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LARINGOTRAQUEÍTE INFECCIOSA EM POEDEIRAS COMERCIAIS NO ESTADO DE SANTA CATARINA: CARACTERIZAÇÃO ANATOMOPATOLÓGICA, MOLECULAR E SOROEPIDEMIOLÓGICA

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RESUMO

A laringotraqueíte infecciosa (LTI) é uma doença respiratória altamente contagiosa e de notificação obrigatória, causada por *Gallid alphaherpesvirus 1* (GaHV-1). Em Santa Catarina, sul do Brasil, a LTI era considerada exótica até 2020, quando foi diagnosticada em uma granja comercial de postura no município de São Ludgero. Este estudo tem por objetivo avaliar a prevalência da doença no estado, determinando sua positividade por ELISA e PCR em tempo real (qPCR), além das alterações anatomopatológicas e os fatores epidemiológicos associados à infecção. O estudo também objetiva caracterizar as estirpes do vírus da laringotraqueíte infecciosa (VLTI) circulantes no surto de 2020-2021 no estado e investigar sua filogenia. O trabalho foi conduzido em duas etapas: a primeira analisou municípios do Bolsão de São Ludgero em 2020, enquanto a segunda avaliou a disseminação do vírus em todo o estado em 2021. Foram amostradas 44 granjas em 2020 e 49 em 2021, abrangendo granjas comerciais e de recria de postura. Em cada granja, 20 aves foram testadas sorologicamente para detecção de anticorpos anti-VLTI, e 10 foram submetidas à eutanásia para necropsia, colheita de órgãos respiratórios e avaliação histopatológica. Amostras de conjuntiva, laringe, traqueia e gânglio trigeminal foram analisadas por qPCR, tendo como alvo a glicoproteína C (gC) do VLTI. As amostras positivas foram submetidas ao sequenciamento Sanger dos genes ICP4 e TK do VLTI. Além disso, um questionário foi aplicado a cada avicultor para identificação de possíveis fatores de risco associados à LTI, através de Teste Exato de Fisher e análise multivariada. Os resultados mostraram que, em 2020, 95,4% das granjas foram soropositivas, com 88,1% de positividade por qPCR. Em 2021, esses índices foram de 65,3% e 21,8%, respectivamente. A infecção por VLTI foi confirmada por exame histopatológico da traqueia e laringe, com detecção de células sinciciais e corpúsculos de inclusão intranucleares em 7,1% das granjas em 2020, mas ausentes em 2021. A análise multivariada identificou a reposição do plantel com aves de 90 dias como fator significativo em 2021 ($p<0,05$) para soropositividade à LTI. Na análise univariada, por sua vez, diversos fatores relacionados à biosseguridade, manejo e doenças prévias apresentaram associação significativa ($p<0,05$) com a positividade por qPCR e a soropositividade, em ambos os anos. O sequenciamento parcial dos genes ICP4 e TK revelou que as sequências de VLTI de Santa Catarina apresentam alta similaridade com a estirpe VFAR-043 do Peru e a estirpe J2 dos Estados Unidos, ambas pertencentes ao genótipo VI. Embora essas estirpes não estejam relacionadas às vacinas CEO e TCO, as sequências catarinenses demonstraram maior proximidade evolutiva com cepas da vacina CEO do que com cepas de campo previamente identificadas no Brasil. Para esclarecer se essas cepas são mais próximas dos vírus do tipo CEO ou do genótipo VI, é necessária uma análise de genoma completo. Esses achados indicam a ampla disseminação da LTI em Santa Catarina e reforçam a importância da biosseguridade na prevenção de surtos em granjas comerciais de postura.

Palavras-chave: Avicultura. Doença infecciosa. Herpesvirus. Sanidade avícola.

SUMMARY

Infectious laryngotracheitis (ILT) is a highly contagious and notifiable respiratory disease caused by *Gallid alphaherpesvirus 1* (GaHV-1). In Santa Catarina, southern Brazil, ILT was considered exotic until 2020, when it was diagnosed in a commercial layer farm in the municipality of São Ludgero. This study assesses the disease prevalence in the state by determining seropositivity, real-time PCR (qPCR) positivity, anatomopathological alterations, and epidemiological factors associated with ILT infection. Additionally, it characterizes the circulating strains of the infectious laryngotracheitis virus (ILTV) during the 2020-2021 outbreak in the state and investigates their phylogeny. The study was conducted in two phases: the first analyzed municipalities in the São Ludgero County in 2020, while the second evaluated the virus spread throughout the state in 2021. A total of 44 farms were sampled in 2020 and 49 in 2021, including commercial layer and rearing farms. In each farm, 20 hens were tested serologically for ILTV antibodies, and 10 were euthanized for necropsy, respiratory organ collection, and histopathological evaluation. Conjunctiva, larynx, trachea, and trigeminal ganglion samples were analyzed by qPCR targeting the ILTV gC glycoprotein. Positive samples were subjected to Sanger sequencing of *the ICP4 and TK* genes. Additionally, a questionnaire was administered to each poultry farmer to identify potential ILT risk factors, using Fisher's Exact Test and multivariate analysis. The results showed that in 2020, 95.4% of farms were seropositive, with a qPCR positivity rate of 88.1%. In 2021, these rates decreased to 65.3% and 21.8%, respectively. ILTV infection was confirmed by histopathological examination of the trachea and larynx, revealing syncytial cells and intranuclear inclusion bodies in 7.1% of farms in 2020, but absent in 2021. The multivariate analysis identified flock replacement with 90-day-old hens as a significant factor for ILT seropositivity in 2021 ($p<0.05$). The univariate analysis revealed significant associations ($p<0.05$) between qPCR positivity, seropositivity, and multiple factors related to biosecurity, management, and previous diseases in both years. Partial sequencing of the ICP4 and TK genes revealed that ILTV sequences from Santa Catarina share high similarity with the VFAR-043 strain from Peru and the J2 strain from the United States, both classified as genotype VI. Although these strains are unrelated to CEO and TCO vaccines, the Santa Catarina sequences showed closer evolutionary proximity to CEO vaccine strains than to previously identified Brazilian field strains. A whole genome analysis is required to determine whether these strains are more closely related to CEO-type viruses or genotype VI. These findings highlight the widespread distribution of ILT in Santa Catarina and reinforce the importance of biosecurity measures in preventing outbreaks in commercial layer farms.

Keywords: Poultry farming. Infectious disease. Herpesvirus. Poultry health.

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1. INTRODUÇÃO

A produção de ovos no Brasil encontra-se em constante expansão, e no ano de 2022 foi registrada a produção de 4,06 bilhões de dúzias de ovos, representando um aumento de 1,2% em relação ao ano anterior, alcançando um recorde histórico. São Paulo é o estado de maior produção nacional, sendo responsável por 1,1 bilhão de dúzias de ovos produzidas em 2022, e Santa Catarina, por sua vez, foi responsável por 196,4 milhões de dúzias de ovos, demonstrando um aumento de 2,8% em relação a 2021 (IBGE, 2022). A mesorregião Sul catarinense aloja mais de 4 milhões de aves, sendo São Ludgero o município desta região com maior número de aves, atingindo um plantel de quase 2 milhões. No ano de 2020, São Ludgero foi enquadrado dentro dos 50 maiores municípios brasileiros na produção de ovos, atingindo a 18ª posição no *ranking*, responsável por 0,83% de toda a produção nacional de ovos e por mais de 25% da produção catarinense (IBGE, 2021). É importante ressaltar que, embora o estado de Santa Catarina não tenha a avicultura de postura como uma produção vital para a economia, esta é uma atividade de grande importância e que merece especial atenção, pois é conduzida por produtores independentes, diferindo da avicultura de corte, que possui cadeias produtivas atreladas a grandes empresas integradoras (GEWEHR et al., 2010).

A avicultura de postura demanda alta concentração de aves por área nos aviários, o que favorece a rápida propagação de doenças infecciosas, especialmente as transmitidas por aerossóis, e, para evitar a transmissão de doenças que possam levar a queda nas taxas de produção ou até mesmo a mortalidade das aves, uma série de medidas de biosseguridade devem ser tomadas, que abrange desde a sanitização do local que receberá as aves, até os cuidados na cadeia final de produção (AMARAL et al., 2016). Um estudo avaliando o perfil das propriedades de produção de ovos de galinha no estado de Santa Catarina, demonstrou que 73% das granjas de postura localizam-se a menos de 5 Km de outra propriedade, e 80% localizam-se em um raio de até 10 Km da granja mais próxima, o que é evidenciado especialmente no município de São Ludgero, que possui grande concentração de avicultores de postura (GEWEHR et al., 2010). Embora dentro das especificações propostas pelo Ministério da Agricultura, Pecuária e Abastecimento pela Instrução Normativa 56

de 4 de dezembro de 2007 e Instrução Normativa 18 de 25 de maio de 2017 (BRASIL, 2007; BRASIL, 2017), a grande concentração de aviários pode facilitar a propagação de doenças infecciosas, favorecendo a disseminação de um foco inicial.

Em Santa Catarina, no ano de 2016, havia 233 estabelecimentos de postura comercial contabilizados no estado, dos quais apenas 12% possuíam certidão de registro emitida pelo Serviço Veterinário Oficial (SVO). A regularização de todos os estabelecimentos é de extrema importância para a garantia da biossegurança e da biosseguridade, preservando a saúde animal e gerando produtos de qualidade (MACIEL et al., 2016). Dentre as enfermidades infecciosas que se tornam facilmente disseminadas em granjas que possuem falhas de biosseguridade, está a laringotraqueíte infecciosa das aves (LTI), que era considerada exótica ao plantel avícola catarinense até agosto de 2020, quando, no mês subsequente, foi detectada em uma granja de postura localizada no município de São Ludgero (WITHOEFT et al., 2021). A LTI é uma doença respiratória altamente contagiosa provocada por *Gallid alphaherpesvirus 1* (GaHV-1), pertencente à família *Herpesviridae* e subfamília *Alphaherpesvirinae* (GUY & GARCÍA, 2008).

A forma leve da doença apresenta morbidade de aproximadamente 5%, com mortalidade variando entre 0,1% e 2% (BAGUST et al., 2000). Já na forma severa, a morbidade pode alcançar 100%, enquanto a mortalidade geralmente varia entre 10% e 20%, podendo atingir até 70%, dependendo da estirpe viral (GOWTHAMAN et al., 2020). A transmissão do vírus se dá de forma horizontal, por meio de sua excreção pelas vias ocular e respiratória, cujos aerossóis e secreções contaminadas entram em contato direto ou indireto com animais susceptíveis. O vírus inicia sua replicação pelo epitélio respiratório, após penetração por meio das vias aéreas, e é transportado para as terminações nervosas dos gânglios trigêmeos, nos quais estabelece uma infecção latente, como é característico entre os *Herpesvirus*, portanto, o agente é capaz de permanecer latente por toda a vida das aves portadoras (GUY & GARCÍA, 2008).

Neste estágio, o genoma permanece inativo e não ocasiona a produção de partículas virais infecciosas. Não há apresentação de sinais clínicos, sendo de difícil detecção, exceto pela produção de anticorpos em resposta à infecção aguda (GUY & GARCÍA, 2008; FLORES, 2012). Nesta circunstância, a detecção

de anticorpos indica a condição de latência, já que todos os infectados permanecem portadores; contudo, nem todos os portadores de infecção latente apresentam anticorpos detectáveis durante este estágio. A condição de latência pode ser revertida em circunstâncias nas quais as aves sejam submetidas a algum tipo de estresse, ocasião em que é retomado o ciclo lítico – quando é reativada a produção de vírus e, consequentemente, a capacidade de provocar doença clínica. Nesta situação, a transmissibilidade entre as aves também é retomada (GUY & GARCÍA, 2008).

A caracterização molecular da estirpe viral por sequenciamento do gene ICP4 é essencial em estudos epidemiológicos que requerem o entendimento da origem do vírus no surto, ou seja, se corresponde a uma estirpe de campo ou vacinal (CHACÓN & FERREIRA, 2009), pois o uso de vacinas vivas atenuadas pode culminar na reversão da virulência, desencadeando surtos graves de LTI (OLDONI et al., 2008). Já o sequenciamento do gene TK é útil na diferenciação de isolados de alta ou baixa virulência, refletindo diretamente na gravidade do surto estudado, variando de casos subclínicos, com baixa mortalidade, até casos graves, levando a uma doença respiratória severa com elevada mortalidade nos aviários (HAN & KIM, 2001).

No Brasil, a LTI requer notificação imediata ao SVO de qualquer caso suspeito (BRASIL, 2021), por causar altos índices de morbidade e mortalidade quando em sua forma grave (GUY & GARCÍA, 2008). Além dos prejuízos econômicos decorrentes da mortalidade das aves, há perdas através da queda de produção de ovos e da predisposição a infecções respiratórias secundárias (GUY et al., 2013), levando a despesas com medicamentos e vacinas, e a restrição de trânsito dos animais e de seus produtos e subprodutos (JOHNSON et al., 2004). A exemplo de um estudo conduzido no estado de Minas Gerais, em granjas poedeiras que foram afetadas por surtos de LTI, o acompanhamento dos aviários pelo SVO demonstrou-se efetivo para o controle da doença, associado a implementação de melhorias de biosseguridade pelos avicultores (HERGOT et al., 2021).

