBV on the poultry sector.

Referências

Abdel-Sabour, M. A., Rohaim, M. A., Salman, O. J. A., Abodalal, S. E., Mohammad, F. F., Madkour, M. S., Abdel-Wanis, N. A., & Munir, M. (2021). Immunogenicity and efficacy of a bivalent vaccine against infectious bronchitis virus. *Comparative Immunology, Microbiology and Infectious Diseases*, *77*, 101670. https://doi.org/10.1016/j.cimid.2021.101670

Associação Brasileira de Proteína Animal (ABPA). (2023). *Annual Report 2023*. https://abpa-br.org/wp-content/uploads/2023/04/Relatorio-Anual-2023.pdf

Britton, P., Armesto, M., Cavanagh, D., & Keep, S. (2012). Modification of the avian coronavirus infectious bronchitis virus for vaccine development. *Bioengineered*, *3*(2), 114–119. https://doi.org/10.4161/bbug.18983

Carranza, C., Astolfi-Ferreira, C. S., Santander Parra, S. H., Nuñez, L. F. N., Penzes, Z., Chacón, J. L., Sesti, L., Chacón, R. D., & Piantino Ferreira, A. J. (2017). Genetic characterisation and analysis of infectious bronchitis virus isolated from Brazilian flocks between 2010 and 2015. *British Poultry Science*, *58*(6), 610–623. https://doi.org/10.1080/00071668.2017.1365116

Cavanagh, D. (2007). Coronavirus avian infectious bronchitis virus. *Veterinary Research*, *38*(2), 281–297. https://doi.org/10.1051/vetres:2006055

Cavanagh, D., Davis, P. J., Pappin, D. J. C., Binns, M. M., Boursnell, M. E. G., & Brown, T. D. K. (1986). Coronavirus IBV: Partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor propolypeptide of IBV strains Beaudette and M41. *Virus Research*, *4*(2), 133–143. https://doi.org/10.1016/0168-1702(86)90037-7

Cavanagh, D., & Gelb, J. J. (2008). Infectious Bronchitis. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, & D. E. Swayne (Eds.), *Diseases of poultry* (12th ed., pp. 117–135). Blackwell Publishing Ltd.

Chacón, J. L., Chacón, R. D., Sánchez-Llatas, C. J., Morín, J. G., Astolfi-Ferreira, C. S., & Piantino Ferreira, A. J. (2023). Antigenic and molecular characterization

of isolates of the Brazilian genotype BR-I (GI-11) of infectious bronchitis virus supports its recognition as BR-I serotype. *Avian Pathology*, *52*(5), 323–338. https://doi.org/10.1080/03079457.2023.2228725

Chacón, R. D., Astolfi-Ferreira, C. S., Chacón, J. L., Nuñez, L. F. N., De la Torre, D. I., & Piantino Ferreira, A. J. (2019). A seminested RT-PCR for molecular genotyping of the Brazilian BR-I Infectious Bronchitis Virus Strain (GI-11). *Molecular and Cellular Probes*, *47*, 101426. https://doi.org/10.1016/j.mcp.2019.101426

Fraga, A. P., Balestrin, E., Ikuta, N., Fonseca, A. S. K., Spilki, F. R., Canal, C. W., & Lunge, V. R. (2013). Emergence of a New Genotype of Avian Infectious Bronchitis Virus in Brazil. *Avian Diseases*, *57*(2), 225–232. https://doi.org/10.1637/10346-090412-Reg.1

Fraga, A. P. de, Gräf, T., Pereira, C. S., Ikuta, N., Fonseca, A. S. K., & Lunge, V. R. (2018). Phylodynamic analysis and molecular diversity of the avian infectious bronchitis virus of chickens in Brazil. *Infection, Genetics and Evolution*, *61*, 77–83. https://doi.org/10.1016/j.meegid.2018.03.014