Visando o enfrentamento e o contingenciamento da doença na região do Bolsão de São Ludgero e no estado de Santa Catarina, o presente estudo foi elaborado e conduzido juntamente à Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina (CIDASC), do Centro de Diagnóstico de Sanidade

Animal (CEDISA), da Empresa Brasileira de Pesquisa Agropecuária (Embrapa Suínos e Aves) e do Instituto Catarinense de Sanidade Agropecuária (ICASA), com o intuito de verificar a situação sanitária referente à LTI nas granjas de postura comercial e recria de postura comercial em todo o estado, elaborando estratégias conjuntas para minimizar os impactos da doença na cadeia produtiva de aves em Santa Catarina.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Determinar a prevalência do vírus da Laringotraqueíte Infecciosa das Aves (LTI) em granjas avícolas comerciais e de recria de postura comercial na região do Bolsão de São Ludgero e no estado de Santa Catarina, identificando os principais fatores associados a infecção, bem como apontar a caracterização filogenética das estirpes circulantes no estado, contribuindo para o entendimento da epidemiologia da doença e fornecendo subsídios para a adoção de medidas mais eficazes de controle e prevenção.

2.2 OBJETIVOS ESPECÍFICOS

a. Determinar a soroprevalência para o vírus da LTI em granjas avícolas comerciais e de recria de postura comercial na região do Bolsão de São Ludgero e no estado de Santa Catarina.

b. Determinar a positividade para o vírus da LTI por meio da Reação em Cadeia da Polimerase em Tempo Real (qPCR) em granjas avícolas comerciais e de recria de postura comercial na região do Bolsão de São Ludgero e no estado de Santa Catarina.

c. Caracterizar a distribuição do vírus da LTI nos diferentes órgãos avaliados (conjuntiva, laringe, traqueia e gânglio trigeminal) com base na detecção por qPCR.

d. Identificar e descrever as alterações histológicas associadas ao vírus da LTI em granjas positivas por qPCR e/ou análise sorológica.

e. Realizar a caracterização epidemiológica das granjas positivas para a LTI em sistemas de postura comercial e recria de postura comercial.

f. Determinar a caracterização filogenética das estirpes circulantes de LTI no estado, por meio do sequenciamento parcial dos genes ICP4 e TK.

3. REVISÃO BIBLIOGRÁFICA

3.1 ETIOLOGIA E HISTÓRICO

O vírus causador da laringotraqueíte infecciosa das aves (LTI) é identificado como *Gallid Herpesvirus 1* (GaHV-1), pertencente ao gênero *Iltovirus*, subfamília *Alphaherpesvirinae* e família *Herpesviridae* (GUY & GARCÍA, 2008; DAVISON et al., 2009). Trata-se de um vírus DNA fita-dupla linear, com genoma total de 150-155Kb, composto por uma região Longa Única (UL) de 120Kb, Curta Única (US) de 17Kb e duas regiões de Repetição Invertida (IR) com 9Kb cada (MORALES RUIZ et al., 2018). O genoma possui cerca de 80 ORFs (*Open Reading Frames)*, dos quais 65 estão localizados na região UL, 9 na região US e 6 nas regiões IR (LEE et al., 2011; GOWTHAMAN et al., 2020). Morfologicamente, os vírions possuem capsídeo icosaédrico de aproximadamente 100nm de diâmetro, envolto por tegumento e envelope, com glicoproteínas em sua superfície (ROIZMAN & PELLETT, 2001) (Figura 1).

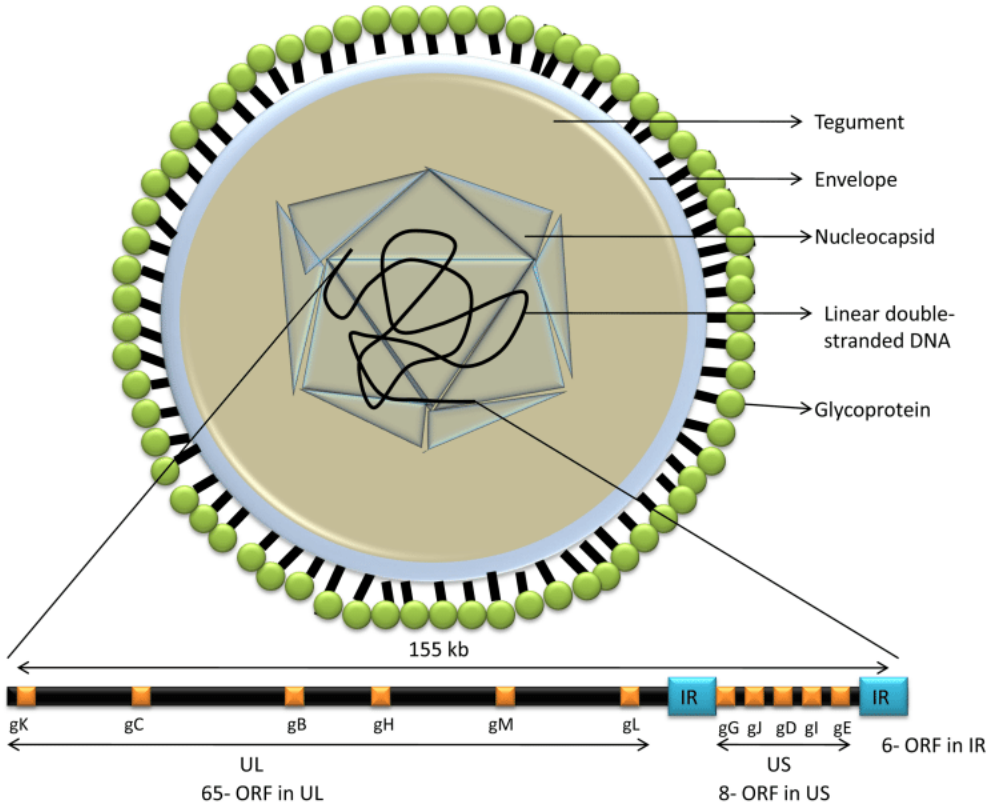

Figura 1. Estrutura e regiões do genoma de Gallid Herpesvirus 1 (GaHV-1), causador de Laringotraqueíte Infecciosa das Aves (LTI).

Fonte: Gowthaman et al. (2020).

As glicoproteínas são denominadas gB, gC, gD, gE, gG, gH, gI, gK, gJ, gL e gM, e são importantes por levar a indução da resposta imune do hospedeiro, e, dentre elas, a gB e a gC são responsáveis pela ligação do vírus às células hospedeiras utilizando um método que independe da heparina, o que distingue o vírus da LTI de outros vírus da mesma família (MO & MO, 2025). A principal característica dos *Herpesvirus* é a manutenção de latência no seu hospedeiro, tornando-o infectado por toda a vida. Durante a latência, não ocorre replicação viral e nem excreção de partículas infecciosas, porém, havendo episódios de imunossupressão ou estresse, a situação pode ser revertida, permitindo a replicação do vírus e ocorrência de sinais clínicos (FRANCO & ROEHE, 2007).

No mundo, acredita-se que a enfermidade tenha ocorrido inicialmente nos Estados Unidos, por volta da década de 1920, no entanto, o termo LTI foi adotado apenas em 1931 (COVER, 1996; GUY & GARCÍA, 2008). Além da América do Norte, sua ocorrência foi amplamente registrada em países da Europa e Oceania, já a partir da década de 1930 (SEDDON & HART, 1935; FRITZSCHE & GERRIERS, 1959; RISLAKKI, 1965). No Brasil, o vírus da LTI foi detectado e isolado pela primeira vez em galinhas no ano de 1974 no estado do Rio de Janeiro (HIPÓLITO et al., 1974), com o primeiro surto grave no Rio de Janeiro descrito entre os anos de 1981 e 1982 (ARAÚJO et al., 1982). A detecção do vírus e o surto não foram notificados às autoridades sanitárias do país, não havendo, portanto, a tomada de nenhuma medida que pudesse levar ao controle da enfermidade no plantel avícola. Em dezembro de 2002, houve a ocorrência de um novo surto grave, agora no estado de São Paulo, na região de Bastos, que atingiu 182 propriedades de avicultura de postura (CHACÓN & FERREIRA, 2009), este devidamente notificado ao Ministério da Agricultura, Pecuária e Abastecimento (MAPA), permitindo estabelecer a investigação epidemiológica e a tomada de medidas para impedir a disseminação da doença para outros estados brasileiros (SÃO PAULO, 2003).

Em 2008, novos focos de LTI foram detectados nos estados do Paraná, Rio Grande do Sul e Distrito Federal, controlados através da intensificação da biosseguridade nas propriedades (BUCHALA et al., 2011). Mais recentemente, em novembro de 2010, o vírus levou a um surto de LTI no sul do estado de Minas Gerais, afetando uma população de aproximadamente oito milhões de galinhas na região das Terras Altas da Mantiqueira (PREIS et al., 2013). Em Santa

Catarina, até agosto de 2020, a LTI era considerada uma enfermidade exótica, ou seja, que não acomete naturalmente o plantel avícola catarinense, no entanto, em setembro de 2020, houve a detecção de um foco da doença no município de São Ludgero (WITHOEFT et al., 2021), com notificação ao serviço veterinário oficial (SANTA CATARINA, 2020).

3.2 TRANSMISSÃO E PATOGENIA

As galinhas (*Gallus gallus domesticus*) são consideradas os hospedeiros naturais do GaHV-1, no entanto, a doença já foi relatada em outros galiformes como faisões (*Phasianus colchicus torquatus*), perdizes (*Rhynchotus rufescens*), pavões (*Pavo cristatus*) e perus (*Meleagris gallopavo* f. *domestica*) (CRAWSHAW & BOICOTT, 1982; PORTZ et al., 2008; GUY et al., 2013). A principal forma de infecção em uma granja é a direta por secreções oronasais, através do contato entre aves que estejam com sinais clínicos e aves suscetíveis, porém o vírus pode ser transmitido de forma indireta, por meio de aerossóis e fômites, como a cama contaminada, veículos, equipamentos, poeira, roupas e objetos de pessoas que possuam acesso às granjas (ZELLEN et al., 1984; GAMA & CANAL, 2009). Animais infectados podem eliminar o vírus em secreções por até 10 dias após a infecção (WILLIAMS et al., 1992). Na ave suscetível, o vírus penetra por via respiratória, ocular ou oral, levando a um período de incubação de seis a 14 dias (JORDAN, 1966; WILLIAMS et al., 1992). O vírus inicia sua replicação a partir do momento em que ocorre a fusão entre o envelope e a membrana celular do hospedeiro, direcionando-se ao núcleo da célula infectada, geralmente replicando durante o período de uma semana (GUY et al., 2013; WILLIAMS et al., 1992). Até o momento, não existem relatos de comprovação de transmissão vertical, e o GaHV-1 não provoca viremia nos hospedeiros durante a infecção, como comumente visualizado em vírus de outras famílias (OLDONI et al., 2009).

A replicação ocorre de forma mais intensa no epitélio da laringe e traqueia, além de conjuntiva, seios nasais, sacos aéreos e pulmões, havendo a sua detecção em secreções do trato respiratório por seis a oito dias após o período de incubação (BRANDÃO & CHACÓN, 2009). Existem evidências em aves experimentalmente infectadas de que o vírus pode replicar em outros órgãos, como encéfalo, timo, coração, pâncreas, fígado, rins e trato gastrointestinal

(WANG et al., 2013). Após a multiplicação do vírus nos tecidos respiratórios, há migração e transporte retrógrado axonal ao gânglio trigeminal, onde o GaHV-1 permanece em latência, como característica de todos *Herpesvirus* (GAMA & CANAL, 2009). O estado de latência impede a atuação do sistema imune pela ausência na expressão de suas proteínas (BRANDÃO & CHACÓN, 2009). Desta forma, o animal permanece com o vírus por toda a sua vida, com a sua reativação espontânea mediante episódios de estresse ou imunossupressão, como doenças intercorrentes, transporte prolongado, alterações bruscas de temperatura, manejo alimentar ou hídrico inadequado, múltiplas idades e alta densidade populacional num mesmo galpão (DUFOUR-ZAVALA et al., 2008).

3.3 SINAIS CLÍNICOS

O curso da doença clínica nas aves é, em geral, de 10 a 14 dias, variando de acordo com a gravidade da doença (JORDAN, 1966), que pode se manifestar de forma leve ou grave. Na forma leve, podem ser visualizados sinais de apatia, descarga nasal, aumento nos seios infraorbitários, espirros, conjuntivite, queda na ingestão de alimentos e água, queda na produção de ovos e atraso no crescimento (SELLERS et al., 2004; MORENO et al., 2010). Essa manifestação da doença é a mais frequente em surtos de LTI de granjas avícolas (GUY et al., 2013), e possui morbidade em torno de 5% e mortalidade variando entre 0,1 e 2,0% (GUY & GARCIA, 2008).

Na forma grave, é relatado um aumento súbito nos índices de mortalidade da granja (AZIZ, 2010), onde visualiza-se letargia, lacrimejamento, edema de pálpebra, dispneia severa, estertor, expectoração de muco sanguinolento, tosse e óbito entre dois e três dias após início dos sinais (MORENO et al., 2010; GUY et al., 2013). É comum, na manifestação grave, a visualização de sangue expectorado em gaiolas, paredes e chão do aviário (GOWTHAMAN et al., 2020). Nestes casos, as taxas de morbidade e mortalidade podem atingir, respectivamente, 100% e 70% (GOWTHAMAN et al., 2020). Em ambas as manifestações clínicas, ocorrem diferentes graus de laringite, traqueíte, conjuntivite e sinusite, com produção acentuada de fibrina e restos celulares, que, em casos graves, pode levar à obstrução das vias aéreas culminando no óbito na ave por asfixia (GUY et al., 2013).

3.4 DIAGNÓSTICO

Como os sinais clínicos visualizados em casos de LTI são inespecíficos e comuns a outras enfermidades do trato respiratório de aves, sua investigação laboratorial é essencial para o diagnóstico conclusivo, aliando técnicas anatomopatológicas, sorológicas e moleculares (GOWTHAMAN et al., 2020).

3.4.1 ACHADOS MACROSCÓPICOS

Como o GaHV-1 é um vírus que acomete predominantemente o sistema respiratório, é comum a visualização de hiperemia em laringe e traqueia, associada ao acúmulo de muco e a edema, também em seios nasais e conjuntiva (GUY et al., 2013). A distribuição e a intensidade destas lesões são influenciadas pela manifestação da doença, variando de alterações discretas na forma leve da doença, com discreto a moderado acúmulo de secreção mucosa ao longo da traqueia e discreta hiperemia da mucosa (TIMURKAAN et al., 2003). As alterações acentuadas em sua forma grave são compostas por hemorragia, ulcerações e necrose do epitélio respiratório, placas diftéricas e acúmulo de material muco-sanguinolento, levando a quadros de laringotraqueíte fibrino-necróticas (CALNEK, 1991; GOWTHAMAN et al., 2014).

O processo inflamatório acentuado pode se estender desde a laringe e traqueia, até os brônquios, pulmões e sacos aéreos, agravando o quadro respiratório da ave e, em alguns casos graves, pode haver a visualização de acúmulo de material caseoso formando um tampão nas vias aéreas, obstruindo o fluxo respiratório, e, nestes casos, em virtude da asfixia, é comum que a ave se encontre cianótica (VILLANUEVA, 2005). Em conjuntiva, pode ser visualizado edema associado a secreção ocular (KIRKPATRICK et al., 2006), e, ainda, em sacos aéreos, pode haver seu espessamento associado a deposição de exsudato caseoso. De maneira mais incomum, pode haver casos de esofagite e faringite ulcerativas (AZIZ, 2010; SARY et al., 2017).

3.4.2 ACHADOS MICROSCÓPICOS

Os órgãos onde há maior frequência de alterações histológicas, concomitantes ou não, são a laringe, a traqueia, as conchas nasais, os pulmões e a conjuntiva, com intensidade e distribuições que variam de acordo com o estágio em que a doença se encontrava no momento da morte do animal, e da

manifestação leve ou grave da LTI (LINARES et al., 1994; PREIS et al., 2013). Ocorre, inicialmente, a visualização de infiltrados inflamatórios compostos por linfócitos, plasmócitos e macrófagos em mucosa e submucosa e perda de células caliciformes (RUSSELL et al., 1983; HAYASHI et al., 1985).

Com o progresso da doença, ocorre necrose seguida de descamação e fusão de células epiteliais, formando sincícios que podem possuir corpúsculos de inclusão intranucleares basofílicos ou eosinofílicos circundados por um halo, especialmente entre o primeiro e o quinto dia após infecção (PURCELL, 1971; RODRÍGUEZ-ÁVILA et al., 2008; SARY et al., 2017). Além disso, diferentes graus de degeneração, necrose e hemorragia são visualizados no epitélio respiratório, bem como exsudato composto por restos celulares, fibrina, heterófilos e hemácias no lúmen das vias respiratórias (HAYASHI et al., 1985; TIMURKAAN et al., 2003). Nas aves que sobrevivem à fase aguda da doença, tem início um processo regenerativo composto por traqueíte hiperplásica a partir de seis dias após a infecção, caracterizada pela proliferação de células basais remanescentes (BAGUST et al., 2000).

Quando presentes, as células sinciciais contendo corpúsculos de inclusão intranucleares basofílicos ou eosinofílicos podem ser consideradas patognomônicas para LTI, mas sua ocorrência nem sempre é visualizada devido ao curto período de apresentação (GARCÍA et al., 2013), desaparecendo poucos dias após, devido ao processo de necrose e descamação de células epiteliais, passando a compor o exsudato das vias aéreas (VILLANUEVA, 2005). É importante ressaltar que a análise histopatológica é o exame de escolha para diagnosticar LTI, pois permite diferenciar a infecção real, que está levando à doença, de casos de animais infectados, dos casos em que há a detecção do DNA viral ou de anticorpos, no entanto, sem alterações aparentes (PREIS et al., 2013).

Devido ao curto período de apresentação de lesões clássicas de LTI, ferramentas como a imuno-histoquímica (IHQ) podem ser úteis na visualização do antígeno viral nos tecidos respiratórios, sendo capaz de demonstrar a distribuição geral do antígeno nos tecidos, bem como sua localização nas células e restos necróticos que compõem exsudatos, mesmo com a ausência de lesões clássicas (PREIS et al., 2014; CARNACCINI et al., 2022). Ainda, em surtos brandos da doença, usualmente provocados por cepas vacinais cultivadas em

embrião de galinha (CEO), é baixa a frequência de células sinciciais com corpúsculos de inclusão clássicas de LTI, e a técnica de IHQ permite confirmar a presença do vírus envolvido inclusive em lesões leves e inespecíficas de LTI, como nestas situações (CARNACCINI et al., 2022). Suas limitações são comuns a todas as técnicas de IHQ, podendo gerar falsos negativos em tecidos armazenados por longos períodos em formalina ou podendo gerar marcações inespecíficas (HOFMAN et al., 2013).

3.4.3 ANÁLISE MOLECULAR

A Reação em Cadeia da Polimerase (PCR) é considerada uma análise de alta especificidade e sensibilidade para detecção de GaHV-1, e durante muitos anos foi aliada à análise de Polimorfismo de Fragmentos de Restrição (RFLP) para distinção entre cepas virais de campo ou vacinais (CREELAN et al., 2006). Essa técnica permite diferenciar se os surtos ocorrem por cepas de campo, ou pela reversão de cepas vacinais vivas atenuadas após a passagem em aves suscetíveis, especialmente em países que permitem a vacinação viva atenuada com a vacina cultivada em embrião de galinha (CEO) e a vacina cultivada em cultura celular (TCO) (OLDONI & GARCIA, 2007).

Posteriormente, para determinar a origem das cepas virais, Chacón & Ferreira (2009), estabeleceram o sequenciamento de dois fragmentos do gene *Infected Cell Protein-4* (ICP4), tratando-se de uma forma mais ágil e acurada, não sendo necessária a amplificação de diversos genes do vírus como necessário para a PCR-RFLP. Conhecer a cepa viral que está acometendo determinado plantel avícola é extremamente importante para detectar e rastrear a sua origem, auxiliando no entendimento epidemiológico de onde possa ter havido a entrada do patógeno na granja para que medidas de prevenção e controle futuras sejam devidamente tomadas (CLAVIJO & ÉVA, 1997).

O gene ICP4 é ligado a regulação da expressão gênica do vírus na célula hospedeira durante a infecção (JOHNSON et al., 1995), e como mencionado, tem se mostrado um importante alvo de sequenciamento para diferenciação de estirpes de campo ou vacinais que sofreram reversão da virulência (CHACÓN & FERREIRA, 2009; COUTO et al., 2015). O gene UL23, que codifica para a proteína TK (*thymidine kinase*), também é um importante alvo de sequenciamento, pois permite executar a diferenciação entre cepas de alta ou

baixa virulência, refletindo diretamente na forma da doença nos aviários, se grave ou leve (HAN & KIM, 2001). Já a determinação do genoma completo traz todas as informações necessárias acerca de possíveis mutações, variações genéticas e evolução do vírus (RADFORD et al., 2012), permitindo comparar com o genoma das cepas do vírus da LTI presentes em outros surtos para detecção das divergências e similaridades. Até o momento, o genoma do GaHV-1 foi sequenciado apenas uma vez no país, após um surto de LTI em São Paulo (LUCIANO, 2021).

Em um estudo comparando *nested* PCR, isolamento viral e histopatologia na detecção de uma cepa de baixa patogenicidade de GaHV-1, a *nested* PCR se mostrou uma técnica sensível, capaz de detectar o DNA viral em traqueias mesmo de aves que já não possuíam mais sinais clínicos (BELTRÃO et al., 2003). Ainda, o desenvolvimento de técnicas recentes de PCR em tempo real (qPCR) mostra-se altamente sensível e específico no diagnóstico da LTI, exigindo menos tempo e um resultado mais objetivo quando comparado ao PCR convencional, permitindo, ainda, a quantificação de cópias do genoma viral, que diferencia aves infectadas de forma aguda e crônica (CALLISON et al., 2007).

3.4.4 ANÁLISE SOROLÓGICA

A sorologia é uma importante ferramenta indireta de detecção da LTI, e o ensaio de imunoabsorção enzimática (ELISA) detém as vantagens de ser um método de alta sensibilidade e especificidade e de permitir a avaliação simultânea de uma grande quantidade de amostras, otimizando os resultados (TESSARI & CARDOSO, 2011). Este é considerado um método de triagem, e sabe-se que galinhas infectadas podem ter anticorpos detectados por este método aos sete dias pós-infecção, com pico aos 21 dias pós-infecção e titulações persistindo por quatro a sete semanas, e sua utilização é essencial em estudos epidemiológicos e no monitoramento dos plantéis avícolas (GAMA & CANAL, 2009).

Por ser um vírus antigenicamente homogêneo, apenas a análise sorológica por *kits* comerciais não consegue diferenciar a origem de campo ou vacinal da infecção, e para tanto, recomenda-se o sequenciamento genético da estirpe viral envolvida no surto (CHACÓN & FERREIRA, 2009; COUTO et al., 2015). No entanto, utilizando a expressão de glicoproteínas específicas do vírus

em *Escherichia coli*, foi possível a elaboração de testes de ELISA específicos para determinadas glicoproteínas do GaHV-1 presentes nas vacinas recombinantes, que podem auxiliar na diferenciação entre anticorpos decorrentes de cobertura vacinal ou desafio de campo (CHANG et al., 2002).

3.5 EPIDEMIOLOGIA

A LTI é uma enfermidade cosmopolita e de ocorrência cíclica em áreas endêmicas, especialmente em locais com alta densidade populacional de galináceos, acometendo aves alocadas em diferentes tipos de criações e alojamentos, com relatos de sua ocorrência em diversos continentes, como nas Américas, Europa, Oceania, Ásia e África (GUY et al., 1991; HIDALGO, 2003; MORENO et al., 2010; COPPO et al., 2012; PARRA et al., 2016). Quanto aos fatores que atuam sobre a transmissão de GaHV-1 entre as aves, pode haver diminuição na suscetibilidade à medida que os animais se tornam mais velhos, e casos de LTI podem ser mais graves em temperaturas mais elevadas (BERCHIERI, 2004). Diferentes idades de aves criadas em um mesmo galpão associadas a falhas de biosseguridade são alguns dos fatores que têm contribuído para a sua ocorrência (GARCÍA & SPATZ, 2019). Aves de idades variando de oito dias de vida a quatro anos de idade são suscetíveis ao desenvolvimento de LTI, mas a idade de maior suscetibilidade gira em torno de três semanas, devido ao sistema imunológico (DUFOUR-ZAVALA, 2008).

Sabe-se que a transmissão direta de ave para ave é a mais efetiva para a manutenção do vírus no local, no entanto a transmissão indireta também possui papel importante na sua disseminação, englobando diversas consequências de falhas na biosseguridade dos aviários (GAMA & CANAL, 2009). Além de fômites contaminados, outra prática indireta que pode atuar na propagação do GaHV-1, é o uso de cama de aviários não compostada pertencente a plantéis avícolas vacinados ou infectados, para fins de fertilização de pastagens (DUFOUR-ZAVALA, 2008). Besouros podem atuar como vetores mecânicos, transportando o vírus entre diferentes criações avícolas, com detecção do vírus neste inseto confirmada através de PCR (OU et al., 2012). Animais domésticos que possuam acesso às aves podem auxiliar na disseminação do vírus, especialmente cães que podem transportar carcaças de aves infectadas de um local a outro (DUFOUR-ZAVALA, 2008). Ainda, aves de

fundo de quintal são apontadas como importantes fontes de infecção de LTI para plantéis aviários comerciais, e granjas afetadas pela doença já foram indicadas a ter 36 vezes maior probabilidade de estarem localizadas a menos de 1Km de criações de aves de fundo de quintal, em relação a granjas negativas, no estado de Delaware, Estados Unidos (JOHNSON et al., 2004). No Brasil, aves de fundo de quintal clinicamente saudáveis, de regiões próximas a granjas de postura comercial, demonstraram PCR positivo em gânglio trigeminal e órgãos do sistema respiratório, indicando um processo de latência do vírus (PREIS et al., 2014).

Coinfecções com outros agentes infecciosos que acometem o trato respiratório como a coriza infecciosa, *Mycoplasma gallisepticum*, *M. synoviae*, bronquite infecciosa, Newcastle e influenza ou, ainda, com agentes imunossupressivos como anemia infecciosa das galinhas e doença de Marek, exacerbam o impacto da LTI na produção avícola (ZAVALA, 2011). Em um trabalho conduzido no Brasil em aviários contendo positividade para GaHV-1, 22.6% das aves apresentaram lesões histológicas sugestivas de coinfecção, e 61.2% das análises de PCR demonstraram positividade para, ao menos, dois agentes etiológicos concomitantes, sendo os mais frequentes GaHV-1 e *Mycoplasma* spp. (COUTO et al., 2016). Em países que fazem uso de vacinas vivas atenuadas, surtos de LTI ocorrem após vacinação inadequada do plantel, seja por erros de aplicação ou falhas de biosseguridade (HIDALGO, 2003). A evolução destas cepas como virulentas em áreas com alta densidade de criação de aves, ocorre devido a presença de reservatórios contínuos para o vírus, assim, posteriormente, estas cepas se estabelecem no campo e acabam levando a surtos de LTI (GUY et al., 1990; KOTIW et al., 1995).