Guzmán, M., & Hidalgo, H. (2020). Live Attenuated Infectious Bronchitis Virus Vaccines in Poultry: Modifying Local Viral Populations Dynamics. *Animals*, *10*(11), 2058. https://doi.org/10.3390/ani10112058

Houta, M. H., Hassan, K. E., El-Sawah, A. A., Elkady, M. F., Kilany, W. H., Ali, A., & Abdel-Moneim, A. S. (2021). The emergence, evolution and spread of infectious bronchitis virus genotype GI-23. *Archives of Virology*, *166*(1), 9–26. https://doi.org/10.1007/s00705-020-04920-z

Ikuta, N., Fonseca, A. S. K., Fernando, F. S., Filho, T. F., Martins, N. R. da S., & Lunge, V. R. (2022). Emergence and molecular characterization of the avian infectious bronchitis virus GI-23 in commercial broiler farms from South America. *Transboundary and Emerging Diseases*, *69*(6), 3167–3172. https://doi.org/10.1111/tbed.14724

Ikuta, N., Kipper, D., Freitas, D. S. S. de, Fonseca, A. S. K., & Lunge, V. R. (2023). Evolution and Epidemic Spread of the Avian Infectious Bronchitis Virus (IBV) GI-23 in Brazil. *Viruses*, *15*(6), 1229. https://doi.org/10.3390/v15061229

Jackwood, M. W. (2012). Review of Infectious Bronchitis Virus Around the World. *Avian Diseases*, *56*(4), 634–641. https://doi.org/10.1637/10227-043012-Review.1

Jackwood, M. W., Clark, R., Cheng, S., & Jordan, B. J. (2020). Protection following simultaneous vaccination with three or four different attenuated live vaccine types against infectious bronchitis virus. *Avian Pathology*, *49*(4), 335–341. https://doi.org/10.1080/03079457.2020.1748173

Jackwood, M. W., & de Wit, S. (2013). Infectious Bronchitis. In *Diseases of Poultry* (pp. 139–159). Wiley. https://doi.org/10.1002/9781119421481.ch4

Jia, W., Karaca, K., Parrish, C. R., & Naqi, S. A. (1995). A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. *Archives of Virology*, *140*(2), 259–271. https://doi.org/10.1007/BF01309861

Jones, R. C., Worthington, K. J., & Gough, R. E. (2005). Detection of the Italy O2 strain of infectious bronchitis virus in the UK. *The Veterinary Record*, *156*(8), 260.

Jordan, B. (2017). Vaccination against infectious bronchitis virus: A continuous challenge. *Veterinary Microbiology*, *206*, 137–143. https://doi.org/10.1016/j.vetmic.2017.01.002

Kariithi, H. M., Volkening, J. D., Leyson, C. M., Afonso, C. L., Christy, N., Decanini, E. L., Lemiere, S., & Suarez, D. L. (2022). Genome Sequence Variations of Infectious Bronchitis Virus Serotypes From Commercial Chickens in Mexico. *Frontiers in Veterinary Science*, *9*. https://doi.org/10.3389/fvets.2022.931272

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Legnardi, M., Franzo, G., Koutoulis, K. C., Wiśniewski, M., Catelli, E., Tucciarone, C. M., & Cecchinato, M. (2019). Vaccine or field strains: the jigsaw pattern of infectious bronchitis virus molecular epidemiology in Poland. *Poultry Science*, *98*(12), 6388–6392. https://doi.org/10.3382/ps/pez473

Lisowska, A., Pikuła, A., Opolska, J., Jasik, A., Kycko, A., & Domańska-Blicharz, K. (2021). Virulence Properties of GI-23 Infectious Bronchitis Virus Isolated in Poland and Efficacy of Different Vaccination strategies. *Pathogens*, *10*(5), 522. https://doi.org/10.3390/pathogens10050522