3.6 CONTROLE E PREVENÇÃO

Não há tratamento eficaz para combater o GaHV-1, e, por sua característica de latência, a ave acometida permanece positiva por toda a sua vida. Por conta disso, as medidas de prevenção e controle se fazem essenciais para a erradicação da LTI em um plantel avícola, como, por exemplo, seguir adequadamente as medidas de biosseguridade e implementar um programa vacinal apropriado (DUFOUR-ZAVALA, 2008). Após o diagnóstico de LTI no município de São Ludgero, o Serviço Veterinário Oficial de Santa Catarina

estabeleceu normas para contenção da enfermidade através de vigilância ativa na região, para o levantamento da situação epidemiológica; seguido do controle e restrição de movimentação de aves e cama de aviário; tornou obrigatório o uso da vacina recombinante no território catarinense e tornou obrigatório também o aprimoramento da biosseguridade nas granjas avícolas situados na região (SANTA CATARINA, 2020).

Dentro dos protocolos vacinais, existem as vacinas vivas atenuadas, utilizadas a partir da década de 1960, e as vacinas recombinantes, aplicadas em plantéis avícolas desde 2004 nos Estados Unidos. As vacinas vivas são conhecidas por induzir resposta imune apropriada nos hospedeiros, no entanto, fatores como aves em estado de latência no momento da vacinação, aplicação inadequada da vacina e falhas de biosseguridade levaram às cepas vacinais a adquirirem virulência, e a se tornarem as causadoras de diversos surtos de LTI. Para evitar este problema, surgiram as vacinas recombinantes, que não possuem a capacidade de sofrer reversão da virulência, nem de provocarem reativação do estado de latência nos animais vacinados (GARCÍA & SPATZ, 2019). As vacinas recombinantes são produzidas através da inserção de genes do GaHV-1 em um *Avipoxvirus fowlpox* (FLP) ou em um *Meleagrid herpesvirus 1* (HVT), que atuam como os vetores virais das vacinas FLP-LT e HVT-LT, respectivamente, e impedem a transmissão do vírus entre aves vacinadas e não-vacinadas, garantindo a proteção desejada (JOHNSON et al., 2010; COPPO et al., 2013).

Quando às vacinas vivas atenuadas, é importante ressaltar que, no território brasileiro, a vacina de múltiplas passagens em ovos embrionados (CEO) é proibida, enquanto a produzida com múltiplas passagens em cultura de tecido (TCO) pode ser autorizada dependendo da situação epidemiológica do local, e seguindo uma série de requisitos para utilização (BRASIL, 2018). No entanto, a vacina CEO já foi utilizada anteriormente no país, na região de Bastos, em São Paulo, para controlar os surtos iniciais de LTI, juntamente com a vacina TCO. Anos depois, entre 2010 e 2011, cepas semelhantes à CEO foram isoladas ou detectadas por PCR e sequenciamento genético do DNA de galinhas poedeiras clinicamente saudáveis, tanto na região de Bastos quanto em granjas avícolas situadas a aproximadamente 350 km da área onde as vacinas CEO e TCO haviam sido permitidas anos antes (CHACÓN et al., 2015).

Quanto às medidas de biosseguridade, cuidados como vazio sanitário adequado, limpeza e desinfecção das instalações, restrição no fluxo de pessoas pelo aviário, uso de uniformes, controle de roedores, insetos, e restrição no acesso de cães aos galpões são essenciais (DUFOUR-ZAVALA, 2008). A restrição de hospedeiros para ocorrência do GaHV-1, sua instabilidade no meio ambiente e a ausência de transmissão vertical tornam as medidas de controle e prevenção ainda mais eficazes no combate à doença (BRANDÃO & CHACÓN, 2009). Deve-se ressaltar para a importância da conscientização de produtores para a notificação obrigatória da enfermidade ao serviço veterinário oficial, pois apenas desta forma, todas as medidas de vigilância e controle poderão ser tomadas, visando a sanidade do plantel avícola (CRUZ et al., 2013).

4. ARTIGO 1

Phylogenetic characterization of the first Infectious Laryngotracheitis (ILT) outbreak in southern Brazil

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ABSTRACT

Infectious laryngotracheitis (ILT) is a highly contagious and reportable respiratory disease of poultry with a worldwide distribution. This study characterizes the infectious laryngotracheitis virus (ILTV) circulating during the 2020–2021 outbreak in Southern Brazil and investigates the phylogeny of these strains. The first case in 2020 was reported in a region densely populated with layer-type chickens, which exhibited increased mortality rates of up to 6%. Epidemiological surveys conducted at this layer production site revealed an ILTV positivity rate of 88.1%, as determined by real-time PCR (qPCR). In 2021, a statewide ILTV positivity rate of 21.8% was recorded for the State of Santa Catarina. Samples with Ct < 25 were subjected to amplification of partial ICP4 and TK gene regions, followed by Sanger sequencing of the amplified products. ILT was confirmed by histopathological examination of tracheal samples. Phylogenetic analysis of the obtained sequences revealed that the partial ICP4 sequence was 100% identical to the VFAR-043 strain from Peru and the J2 strain from the United States. Based on whole-genome sequences, the VFAR-043 and J2 strains belong to genotype (GT) VI, which is not related to the chicken embryo origin (CEO) or tissue culture origin (TCO) live-attenuated ILT vaccines. In this study, based on partial ICP4 and TK gene sequences, the cluster of sequences from Santa Catarina strains appears to be more closely related to CEO vaccine strains than to TCO vaccine strains or previously identified field strains from Brazil. To determine whether the Santa Catarina strains are more closely related to CEO-like viruses than to GT VI field strains, whole-genome sequence comparisons are necessary.

Keywords: Layer poultry farming. Herpesvirus. ICP4. Sanger.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease with a global distribution, frequently recurring in regions with dense poultry populations. It affects birds across various farm types and housing systems (GUY et al., 1991; HIDALGO, 2003; MORENO et al., 2010; COPPO et al., 2012; PARRA et al., 2016). The disease is caused by *Gallid alphaherpesvirus 1* (GaHV-1), a herpesvirus belonging to the genus *Iltovirus* within the subfamily *Alphaherpesvirinae* (GUY & GARCÍA, 2008; DAVISON et al., 2009).

Since the 1960s, live-attenuated vaccines, such as the chicken embryo origin (CEO) and tissue culture origin (TCO) vaccines, have been incorporated into vaccination protocols to elicit an immune response. However, in countries where these vaccines are used, ILT outbreaks still occur due to insufficient flock immunity, often resulting from improper vaccine application or lapses in biosecurity (HIDALGO, 2003). The persistence of vaccine strains in the field establishes viral reservoirs in areas with high poultry density, which can drive the evolution of virulent strains and trigger ILT outbreaks (GUY et al., 1990; KOTIW et al., 1995). To provide safer vaccination strategies against ILT, recombinant viral vector vaccines were developed using *Fowlpoxvirus* (FPV) or *Meleagrid alphaherpesvirus 1* (HVT) as viral vectors to express infectious laryngotracheitis virus (ILTV) glycoprotein antigens. ILT recombinant vaccines were first introduced in the United States in 2004. These vaccines do not revert to virulence and have limited transmission from vaccinated to non-vaccinated chickens, providing a safer alternative for disease control (JOHNSON et al., 2010; COPPO et al., 2013; GARCÍA & SPATZ, 2019).

In Brazil, according to the Ministry of Agriculture, Livestock, and Supply (2018), CEO vaccines are completely banned, while the TCO vaccine can be authorized under specific use requirements. During ILT outbreaks, sequencing tools are extensively used to determine whether the virus originates from a field strain or a vaccine strain. Partial sequencing of the ICP4 (*infected cell protein 4*) gene has been employed to distinguish between field and vaccine strains. The ICP4 gene spans approximately 4,386 bp, with two highly conserved regions serving as sequencing targets (JOHNSON et al., 1995), enabling strain differentiation (CHACÓN & FERREIRA, 2009; COUTO et al., 2015). Additionally, the UL23 gene, encoding the thymidine kinase (TK) protein, were used in the

1990's to distinguish non-virulent and virulent strains in Korea, correlating directly with disease severity (HAN & KIM, 2001). However, TK sequencing alone is insufficient to determine strain virulence, as other genomic mutations must be analyzed in conjunction with field observations (MENENDEZ et al., 2014).

According to genome analysis, especially TK sequencing, ILT outbreaks in Brazil have been predominantly caused by low-virulence strains (COUTO et al., 2015; SANTOS et al., 2022). However, highly virulent field strains were detected in São Paulo during outbreaks in 2002–2003 and 2015–2016, and in the same state, low-virulence TCO-derived strains were also reported in circulation in 2015–2016 (CHACÓN & FERREIRA, 2009; SANTANDER-PARRA et al., 2022). Additionally, strains related to the CEO vaccine were identified in clinically healthy chickens in a quarantine area in São Paulo, as well as in regions outside the controlled area, where they caused disease (CHACÓN et al., 2015). Recently, strains very similar to those from Santa Catarina were reported in Paraná, a neighboring state (CHACÓN et al., 2025).

In Santa Catarina, ILT was introduced into a region with a high density of layer farms in 2020, leading to an outbreak. In response, epidemiological surveys were conducted in 2020 and 2021 at the regional and state levels to establish containment measures, including active surveillance, movement restrictions on birds and poultry litter, mandatory use of recombinant vaccines, and enhanced biosecurity protocols on poultry farms (SANTA CATARINA, 2022).

Therefore, this study aims to describe the 2020 ILT outbreak and conduct a molecular characterization of GaHV-1 strains detected in epidemiological surveys from 2020 and 2021, focusing on the phylogenetic relationships of these strains through ICP4 and TK gene sequencing.

MATERIAL AND METHODS

DESCRIPTION OF THE INFECTIOUS LARYNGOTRACHEITIS OUTBREAK

In 2020, a commercial laying farm in São Ludgero (lat. 28°19'33''; long. 49°10'37'') experienced a significant ILT outbreak in a farm with a total flock of 42,650 hens. The Official Veterinary Service (SVO) of Santa Catarina carried out a first technical visit, taking blood samples and swabs from the trachea and cloaca of 30 hens to screen for high mortality diseases such as Avian Influenza and Newcastle Disease, as well as to test for the ILT virus using the polymerase

chain reaction (PCR). The tests were performed at the Federal Agricultural Defense Laboratory in São Paulo (LFDA-SP), located in Campinas, Brazil. Three weeks later, during a follow-up visit, 10 hens nearing the end of the production cycle were selected for euthanasia by cervical dislocation. Necropsies were performed, and tissue samples from the conjunctiva, nasal sinuses, larynx, trachea, and lungs were collected in 10% buffered formalin and stained using the hematoxylin and eosin (HE) technique for histological examination

REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

After confirming the ILT outbreak in São Ludgero, two epidemiological studies were conducted in Santa Catarina. The first study, in 2020, focused on the municipalities in the region where the outbreak occurred, while the second study, in 2021, expanded to cover cities across all regions of the state (Data in pre-publication phase). Sampling was performed on laying farms through active surveillance, with chickens that were either clinically healthy or showing mild respiratory signs. To detect the virus, pools of 10 hens per farm were created, consisting of conjunctiva, larynx, trachea, and trigeminal ganglia tissue. In the 2020 study (Study 1), 42 pools were analyzed for molecular diagnosis, and in the 2021 statewide survey (Study 2), 32 pools were examined. Each pool originated from commercial laying or rearing farms included in the respective epidemiological surveys. The samples, previously frozen at -20°C, were thawed at room temperature (21°C) for 30 minutes and homogenized for DNA extraction. Total DNA was extracted using the IndiMag 48 automatic extractor (Indical Bioscience GmbH, Leipzig, Germany) with the IndiMag Pathogen Kit® (Indical Bioscience GmbH, Leipzig, Germany), following the manufacturer's protocols.

For the qPCR technique, the ILT virus glycoprotein C (gC) gene was amplified using the primers ILTVgCU771 (5'-CCTTGCGTTTGAATTTTTCTGT-3'), ILTVgCL873 (5'-TTCGTGGGTTAGAGGTCTGT-3'), and the Taqman probe ILTVprobe817 (5'-FAM-CAGCTCGGTGACCCCATTCTABHQ1-3') (CALLISON et al., 2007). The reaction was carried out using the GoTaq Probe qPCR Master Mix (Promega, Madison, USA) in a QuantiStudio 6 thermocycler (ThermoFisher, Waltham, USA) under the following conditions: 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 32 seconds. A positive control,

consisting of a sample from the ILT agar gel immunodiffusion (AGID) antigen, was used, with autoclaved ultrapure water serving as the negative control.

CONVENTIONAL POLYMERASE CHAIN REACTION

Positive qPCR samples with a Ct (cycle threshold) value below 25 were selected for conventional PCR. The technique was performed on two fragments of the ICP4 gene using two primer pairs, which amplified fragments of 923 bp and 747 bp, respectively: 3F (5'-GGGTCTTGTTCTGCAGGATTCT-3') / R1 (5'-CATCGGGACATTCTCCAGGTAGCA-3') and F2 (5'-CTTCAGACTCCAGCTCATCTG-3') / 3R (5'-AATGAGCACGCAACCAGAAGTAA-3'). For the other gene of TK protein, PCR utilized a primer pair that amplified a 783 bp product: TKIP (5'-CTTAGCGGAACCTATGCAAG-3') / TK-R (5'-GAGGCCATGTGCTGGTAAGT-3'). The technique, along with the use of the three primer pairs for amplifying both genes, was adapted from Couto et al. (2015). The positive control consisted of a laryngeal sample confirmed to be positive from Study 1, while the negative control used autoclaved ultrapure water.

PCR amplification of the ICP4 and TK genes in positive samples was performed with a reaction mix containing 1x buffer, 0.2 mM dNTP, 0.5 µM of each primer, 1.5 mM MgCl2, 10.2 µL of ultrapure water, and 1.25 U of Taq DNA Polymerase (GoTaq G2 Hot Start Polymerase Kit, Promega Corporation®, Madison, Wisconsin, USA) for a final volume of 25 µL (1.25 µL dNTP, 5.0 µL buffer, 3.75 µL MgCl2, 0.3 µL Taq, 1.25 µL Primer F, 1.25 µL Primer R, 2 µL DNA, 10.2 µL ultrapure water). The reactions were carried out in an ABI Veriti thermocycler (Applied Biosystems®, Foster City, California, USA) under the following conditions: 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 10 minutes. The amplified products were then subjected to 1% agarose gel electrophoresis and visualized under ultraviolet light.

PURIFICATION AND DNA SEQUENCING

The conventional PCR products were purified using a manual method. Each reaction was treated with 2.5 µL of 3M sodium acetate (10% of the reaction volume) and 100 µL of cold 100% ethanol per sample. After an overnight

incubation at -20°C, the samples were centrifuged at 14,000 rpm for 15 minutes at 4°C, and the ethanol was removed by inversion. The pellets were then resuspended in cold 70% ethanol and subjected to a second centrifugation under the same conditions. Following this, the ethanol was again removed by inversion, and the pellet was resuspended in 20 µL of autoclaved ultrapure water. The DNA concentration and quality were assessed using a BioDrop DUO+™ (BioChrom®, Cambridge, UK), and the final volume was adjusted to achieve a DNA concentration between 50 and 70 ng/µL. The samples were then subjected to Sanger sequencing using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, California, USA), following the manufacturer's protocol on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems®, Foster City, California, USA). Each sample was sequenced in both forward and reverse directions.

ALIGNMENT AND PHYLOGENETIC ANALYSIS

The raw sequences were trimmed and assembled using the Phred/Phrap/Consed package (EWING et al., 1998; GORDON et al., 1998; GORDON et al., 2001) to create a consensus sequence with a Phred quality score ≥ 20. These consensus sequences were submitted to the online NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov) to identify similarities with previously deposited GaHV-1 sequences in GenBank. The alignment of the two regions of the ICP4 gene and the TK gene was performed separately, selecting the highest quality samples with the most nucleotides available from both farm visits.

In addition to the sequences obtained in this study, sequences from GenBank were included in the alignment, representing field strains and live attenuated vaccine strains of the TCO and CEO types for the ICP4 gene, as well as strains previously identified with high and low-virulence genotype for the TK gene. Sequences from Brazil and other countries were used (CHACÓN & FERREIRA, 2009; CHACÓN et al., 2015; COUTO et al., 2015; LONCOMAN et al., 2017; RUIZ et al., 2018a; RUIZ et al., 2018b; THILAKARATHNE et al., 2020; SANTANDER-PARRA et al., 2022; SANTOS et al., 2022). For the ICP4 gene, a phylogenetic tree was constructed based on concatenated sequences from fragments 1 and 2, following the methodology of Couto et al. (2015). Phylogenetic trees were generated using the Neighbor-Joining method (SAITOU & NEI, 1987)

with 1,000 bootstrap replications, and evolutionary distances between sequences were calculated using the Tamura 3-parameter method (TAMURA, 1992), all performed in MEGA 11 Software (TAMURA et al., 2021).

ETHICS COMMITTEE

The study was approved by the Animal Ethics Committee **(CEUA)** of the Universidade do Estado de Santa Catarina **(UDESC)** under approval number 2793260221.

RESULTS

DESCRIPTION OF THE INFECTIOUS LARYNGOTRACHEITIS OUTBREAK

On a first exploratory visit to a farm, the clinical signs observed in the laying hens included epistaxis, dyspnea, facial swelling and mucus with streaks of blood on the premises, along with a mortality rate of approximately 6% within a week, a drop in egg production and a reduction in food and water intake. Tests for Avian Influenza and Newcastle Disease returned negative, but ILT virus detection in tracheal swabs was positive. During the second visit, clinical signs from 10 hens that were sampled included mild eyelid edema (20% - 2/10) (Figure 2A), mild comb cyanosis (10% - 1/10) (Figure 2B), and mucous secretion in the laryngeal region (10% - 1/10) (Figure 2C). The necropsy revealed diffuse mucosal hyperemia in the trachea, ranging from mild to moderate (60% - 6/10), occasionally accompanied by blood-tinged secretion in the lumen (10% - 1/10) (Figure 2D).

Microscopically, lymphoplasmacytic laryngitis (80% - 8/10) (Figure 2E) and lymphoplasmacytic tracheitis (60% - 6/10) (Figure 2F) were evident, characterized by diffuse hyperplasia, ranging from mild to severe, and associated with the formation of syncytial cells containing eosinophilic intranuclear inclusion bodies in the larynx (10% - 1/10) and trachea (20% - 2/10), occasionally accompanied by epithelial desquamation, necrosis, hemorrhage, and fibrin deposition in the lumen. The lungs exhibited mild diffuse lymphoplasmacytic bronchitis (40% - 4/10) with rare syncytial cells (20% - 2/10) and fibrinous exudate (20% - 2/10). In addition, lymphoplasmacytic conjunctivitis was observed in 30% (3/10) of the samples.

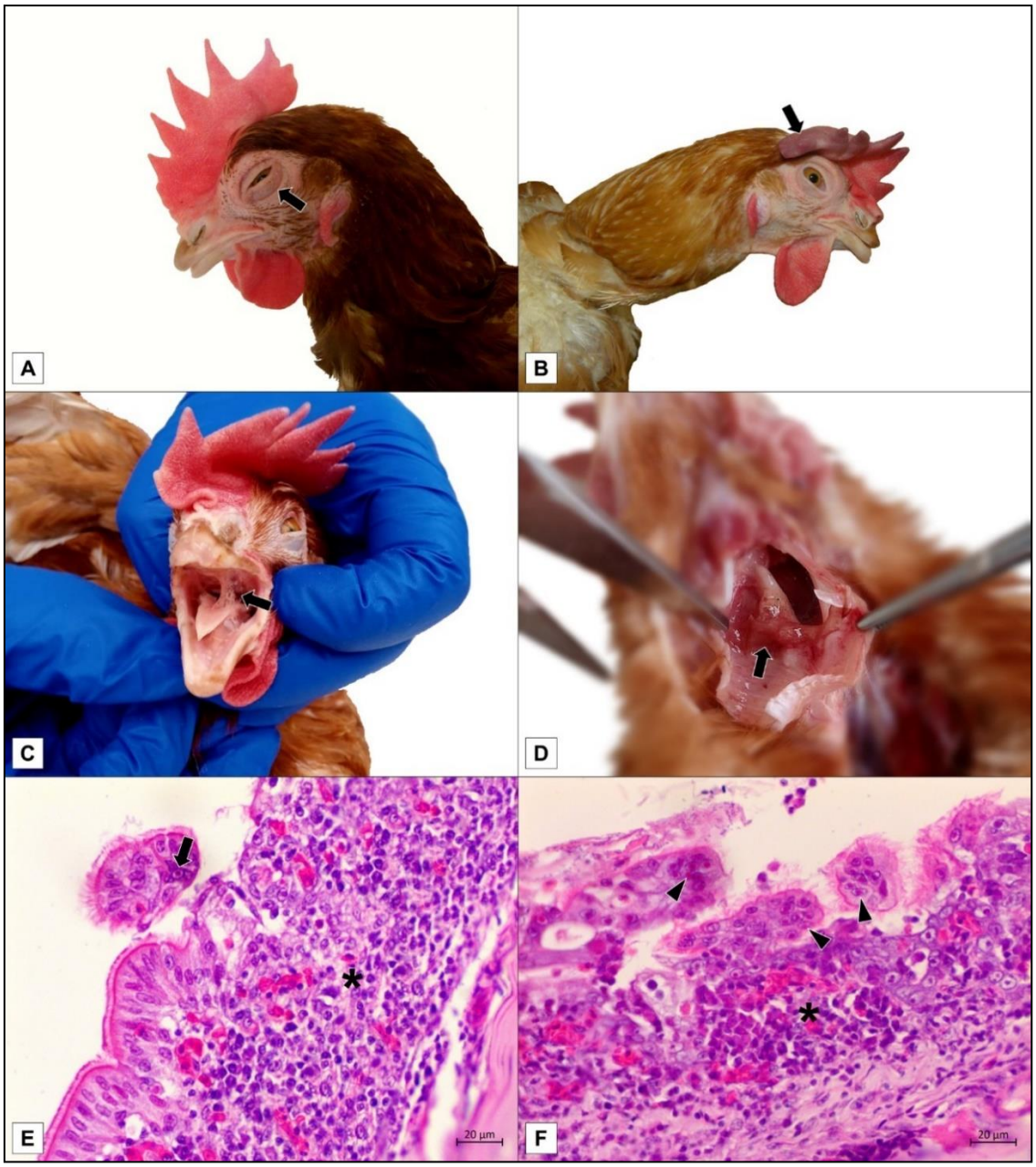

Figure 2. Outbreak of Infectious Laryngotracheitis (ILT) in commercial laying hens in the municipality of São Ludgero, Santa Catarina, Brazil. **A)** Mild conjunctiva edema (arrow). **B)** Mild comb cyanosis (arrow). **C)** Accumulation of mucous secretion in the laryngeal region (arrow). **D)** Accumulation of blood-tinged secretion in the proximal laryngeal region (arrow). **E) Larynx:** diffuse moderate lymphoplasmacytic laryngitis (asterisk), associated with syncytial cells with eosinophilic intranuclear inclusion bodies (arrow). H&E, Obj: 40x. **F) Trachea:** diffuse moderate lymphoplasmacytic tracheitis (asterisk), associated with syncytial cells with numerous eosinophilic intranuclear inclusion bodies (arrowheads). H&E, Obj: 40x.

SEQUENCING OF THE ICP4 AND TK GENES FROM EPIDEMIOLOGICAL STUDIES

The 2020 study yielded an 88.1% (37/42) positivity rate for ILT in qPCR, while the 2021 study found 21.8% (7/32) of farms testing positive. Based on qPCR results, this included 22 samples from the first collection and four samples from the second collection (Table 1). The 22 samples from the 1st collection accounted for 59.5% positive pools (22/37), while the four samples from the 2nd collection represented 57.1% of the positive pools (4/7). A total of 25 sequences for fragment one of the ICP4 gene, 10 sequences for fragment two of the ICP4 gene, and 23 sequences for the TK gene were deposited in GenBank. The availability of sequences was determined by the number of high-quality samples obtained after sequencing. The identification of each sequence, along with their respective GenBank accession numbers for each gene, is detailed in Table 2.

Table 1. Positive samples for Infectious Laryngotracheitis Virus (ILT) selected for sequencing from epidemiological studies conducted in the São Ludgero region and across the state of Santa Catarina, southern Brazil.

Samples	Study	Municipality	Organ *pool*	Ct
01	1	Urussanga	Larynx	17,73
02	1	Orleans	Trachea	21,8
03	1	Orleans	Larynx	23,95
04	1	São Ludgero	Larynx	22,23
05	1	São Ludgero	Conjunctiva	20,32
06	1	São Ludgero	Larynx	19,15
07	1	São Ludgero	Larynx	18,74
08	1	São Ludgero	Larynx	19,24
09	1	São Ludgero	Larynx	20,2
10	1	Braço do Norte	Larynx	17,79
11	1	Braço do Norte	Larynx	20,49
12	1	Braço do Norte	Trachea	19,25
13	1	Braço do Norte	Larynx	21,56
14	1	São Ludgero	Larynx	23,16
15	1	São Ludgero	Conjunctiva	19,78
16	1	São Ludgero	Conjunctiva	20,68
17	1	São Ludgero	Larynx	22,41
18	1	São Ludgero	Larynx	18,09
19	1	Braço do Norte	Conjunctiva	18,54
20	1	São Ludgero	Larynx	21,81
21	1	São Ludgero	Larynx	20,31
22	1	São Ludgero	Larynx	21
23	2	Iraceminha	Larynx	23,43
24	2	Cunha Porã	Larynx	24,26
28	2	Braço do Norte	Conjunctiva	17,12
29	2	Grão Pará	Conjunctiva	20,21

Ct: cycle threshold.

Table 2. Sequences and corresponding GenBank accession numbers for fragments 1 and 2 of the ICP4 gene, and the TK gene of the Infectious Laryngotracheitis Virus (ILT), derived from samples collected in two epidemiological studies conducted in the state of Santa Catarina, southern Brazil.