Lisowska, A., Sajewicz-Krukowska, J., Fusaro, A., Pikula, A., & Domanska-Blicharz, K. (2017). First characterization of a Middle-East GI-23 lineage (Var2-like) of infectious bronchitis virus in Europe. *Virus Research*, *242*, 43–48. https://doi.org/10.1016/j.virusres.2017.09.010

Masters, P. S. (2006). The Molecular Biology of Coronaviruses. In *Advances in Virus Research* (pp. 193–292). https://doi.org/10.1016/S0065-3527(06)66005-3

Mendoza-González, L., Marandino, A., Panzera, Y., Tomás, G., Williman, J., Techera, C., Gayosso-Vázquez, A., Ramírez-Andoney, V., Alonso-Morales, R., Realpe-Quintero, M., & Pérez, R. (2022). Research Note: High genetic diversity of infectious bronchitis virus from Mexico. *Poultry Science*, *101*(10), 102076. https://doi.org/10.1016/j.psj.2022.102076

Molenaar, R. J., Dijkman, R., & de Wit, J. J. (2020). Characterization of infectious bronchitis virus D181, a new serotype (GII-2). *Avian Pathology*, *49*(3), 243–250. https://doi.org/10.1080/03079457.2020.1713987

Moreno, A., Franzo, G., Massi, P., Tosi, G., Blanco, A., Antilles, N., Biarnes, M., Majó, N., Nofrarías, M., Dolz, R., Lelli, D., Sozzi, E., Lavazza, A., & Cecchinato, M. (2017). A novel variant of the infectious bronchitis virus resulting from recombination events in Italy and Spain. *Avian Pathology*, *46*(1), 28–35. https://doi.org/10.1080/03079457.2016.1200011

Saraiva, G. L., Santos, M. R., Pereira, C. G., Vidigal, P. M. P., Fietto, J. L. R., de Oliveira Mendes, T. A., Bressan, G. C., Soares-Martins, J. A. P., de Almeida, M. R., & Silva-Júnior, A. (2018). Evaluation of the genetic variability found in

Brazilian commercial vaccines for infectious bronchitis virus. *Virus Genes*, *54*(1), 77–85. https://doi.org/10.1007/s11262-017-1515-2

Sjaak de Wit, J. J., Cook, J. K. A., & van der Heijden, H. M. J. F. (2011). Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathology*, *40*(3), 223–235. https://doi.org/10.1080/03079457.2011.566260

Trevisol, I. M., Caron, L., Mores, M. A. Z., Voss-Rech, D., da Silva Zani, G., Back, A., Marchesi, J. A. P., & Esteves, P. A. (2023). Pathogenicity of GI-23 Avian Infectious Bronchitis Virus Strain Isolated in Brazil. *Viruses*, *15*(5), 1200. https://doi.org/10.3390/v15051200

Valastro, V., Holmes, E. C., Britton, P., Fusaro, A., Jackwood, M. W., Cattoli, G., & Monne, I. (2016). S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infection, Genetics and Evolution*, *39*, 349–364. https://doi.org/10.1016/j.meegid.2016.02.015

Wang, X., & Khan, M. I. (2000). Use of reverse transcriptase-polymerase chain reaction-restriction fragment length polymorphism to examine the interaction between infectious bronchitis virus strains Massachusetts 41 and JMK *in ovo*. *Avian Pathology*, *29*(5), 441–448. https://doi.org/10.1080/030794500750047199

Woo, P. C. Y., Lau, S. K. P., Lam, C. S. F., Lai, K. K. Y., Huang, Y., Lee, P., Luk, G. S. M., Dyrting, K. C., Chan, K.-H., & Yuen, K.-Y. (2009). Comparative Analysis of Complete Genome Sequences of Three Avian Coronaviruses Reveals a Novel Group 3c Coronavirus. *Journal of Virology*, *83*(2), 908–917. https://doi.org/10.1128/JVI.01977-08

Yehia, N., Salem, H. M., Mahmmod, Y., Said, D., Samir, M., Mawgod, S. A., Sorour, H. K., AbdelRahman, M. A. A., Selim, S., Saad, A. M., El-Saadony, M. T., El-Meihy, R. M., Abd El-Hack, M. E., El-Tarabily, K. A., & Zanaty, A. M. (2023). Common viral and bacterial avian respiratory infections: an updated review. *Poultry Science*, *102*(5), 102553. https://doi.org/10.1016/j.psj.2023.102553

6. CONSIDERAÇÕES FINAIS

As projeções publicadas pela ONU, estimam um crescimento de 22% da população até 2025, ou seja, aproximadamente 9,8 milhões de pessoas habitarão o planeta terra. As tendências demográficas variam significativamente de uma região para outra. Algumas áreas, especialmente na África Subsaariana, ainda experimentarão um crescimento populacional substancial, enquanto outras, como partes da Europa e Ásia, podem enfrentar um declínio populacional.

Nesse sentido, o crescimento populacional tem uma correlação significativa com a questão da falta de alimentos, e essa relação é complexa. O aumento da população mundial coloca uma pressão crescente sobre os recursos naturais, incluindo produção agrícola e produção de proteína animal. À medida que a demanda por alimentos aumenta com o crescimento populacional, há uma necessidade correspondente de expandir a produção agrícola e de proteína animal para alimentar uma população maior.

O crescimento populacional pode aumentar os desafios relacionados à segurança alimentar, especialmente em áreas onde a infraestrutura agrícola é limitada. Garantir que a quantidade e a qualidade dos alimentos sejam adequadas para atender às necessidades crescentes da população torna-se uma preocupação crucial. O aumento da população destaca a necessidade de inovação na agricultura para aumentar a produtividade de forma sustentável e produzir alimentos seguros e saudáveis. Isso inclui o desenvolvimento de práticas agrícolas mais eficientes, o uso de tecnologias agrícolas avançadas e a promoção de métodos de produção que sejam ambientalmente sustentáveis, evitando assim, a transmissão de doenças através dos alimentos.

Em suma, a correlação entre o crescimento populacional e a falta de alimentos destaca a importância de abordagens integradas que considerem fatores sociais, econômicos, ambientais e tecnológicos para garantir a segurança alimentar global. Estratégias que visam a sustentabilidade, inovação e equidade na distribuição de alimentos são cruciais para enfrentar os desafios associados ao crescimento demográfico, evidenciando o conceito de saúde única.

A epidemiologia e monitoramento das doenças nos plantéis avícolas brasileiro necessitam de uma melhor compreensão, principalmente das formas de diagnóstico e a história evolutiva viral. A coinfecção entre agente viral e bacteriano podem

exacerbar os quadros clínicos aumentando assim, as perdas produtivas em vários níveis, no campo até o abatedouro.

Os resultados encontrados na presente tese doutoral apontam para busca de uma melhor compreensão de patógenos virais, assim como aperfeiçoamento de técnicas de diagnóstico, para caracterização viral e desenvolvimento de possíveis vacinas ou ferramentas para prevenção e controle. Torna-se relevante ressaltar a importância de estudos que considerem a evolução e biologia viral, uma vez que os vírus vão sofrendo mutações e acumulando tais mutações ao longo das infecções nos seus hospedeiros. Uma modificação ou mutação pode realmente não imprimir patogenia imediata, mas ao longo do tempo isso pode acumular-se, surgindo variantes patogênicas e/ou escape imune/vacinal. De tal forma, caracterizar regiões virais responsáveis pelo reconhecimento celular torna-se biotecnologicamente importante para controle sanitário e aperfeiçoamento, especialmente na avicultura, podendo evitar eventos epidemiológicos relevantes.

7. CONCLUSÃO

O **primeiro capítulo** discute a circulação e subtipagem de aMPV em diferentes países. Evidencia-se a necessidade de implementação de novas técnicas de diagnósticos para melhor compreensão da circulação desse agente nos plantéis de aves, além disso, a identificação do agente pode facilitar os programas imunoprofiláticos até mesmo, prospectar novas vacinas para uso em aves.