ID	ICP4 gene Fragment 1	ICP4 gene Fragment 2	TK gene
	GenBank accession numbers		
SC/01/2020/BRA	OR184166	OR184133	OR184143
SC/03/2020/BRA	OR184167	OR184134	OR184144
SC/04/2020/BRA	OR184168	OR184135	OR184145
SC/05/2020/BRA	OR184169	-	OR184146
SC/06/2020/BRA	OR184170	-	-
SC/07/2020/BRA	OR184171	OR184136	OR184147
SC/08/2020/BRA	OR184172	-	OR184148
SC/09/2020/BRA	OR184173	OR184137	OR184149
SC/10/2020/BRA	OR184174	OR184138	OR184150
SC/11/2020/BRA	OR184175	-	OR184151
SC/12/2020/BRA	OR184176	-	OR184152
SC/13/2020/BRA	OR184177	OR184139	OR184153
SC/14/2020/BRA	OR184178	-	OR184154
SC/15/2020/BRA	OR184179	-	OR184155
SC/16/2020/BRA	OR184180	OR184140	OR184156
SC/17/2020/BRA	OR184181	-	-
SC/18/2020/BRA	OR184182	-	OR184157
SC/19/2020/BRA	OR184183	OR184141	OR184158
SC/20/2020/BRA	OR184184	-	OR184159
SC/21/2020/BRA	OR184185	-	OR184160
SC/22/2020/BRA	OR184186	-	OR184161
SC/23/2020/BRA	OR184187	-	OR184162
SC/27/2021/BRA	OR184188	-	-
SC/28/2021/BRA	-	-	OR184163
SC/29/2021/BRA	OR184189	-	OR184164
SC/30/2021/BRA	OR184190	OR184142	OR184165

Aligned sequences of the ICP4-1 fragment encompassed nucleotide positions 204-761 bp of the gene. Sequences from the 1st and 2nd collection demonstrated 100% identity with each other up to position 690 bp. Beyond this point, sequences from 2nd collection were truncated due to trimming of lower-quality ends and were excluded from the ICP4 phylogeny. When compared with sequences previously deposited in GenBank, partial sequencing of the ICP4-1 gene from both studies showed 100% identity with the VFAR-043 strain from Peru (MG775218.1) and the J2 strain from the United States (MF417808.1), 99.64% similarity with the TCO vaccine (EU104908.1), 99.25% similarity with field strains isolated in São Paulo in 2015 and 2016 (MF678664.1 to MF678669.1), and 99.1% similarity with field strains from Minas Gerais in 2015 and 2018 (MN689092.1 to MN689095.1). The Santa Catarina circulating viruses share an adenosine at positions 438, 456, and 598 of the ICP4-1 gene with the CEO vaccines and other CEO-like viruses from Brazil and other countries. In contrast,

other strains, whether derived from the TCO vaccine or field strains from Brazil, had a guanine at positions 438, 456 and 598 of the ICP4-1 gene (Table 3).

Table 3. Infectious Laryngotracheitis in commercial laying hens in the state of Santa Catarina, Brazil. Nucleotide alignment of the ICP4-1 fragment from samples from two epidemiological studies in Santa Catarina, vaccine strains, and field strains previously published from other Brazilian states and countries.

Strain	Gene position								
	284	430	438	456	539	598	606	623	685
OR184166.1 SC/01/2020/BRA	T	G	A	A	G	A	A	C	A
OR184168.1 SC/04/2020/BRA	-	-	-	-	-	-	-	-	-
OR184171.1 SC/07/2020/BRA	-	-	-	-	-	-	-	-	-
OR184173.1 SC/09/2020/BRA	-	-	-	-	-	-	-	-	-
OR184177.1 SC/13/2020/BRA	-	-	-	-	-	-	-	-	-
OR184183.1 SC/19/2020/BRA	-	-	-	-	-	-	-	-	-
OR184190.1 SC/30/2021/BRA	-	-	-	-	-	-	-	-	-
MG775218.1 VFAR-043	-	-	-	-	-	-	-	-	-
MF417808.1 J2	-	-	-	-	-	-	-	-	-
EU104900.1 CEO vaccine	-	-	-	-	-	-	-	-	-
FJ477350.1 CEO vaccine Laryngo-Vac	-	-	-	-	-	-	-	-	G
FJ477351.1 CEO vaccine Nobilis-ILT	-	-	-	-	-	-	-	-	-
HQ630064.1 Strain live attenuated Serva	-	-	-	-	-	-	-	-	-
JX646898.1 strain V1-99	C	-	-	-	-	-	-	-	-
KP677885.1 strain 4787/80	-	-	-	-	-	-	-	-	-
JN542536.1 strain 63140/C/08/BR	-	-	-	-	-	-	-	-	-
OL661344.1 CAN/QC-2307414	-	-	-	-	-	-	-	-	-
FJ477372.1 ILTV/Brazil/2005/USP-50	-	-	-	-	-	-	-	-	G
FJ477373.1 ILTV/Brazil/2005/USP-51	-	-	-	-	-	-	-	-	G
FJ477374.1 ILTV/Brazil/2005/USP-52	-	-	-	-	-	-	-	-	-
FJ477375.1 ILTV/Brazil/2007/USP-57	-	-	-	-	-	-	-	-	-
FJ477376.1 ILTV/Brazil/2007/USP-59	-	-	-	-	-	-	-	-	-
FJ477377.1 ILTV/Brazil/2007/USP-62	-	-	-	-	-	-	-	-	G
FJ477378.1 ILTV/Brazil/2007/USP-64	-	-	-	-	-	-	-	-	-
FJ477379.1 ILTV/Brazil/2007/USP-65	-	-	-	-	-	-	-	-	-
KJ028222.1 ILTV/Brazil/2010/USP-82	-	-	-	-	-	-	-	-	-
KJ028223.1 ILTV/Brazil/2010/USP-83	-	-	-	-	-	-	-	-	-
KJ028224.1 ILTV/Brazil/2010/USP-84	-	-	-	-	-	-	-	-	-
KJ028225.1 ILTV/Brazil/2010/USP-85	-	-	-	-	-	-	-	-	-
KJ028226.1 ILTV/Brazil/2011/USP-86	-	-	-	-	-	-	-	-	G
EU104908.1 TCO vaccine	-	-	G	-	-	G	-	-	-
FJ477349.1 TCO vaccine LTI-IVAX	-	-	G	-	-	G	-	-	-
DQ995291.1 WangGang	-	-	G	G	-	G	-	-	-
EU104909.1 USDA	-	-	G	-	-	G	-	-	-
JN542535.1 81658	-	-	G	-	-	G	-	-	-
FJ477352.1 ILTV/Brazil/2002/USP-01	-	-	G	G	-	G	-	-	-
FJ477353.1 ILTV/Brazil/2002/USP-02	-	-	G	G	-	G	-	-	-
FJ477356.1 ILTV/Brazil/2003/USP-06	-	-	G	G	-	G	-	-	-
FJ477357.1 ILTV/Brazil/2003/USP-09	-	-	G	G	-	G	-	-	-
FJ794467.1 ILTV/Brazil/2008/USP-74	-	A	G	G	-	G	-	-	-
FJ794468.1 ILTV/Brazil/2008/USP-80	-	A	G	G	-	G	-	-	-
MF678664.1 ILTV/Brazil/2015/USP-657-3	-	-	G	G	-	G	C	-	-
MF678665.1 ILTV/Brazil/2015/USP-657-4T	-	-	G	G	-	G	C	-	-
MF678667.1 ILTV/Brazil/2015/USP-657-5	-	-	G	G	-	G	C	-	-
MF678668.1 ILTV/Brazil/2015/USP-657-7	-	-	G	G	-	G	C	-	-
MF678669.1 ILTV/Brazil/2016/USP-659-1	-	-	G	G	T	G	-	T	-
KF786292.1 2011/UFMG	-	-	G	G	T	G	-	T	-
KF786293.1 2012/UFMG-1	-	-	G	G	T	G	-	T	-
KF786294.1 2012/UFMG-2	-	-	G	G	T	G	-	T	-
KF786295.1 2013/UFMG-1	-	-	G	G	T	G	-	T	-
KF786296.1 2013/UFMG-2	-	-	G	G	T	G	-	T	-
MN689092.1 MG/FarmE-P871/Summer/2016	-	-	G	G	T	G	-	T	-
MN689093.1 MG/FarmG-P982/Summer/2016	-	-	G	G	T	G	-	T	-
MN689094.1 MG/FarmF-P1253/Spring/2016	-	-	G	G	T	G	-	T	-
MN689095.1 MG/FarmF-P1369/Summer/2018	-	-	G	G	T	G	-	T	-

Signs of – indicate regions with nucleotides identical to those in sample SC/01/2020/BRA.

Despite these similarities, the ICP4-1 gene from Santa Catarina sequences exhibited 97.83% similarity with the CEO vaccine strain (EU104900.1) and the CEO-like strains, such as strain 63140 from the United States (JN542536.1) and strain CAN/QC-2307414 from Canada (OL661344.1). Sequences from São Paulo samples (FJ477372.1 to FJ477379.1 and KJ028222 to KJ028226) were 97.65% similar to CEO vaccines and CEO-like strains. The difference in the identity from ICP4-1 gene between Santa Catarina samples and the sequences originating from CEO vaccine and CEO-like strains is attributed to the absence of a deletion at positions 272-283 of the ICP4-1 gene region in the Santa Catarina samples sequences, a deletion that was observed in the ICP4 gene sequences of the CEO vaccine and its derivatives (COUTO et al., 2015).

Aligned sequences of the ICP4-2 fragment were analyzed between positions 3895-4376 bp. This fragment demonstrated 100% identity with the previously mentioned VFAR-043 and J2 strains, as well as 99.79% similarity with the CEO vaccine strain (EU104900.1) and the CEO-like strains 63140 and CAN/QC-2307414. The similarity was also 99.79% with CEO-derived vaccine strains detected in São Paulo, as well as with field strains found in São Paulo and Minas Gerais. The TCO vaccine sequence (EU104908.1) exhibited 98.96% similarity. At position 3,905 bp, the Santa Catarina sequences shared a thymine nucleotide with the CEO-derived strains, while other field and TCO-derived strains had cytosine at this position. Additionally, variability was found at position 4,047 bp of the ICP4-2 fragment, where CEO vaccine reference sequences and some strains derived from this vaccine had an adenine nucleotide, while sequences from studies 1 and 2 and other CEO-derived sequences had guanine (Table 4).

Table 4. Infectious Laryngotracheitis in commercial laying hens in the state of Santa Catarina, Brazil. Nucleotide alignment of the ICP4-2 fragment from samples from two epidemiological studies in Santa Catarina, vaccine strains, and field strains previously published from other Brazilian states and countries.

Strain	Gene position								
	39 05	39 57	39 81	40 12	40 16	40 47	42 98	43 11	43 39
OR184166.1 SC/01/2020/BRA	T	C	C	A	A	G	C	A	T
OR184168.1 SC/04/2020/BRA	-	-	-	-	-	-	-	-	-
OR184171.1 SC/07/2020/BRA	-	-	-	-	-	-	-	-	-
OR184173.1 SC/09/2020/BRA	-	-	-	-	-	-	-	-	-
OR184177.1 SC/13/2020/BRA	-	-	-	-	-	-	-	-	-
OR184183.1 SC/19/2020/BRA	-	-	-	-	-	-	-	-	-
OR184142.1 SC/30/2021/BRA	-	-	-	-	-	-	-	-	-
MG775218.1 VFAR-043	-	-	-	-	-	-	-	-	-
MF417808.1 J2	-	-	-	-	-	-	-	-	-
DQ995291.1 WangGang	C	T	-	-	-	-	T	-	-
EU104900.1 CEO vaccine	-	-	-	-	-	A	-	-	-
FJ477350.1 CEO vaccine Laryngo-Vac	-	-	-	-	-	A	-	-	-
FJ477351.1 CEO vaccine Nobilis-ILT	-	-	-	-	-	A	-	G	-
HQ630064.1 strain live attenuated Serva	-	-	-	-	-	A	-	G	-
JX646898.1 strain V1-99	C	-	-	-	-	-	-	-	-
KP677885.1 strain 4787/80	-	-	-	-	-	A	-	-	-
JN542536.1 strain 63140/C/08/BR	-	-	-	-	-	A	-	-	-
OL661344.1 CAN/QC-2307414	-	-	-	-	-	A	-	-	-
FJ477372.1 ILTV/Brazil/2005/USP-50	-	-	-	-	-	A	-	-	-
FJ477373.1 ILTV/Brazil/2005/USP-51	-	-	-	-	-	A	-	-	-
FJ477374.1 ILTV/Brazil/2005/USP-52	-	-	-	-	-	A	-	G	-
FJ477375.1 ILTV/Brazil/2005/USP-57	-	-	-	-	-	A	-	G	-
FJ477376.1 ILTV/Brazil/2005/USP-59	-	-	-	-	-	A	-	-	-
FJ477377.1 ILTV/Brazil/2005/USP-62	-	-	-	-	-	A	-	-	-
FJ477378.1 ILTV/Brazil/2005/USP-64	-	-	-	-	-	A	-	G	-
FJ477379.1 ILTV/Brazil/2005/USP-65	-	-	-	-	-	A	-	-	-
KJ028222.1 ILTV/Brazil/2010/USP-82	-	-	-	-	-	A	-	G	-
KJ028223.1 ILTV/Brazil/2010/USP-83	-	-	-	-	-	A	-	G	-
KJ028224.1 ILTV/Brazil/2010/USP-84	-	-	-	-	-	A	-	-	-
KJ028225.1 ILTV/Brazil/2010/USP-85	-	-	-	-	-	A	-	G	-
KJ028226.1 ILTV/Brazil/2010/USP-86	-	-	-	-	-	A	-	-	-
EU104908.1 TCO vaccine	C	T	T	G	-	-	-	-	A
FJ477349.1 TCO vaccine LTI-IVAX	C	T	T	G	-	-	-	-	A
EU104909.1 strain USDA	C	T	T	G	G	-	-	-	A
JN542535.1 strain 81658	C	T	T	G	-	-	-	-	A
FJ477352.1 ILTV/Brazil/2002/USP-01	C	-	-	-	-	-	-	-	-
FJ477353.1 ILTV/Brazil/2002/USP-02	C	-	-	-	-	-	-	-	-
FJ477356.1 ILTV/Brazil/2003/USP-06	C	-	-	-	-	-	-	-	-
FJ477357.1 ILTV/Brazil/2003/USP-09	C	-	-	-	-	-	-	-	-
FJ794467.1 ILTV/Brazil/2008/USP-74	C	-	-	-	-	-	-	-	-
FJ794468.1 ILTV/Brazil/2008/USP-80	C	-	-	-	-	-	-	-	-
MF678673.1 ILTV/Brazil/2015/USP-657-3	C	-	-	-	-	-	-	-	-
MF678674.1 ILTV/Brazil/2015/USP-657-4	C	-	-	-	-	-	-	-	-
MF678676.1 ILTV/Brazil/2015/USP-657-5	C	-	-	-	-	-	-	-	-
MF678677.1 ILTV/Brazil/2015/USP-657-7	C	T	T	G	-	-	-	-	A
MF678678.1 ILTV/Brazil/2015/USP-659-1	C	T	T	G	-	-	-	-	A
KF786297.1 2011/UFMG	C	-	-	-	-	-	-	-	-
KF786298.1 2012/UFMG-1	C	-	-	-	-	-	-	-	-
KF786299.1 2012/UFMG-2	C	-	-	-	-	-	-	-	-
KF786300.1 2013/UFMG-1	C	-	-	-	-	-	-	-	-
KF786301.1 2013/UFMG-2	C	-	-	-	-	-	-	-	-
MN689802.1 MG/FarmE-P871/Summer/2016	C	-	-	-	-	-	-	-	-
MN689803.1 MG/FarmG-P982/Summer/2016	C	-	-	-	-	-	-	-	-
MN689804.1 MG/FarmF-P1253/Spring/2016	C	-	-	-	-	-	-	-	-
MN689805.1 MG/FarmF-P1369/Summer/2018	C	-	-	-	-	-	-	-	-

Signs of – indicate regions with nucleotides identical to those in sample SC/01/2020/BRA.

Translation of ICP4-1 and ICP4-2 sequences revealed that nucleotide changes resulted in methionine at position 200 and leucine at position 1,304 in the Santa Catarina samples and CEO-derived strains, while field strains and

TCO-derived strains had valine and proline at these same positions (M200V and L1304P). Concatenation of the ICP4-1 and ICP4-2 fragments generated a 1,039 bp sequence, clustering all sequences from Santa Catarina alongside the VFAR-043 and J2 strains. This cluster was identified as part of the phylogenetic lineage that includes CEO vaccine sequences and their derivatives, both from Brazil and other countries. The Santa Catarina sequences were found to be evolutionarily more distant from previously described field strains in São Paulo and Minas Gerais, as well as from TCO-derived sequences (Figure 3).

Aligned sequences of the UL23 gene, encoding the TK protein, were analyzed between positions 277-799 bp. Sequences among Santa Catarina samples exhibited 100% identity within this gene fragment. Compared to previously reported sequences, the Santa Catarina strains showed 100% identity with the VFAR-043 strain from Peru (MG775218.1), and the J2 and 1874C5 strains from the United States (MF417808.1 and JN542533.1). A threonine-to-methionine substitution at position 252 (T252M) is a mutation that contributes to the characterization of a strain with a virulent genotype; however, this substitution was not observed in the Santa Catarina strains.

Only ILT strains from outbreaks in São Paulo, previously identified as virulent, based on clinical signs and genome analysis, carried methionine at this position (MF678655.1 to MF678659.1, FJ444833.1, FJ444834.1, FJ444836.1, DQ786401.1, FJ444838.1 to FJ444845.1), along with the virulent CL9 strain from Australia (JN804827.1) (Table 5). Phylogenetic analysis of the TK gene showed that the Santa Catarina samples clustered with previously reported non-virulent strains, due to the absence of the T252M mutation, as did the vast majority of strains reported in outbreaks in Brazil, being evolutionarily distant from virulent strains found in the state of São Paulo, Brazil, and other countries (Figure 4).

Table 5. Infectious Laryngotracheitis in commercial laying hens in the state of Santa Catarina, Brazil. Alignment of amino acids of the thymidine kinase (TK) protein from samples from two epidemiological studies in Santa Catarina, vaccine strains, and field strains previously published from other Brazilian states and countries.

Samples	Amino acid position						
	84	190	212	217	252	265	266
OR184143.1 SC/01/2020/BRA	E	M	N	C	T	T	V
OR184145.1 SC/04/2020/BRA	-	-	-	-	-	-	-
OR184147.1 SC/07/2020/BRA	-	-	-	-	-	-	-
OR184149.1 SC/09/2020/BRA	-	-	-	-	-	-	-
OR184153.1 SC/13/2020/BRA	-	-	-	-	-	-	-
OR184158.1 SC/19/2020/BRA	-	-	-	-	-	-	-
OR184165.1 SC/30/2021/BRA	*	-	-	-	-	-	-
MG775218.1 strain VFAR-043	-	-	-	-	-	-	-
MF417808.1 strain J2	-	-	-	-	-	-	-
OL661344.1 CAN/QC-2307414	-	-	D	-	-	-	-
JN542534.1 strain USDA	-	-	D	-	-	-	-
JN542533.1 strain 1874C5	-	-	-	-	-	-	-
DQ522949.1 strain CG	-	-	D	-	-	-	-
DQ522947.1 strain Samberg	-	-	D	-	-	-	-
JX646898.1 strain V1-99	-	-	D	-	-	-	-
JN804827.1 strain CL9	-	-	D	-	M	-	-
FJ444832.1 LTI-IVAX	-	-	D	-	-	-	G
HM230801.1 CEO vaccine strain Merial	-	-	D	-	-	-	-
EU423897.1 strain SP-Trachivax	-	-	D	Y	-	-	-
EU360950.1 isolate TCO	-	-	D	-	-	-	-
MF678655.1 ILTV/Brazil/2015/USP-657-3	-	-	D	-	M	-	-
MF678656.1 ILTV/Brazil/2015/USP-657-4T	V	-	D	-	M	-	-
MF678657.1 ILTV/Brazil/2015/USP-657-4	-	-	D	-	M	-	-
MF678658.1 ILTV/Brazil/2015/USP-657-5	-	-	D	-	M	-	-
MF678659.1 ILTV/Brazil/2015/USP-657-7	-	-	D	-	M	-	-
MF678660.1 ILTV/Brazil/2016/USP-659-1	-	L	D	-	-	-	-
MF678661.1 ILTV/Brazil/2016/USP-659-2	-	L	D	-	-	-	-
MF678662.1 ILTV/Brazil/2016/USP-659-3	-	L	D	-	-	-	-
MF678663.1 ILTV/Brazil/2016/USP-659-729	-	L	D	-	-	-	-
FJ444833.1 ILTV/Brazil/2002/USP-01	-	-	D	-	M	-	-
FJ444834.1 ILTV/Brazil/2002/USP-02	-	-	D	-	M	-	-
GQ499341.1 ILTV/Brazil/2003/USP-07	-	-	D	-	-	-	-
FJ444836.1 ILTV/Brazil/2003/USP-09	-	-	D	-	M	-	-
DQ786401.1 ILTV/Brazil/2003/USP-27	-	-	D	-	M	-	-
FJ444838.1 ILTV/Brazil/2004/USP-29	-	-	D	-	M	P	-
FJ444839.1 ILTV/Brazil/2004/USP-32	-	-	D	-	M	-	-
FJ444840.1 ILTV/Brazil/2004/USP-38	-	-	D	-	M	-	-
FJ444841.1 ILTV/Brazil/2004/USP-39	-	-	D	-	M	-	-
FJ444842.1 ILTV/Brazil/2004/USP-41	-	-	D	-	M	-	-
FJ444843.1 ILTV/Brazil/2004/USP-44	-	-	D	-	M	-	-
FJ444844.1 ILTV/Brazil/2004/USP-45	-	-	D	-	M	-	-
FJ444845.1 ILTV/Brazil/2004/USP-47	-	-	D	-	M	-	-
FJ444846.1 ILTV/Brazil/2004/USP-55	-	-	D	-	-	-	-
FJ444847.1 ILTV/Brazil/2004/USP-57	-	-	D	-	-	-	-
FJ444848.1 ILTV/Brazil/2004/USP-59	-	-	D	-	-	-	-
FJ444849.1 ILTV/Brazil/2004/USP-62	-	-	D	-	-	-	-
FJ444850.1 ILTV/Brazil/2004/USP-65	-	-	D	-	-	-	-
KF786287.1 2011/UFMG	-	L	D	-	-	-	-
KF786288.1 2012/UFMG-1	-	L	D	-	-	-	-
KF786289.1 2012/UFMG-2	-	L	D	-	-	-	-
KF786290.1 2013/UFMG-1	-	L	D	-	-	-	-
KF786291.1 2013/UFMG-2	-	L	D	-	-	-	-
MN643590.1 H1096 UFMG/Brazil	-	L	D	-	-	-	-
MN643591.1 P871 UFMG/Brazil	-	L	D	-	-	-	-
MN643592.1 P982 UFMG/Brazil	-	L	D	-	-	-	-
MN643593.1 P1253 UFMG/Brazil	-	L	D	-	-	-	-
MN643594.1 P1369 UFMG/Brazil	-	L	D	-	-	-	-

Signs of – indicate regions with amino acids identical to those in sample SC/01/2020/BRA. Signs of * indicate areas of deletions in the translation. Amino acid legends: E = Glutamic acid; V = Valine; R = Arginine; C = Cysteine; M = Methionine; L = Leucine; N = Asparagine; D = Aspartic acid; Y = Tyrosine; T = Threonine; G = Glycine; P = Proline

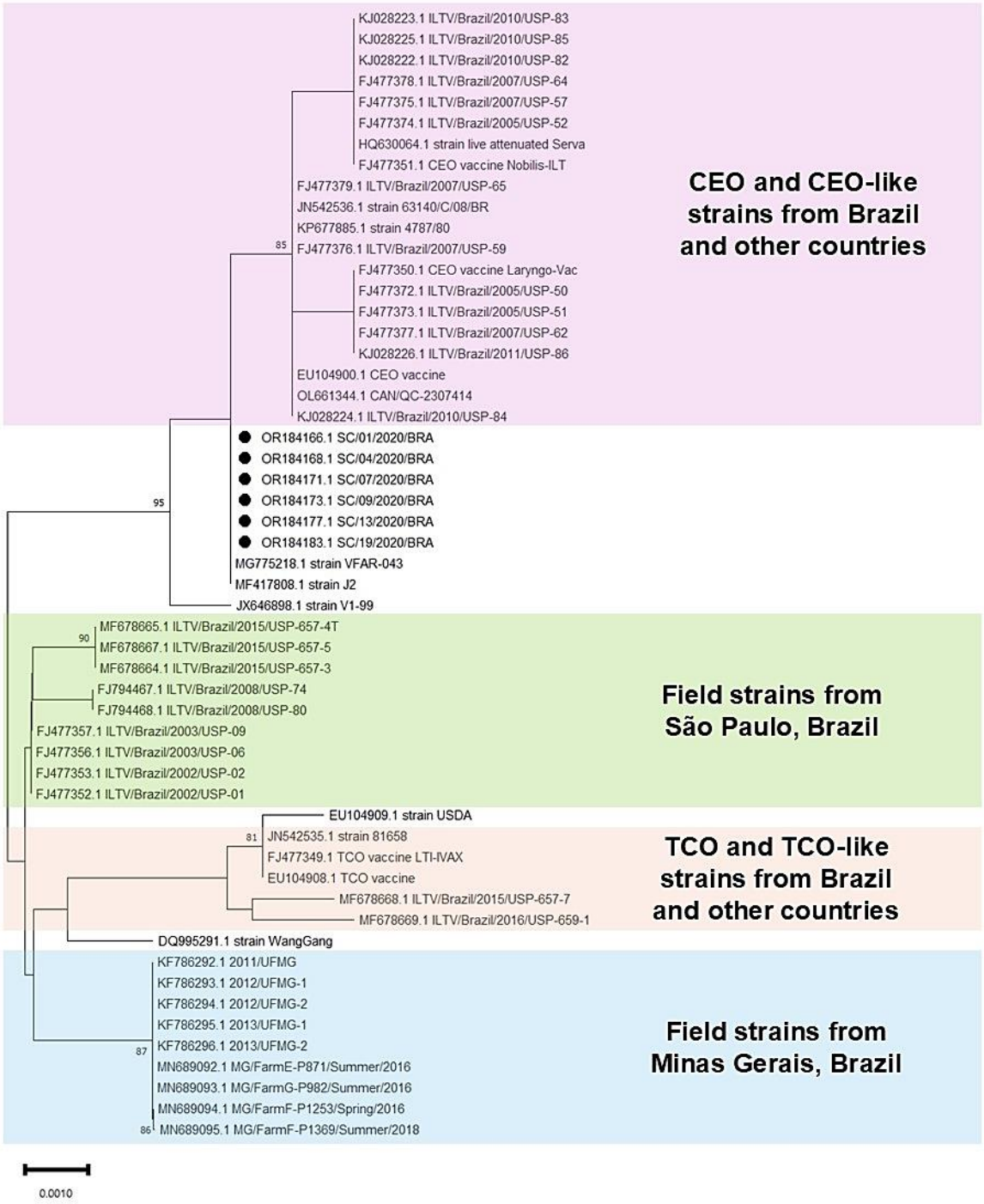

Figure 3. Phylogenetic tree generated using concatenated fragments from ICP4 gene of Infectious Laryngotracheitis (ILT) virus, including Santa Catarina strains (circle), field strains, and strains derived from attenuated live CEO and TCO vaccines. The tree was generated using the Neighbor-Joining method with 1,000 bootstraps and the Tamura 3-parameter statistical model.