O **segundo capítulo** discutiu a soroprevalência de aMPV e identificação e caracterização molecular para subitpagem de aMPV. Além disso, foi discutido os impactos relacionados à coinfecção entre agente viral (aMPV) e bacteriano (APEC) podem exacerbar os quadros clínicos aumentando assim, as perdas produtivas em vários níveis, no campo até o abatedouro.

O **terceiro capítulo** evidenciou a presença de uma nova linhagem de IBV no plantel avícola brasileiro, sugerindo sua circulação em 2021 nos estados do Rio Grande do Sul e Paraná. Mas estudos adicionais são necessários para explorar as propriedades e avaliar o impacto potencial dessa variante na saúde das aves e para investigar as propriedades e o impacto potencial dessa variante na saúde das aves. Além disso, considerando o grande número de cepas derivadas de vacinas

identificadas, surgem questões sobre a capacidade das vacinas vivas se espalharem e persistirem.

Em geral, conclui-se que foi possível monitorar o cenário nacional frente ao aMPV e IVB nas regiões produtoras de frango no Brasil, sendo que os resultados podem contribuir no desenvolvimento de estratégias mais eficazes de controle e prevenção do IBV, aMPV, bem como das coinfecções com APEC na indústria avícola.

8. PERSPECTIVAS DO ESTUDO

Abre-se a possibilidade de estudo relacionado a Viroma aviário, sendo que esse método poderá auxiliar na identificação de vírus e nas projeções de programas de vigilância e prevenção viral mais apropriadas mitigando os efeitos nas aves e futuras zoonoses.

9. ATIVIDADES COMPLEMENTARES REALIZADAS AO LONGO DO DOUTORAMENTO

Artigos Científicos publicados em Revistas Internacionais Qualis "*A*":

- A.L. Kraieski, **G.B.C. Salles,** E.C. Muniz, D.V.J. Nascimento, A.J. Lima Neto, I.L. Santos, A.M.B.N. Madeira, Sensitivity of field isolates of Eimeria acervulina and E. maxima from three regions in Brazil to eight anticoccidial drugs, Poultry Science,Volume 100, Issue 8, 2021, 101233, ISSN 0032-5791, https://doi.org/10.1016/j.psj.2021.101233.

- Pilati GVT, Cadamuro RD, Filho VB, Dahmer M, Elois MA, Savi BP, **Salles GBC,** Muniz EC, Fongaro G. Bacteriophage-Associated Antimicrobial Resistance Genes in Avian Pathogenic *Escherichia coli* Isolated from Brazilian Poultry. *Viruses*. 2023; 15(7):1485. https://doi.org/10.3390/v15071485.

Cursos e Palestras Ministrados

- PALESTRA NO CURSO 28º CURSO DE SANIDADE – PREVENÇÃO E CONTROLE DE SALMONELLA, 2022
- PALESTRA NO CURSO 28º CURSO DE SANIDADE – PREVENÇÃO E CONTROLE DE E. COLI, 2022
- PALESTRA NO CURSO 29º CURSO DE SANIDADE – PREVENÇÃO E CONTROLE DE E. COLI, 2023
- PALESTRA NO CURSO 29º CURSO DE SANIDADE – DOENÇAS RESPIRATÓRIAS EM AVICULTURA, 2023

- PALESTRA NO SEGUNDO CURSO DE ATUALIZAÇÕES TÉCNICAS FACTA – DOENÇA DE GUMBORO.

Capítulo de livro publicado

- Salles GBC, Muniz EC, capítulo: Vacinação em frangos de corte, Produçao de frangos de corte. FACTA WPSA-BR, 2023.

Apresentação de posteres em Evento Científico

- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA 2021: Teste de sensibilidade aos anticoccidianos realizado em integração de frango de corte na região sudeste do Brasil com *Eimeria Maxima,*
- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA 2021: Anticoccidial sensitivity testing para *Eimeira Maxima* em uma integração de frangos de corte no nordeste do Brasil
- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA 2021: Avaliação sorológica de uma vacina imunocomplexo em matrizes de corte no estado de Santa Catarina
- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA 2022: Comparação a campo dos resultados zootécnicos em frango de corte entre a vacina para doença de Gumboro de imunocomplexo com a cepa V877 e a vacina com a cepa M.B.
- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA 2022: Comparação entre dois métodos de análise sorológica em amostras para Gumboro e Reovírus
- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA 2022: Avaliação sorológica de uma vacina de Encefalomielite aviária utilizando diferentes kits de diagnóstico e vias de aplicação
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2022: Correlação entre o teste de sensibilidade aos anticoccidianos para *Eimeria maxima* e OPG (número de oocistos por grama de fezes) em granja de frango de corte

- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2022: Avaliação de eficácia da Poulvac ST e do Lincospectin 440 na redução da *Salmonella* Minnesota em frangos experimentalmente desafiados
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2022: Prevalência e perfil de resistência à antimicrobianos de *Escherichia coli* patogênica aviária isolada em aves brasileiras
- PUBLICAÇÃO DE POSTER NO CONGRESSO FACTA: Proteção cruzada da vacina viva com sorovar *Salmonella* Typhimurium em aves desafiadas por *Salmonella* Minnesota, 2022
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2022: Soroprevalência do Metapneumovírus Aviário em frangos de corte não vacinados no Brasil
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: resultados de um ast (teste de sensibilidade aos anticoccidianos) para *eimeria maxima* no estado do paraná
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: avaliação da eficácia de dois programas vacinais nos parâmetros zootécnicos de frangos de corte
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: impacto no custo de produção e desempenho zootécnicode frangos de corte submetidos a dois diferentes programas vacinais
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: avaliação temporal da positividade para *salmonella*com uso de vacina viva em granjas de frango de corte - estudo de campo
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: ocorrência de *escherichia coli* patogênica aviária (apec) em frangos de corte dos estados de minas gerais e da bahia
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: coinfecção entre metapneumovírus aviário (ampv) e *escherichia coli* patogênica aviária (apec) em frangos de corte em diferentes regiões do brasil
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: teste de sensibilidade anticoccidiana (ast) como ferramenta para escolha de programas com base na sensibilidade das moléculas
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: avaliação de índices zootécnicos de frangos de corte após a utilização de uma vacina viva de *escherichia coli*
- PUBLICAÇÃO DE PÔSTER NO CONGRESSO FACTA 2023: avaliação de índices zootécnicos de frangos de corte após a utilização de uma vacina viva de *escherichia coli*
- A comparison of zootechnical results in broilers vaccinated with a live attenuated IBD vaccine containing mb strain or an immunocomplex IBD v877 strain vaccine, XXII congress of the WVPA, 2023
- PUBLICAÇÃO EM ANAIS (Congresso Brasileiro de Virologia & XV Encontro de Virologia do Mercosul), 2022.

Participação em congressos nacionais e internacionais

- PARTICIPAÇÃO SIMPÓSIO BRASIL SUL DE AVICULTURA, 2021
- PARTICIPAÇÃO SIMPÓSIO BRASIL SUL DE AVICULTURA, 2022
- PARTICIPAÇÃO SIMPÓSIO BRASIL SUL DE AVICULTURA, 2023
- ORGANIZADOR DO SIMPÓSIO ACAV, 2021
- ORGANIZADOR DO SIMPÓSIO ACAV, 2022
- ORGANIZADOR DO SIMPÓSIO ACAV, 2023
- PARTICIPAÇÃO DO SIMPÓSIO INTERNACIONAL DE SAÚDE INTESTINAL DE AVES, 2023.