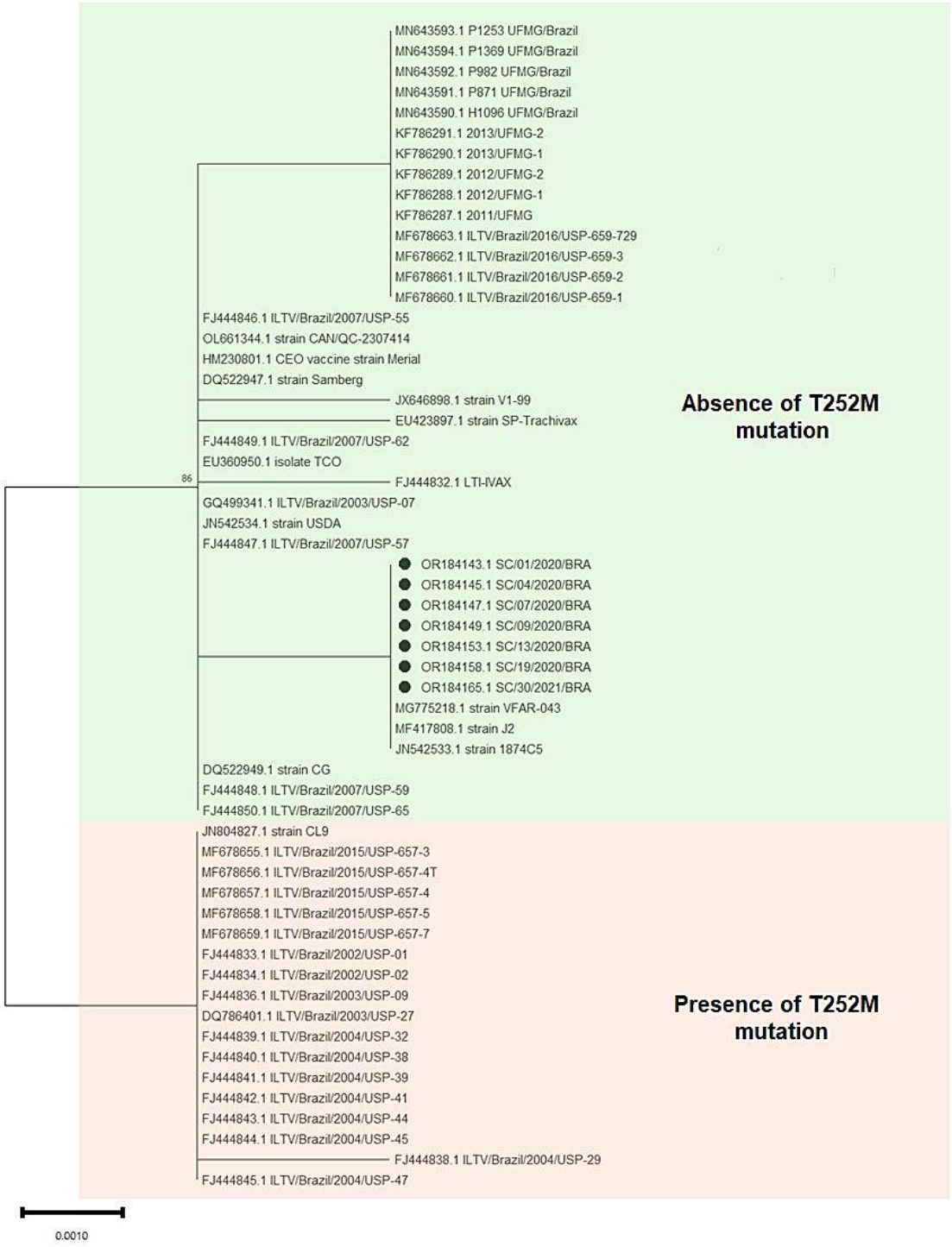

Figure 4. Phylogenetic tree generated using the UL23 gene, which encodes the TK protein of the Infectious Laryngotracheitis (ILT) virus, including Santa Catarina strains (circle), and strains with presence or absence of T252M mutation, previously reported as virulent and non-virulent strains, respectively. The tree was generated using the Neighbor-Joining method with 1,000 bootstraps and the Tamura 3-parameter statistical model.

DISCUSSION

In Brazil, ILT outbreaks have been reported in other states, with the most significant ones occurring in São Paulo in 2002, affecting around 182-layer poultry farms (CHACÓN & FERREIRA, 2009), and in Minas Gerais in 2010, impacting a region with approximately eight million chickens (PREIS et al., 2013), both states belonging to the southeastern region of the country. In Santa Catarina, in the southern region of Brazil, until the occurrence of the present outbreak in a layer hen region in the municipality of São Ludgero, ILT was considered exotic, not affecting the poultry population in this poultry production region of Brazil.

The six percent mortality rate observed in the current outbreak is higher than the rates typically associated with the mild form of the disease, which usually presents 5% morbidity and mortality ranging from 0.1% to 2.0% (GUY & GARCIA, 2008). While the São Ludgero outbreak exhibited a slightly elevated mortality rate for the mild form, when compared to previous outbreaks in Brazil the mortality rate in Santa Catarina remained lower than that reported in São Paulo, where rates ranged from 2-20% (CHACÓN et al., 2007). However, it was like the rates documented in Minas Gerais, which varied between 1-9% (PREIS et al., 2013). More recently, in 2024, ILT outbreaks were reported in the state of Paraná, neighboring Santa Catarina, with higher mortality rates ranging from 7–12.5% in broilers and 3.5–48% in broiler breeder flocks (CHACÓN et al., 2025). Notably, the state of Santa Catarina had not implemented vaccination against ILT prior to the first reported outbreak. This lack of immunity likely contributed to the relatively high mortality rate, and it is known that naïve populations tend to exhibit more prolonged and severe diseases (WOLFRUM, 2020). Following the outbreak, Santa Catarina introduced mandatory recombinant vaccination against the virus (SANTA CATARINA, 2022).

During the first visit to the farm, the clinical signs were considered alarming, as sudden mortality, dyspnea, epistaxis, and the observation of mucus and expectorated blood on cages, walls, and the aviary floor are reported in severe forms of ILT (MORENO et al., 2010; GUY et al., 2013; GOWTHAMAN et al., 2020). Experimental inoculation studies demonstrate that the peak of ILT clinical signs occurred on the fourth day after virus inoculation (KIRKPATRICK et

al., 2006). It is known that the clinical course of the disease in chickens that do not succumb to it varies from 10 to 14 days, and the signs become milder during disease remission, consisting of nasal discharge, eyelid edema, lethargy, and cyanosis of the comb (SELLERS et al., 2004; MORENO et al., 2010), which are compatible with the signs observed during the second visit to the farm, demonstrating the regression of clinical signs.

The milder gross findings during necropsy can also be explained by the remission phase of the disease, and commonly observed mild findings include hyperemia of the larynx and trachea and mucus accumulation in the lumen (GUY et al., 2013). In more severe cases of ILT, hemorrhage, ulcerations, and necrosis of the respiratory epithelium, along with diphtheritic plaques and fibrinonecrotic material accumulation, with mucus and blood in the lumen are observed (GOWTHAMAN et al., 2014), none of which were seen in the necropsied hens. The gross findings observed in the Minas Gerais outbreak were considered more severe than the Santa Catarina outbreak, with the visualization of fibrinous and caseous exudate in the airways, sometimes completely obstructing the airway (PREIS et al., 2013). Macroscopic findings were also more severe in broilers and broiler breeders from the state of Paraná, where mucous exudate, blood clots, and caseous plaques were found in the tracheal and laryngeal lumen, partially or completely obstructing the airways (CHACÓN et al., 2025).

Microscopically, the organs most frequently affected, either concomitantly or not, are the larynx, trachea, nasal conchae, lungs, and conjunctiva (LINARES et al. 1994, PREIS et al., 2013), and in the Santa Catarina outbreak, except for the nasal conchae, alterations were observed in all other organs. The visualization of syncytial cells with eosinophilic intranuclear inclusion bodies is considered a classic sign of ILT, but its visualization is restricted to about 1-5 days after infection (CARNACCINI et al., 2022), disappearing a few days later due to the necrosis and desquamation of epithelial cells, which become part of the airway exudate, which explains the identification of this alteration in only a few of the necropsied chickens in this study.

In this study, syncytial cells containing eosinophilic intranuclear inclusion bodies were found in the larynx, trachea, and bronchi, as in the study by Preis et al. (2013) in Minas Gerais, where they were found in 70% of tracheas, 50% of larynges, and 10% of primary and secondary bronchi. In the Minas Gerais

outbreak, they were also found in the nasal sinuses; however, in the Santa Catarina outbreak, these cells were not found in the nasal sinuses. On the other hand, as the clinical course progresses, epithelial hyperplasia of the respiratory tract commonly occurs in cases where necrosis and desquamation do not occur, in addition to lymphocyte and plasma cell infiltration (SIVASEELAN et al., 2014; CARNACCINI et al., 2022), reflecting the survival of chickens during the acute phase of the disease, as part of a regenerative process characterized by hyperplastic tracheitis from the sixth day after infection, marked by the proliferation of remaining basal cells (BAGUST et al., 2000), widely observed in this ILT outbreak.

It is important to emphasize that histopathological analysis is the diagnostic method of choice for identifying ILT, as it allows for distinguishing real infections leading to disease from cases of infected animals where viral DNA or antibodies are detected without apparent changes (PREIS et al., 2013). However, in cases where classic lesions are not observed, complementary analyses are essential for diagnosing ILT and ruling out diseases that may present with similar clinical signs and gross and microscopic alterations, such as *Mycoplasma* spp. infection, infectious bronchitis, the diphtheritic form of avian pox, avian pneumovirus, Newcastle Disease, and Avian Influenza virus, as previously investigated in ILT outbreaks in Brazil (CHACÓN et al., 2007; COUTO et al., 2016). In the present case, due to the sudden mortality observed during the first week, it was crucial to rule out severe diseases such as Newcastle Disease and Avian Influenza virus.

Considering the economic losses caused by ILT virus infection, such as poultry mortality, decreased egg production, and increased susceptibility to secondary respiratory infections (GUY et al., 2013), it is of great importance to conduct epidemiological surveys in regions affected by ILT outbreaks, especially for diagnosis, investigation of the outbreak’s origin, and assessment of the strain’s virulence level. These are considered the first steps in establishing control measures to contain and prevent the virus's spread to other regions (CHACÓN et al., 2007). A study conducted in the state of Minas Gerais demonstrated that monitoring by the Official Veterinary Service, combined with improved biosecurity measures by poultry farmers, was effective in controlling the disease (HERGOT et al., 2021).

To achieve this goal, the origin of the strains in the ILT outbreak in Santa Catarina was investigated by sequencing two fragments of ICP4 gene, a methodology established by Chacón & Ferreira (2009) during ILT outbreaks in the state of São Paulo, considered faster and more accurate than PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), which had been used until then. These same regions were also amplified during ILT outbreaks in the state of Minas Gerais, with the aim of differentiating whether the strain involved was a field strain or a reverted virulent CEO or TCO vaccine strain (COUTO et al., 2015). The evaluation of the Santa Catarina strains was complemented with the sequencing of the UL23 gene, which encodes the TK protein, that changes among strains and have been previously related to strain virulence, which directly impacts the severity of the disease in poultry farms (HAN & KIM, 2001; COUTO et al., 2015). The methodology used in the Santa Catarina outbreak is effective, particularly in cases where it is not possible to immediately perform full genome sequencing of the GaHV-1 involved in the outbreak. To date, the complete genome of GaHV-1 has been sequenced only once in the country, following ILT outbreaks in the state of São Paulo (LUCIANO, 2021).

The selection of tissues for molecular analysis is based on the preferred sites of viral replication, including the larynx, trachea, and conjunctiva, which are widely used in studies for molecular characterization of ILT virus (CHACÓN et al., 2007; PREIS et al., 2014; COUTO et al., 2015; SANTANDER-PARRA et al., 2022), as well as the trigeminal ganglion, the main tissue where the virus establishes its latency period in the host (WILLIAMS et al., 1992). Due to the sampling in studies 1 and 2 being conducted through active surveillance, often in clinically healthy chickens, the selection of these tissues becomes even more important because the virus can remain latent in these tissues, allowing for its identification (CHACÓN et al., 2015). The partial sequences obtained in studies 1 and 2 from the state of Santa Catarina showed 100% identity with each other, even though they were collected from different municipalities within the state, indicating a rapid and consistent spread of the virus in the region.

Regarding the partial sequencing of the ICP4 gene, when compared with sequences previously deposited in GenBank, it is noteworthy that the strains from Santa Catarina share 100% identity with the VFAR-043 strain, which was isolated in December 2014 during an ILT outbreak in Chincha Alta, Ica, Peru, and is

considered the first strain to have its complete genome sequenced in South America (RUIZ et al., 2018a). This strain, in turn, shows 99.98% similarity with the J2 strain, a parental virus of North American strains (LONCOMAN et al., 2017), with which the partial sequencing from Santa Catarina also demonstrated 100% identity. It is known that the first report of ILT in Peru occurred in 2008 in gamecocks and poultry farms (ALVARADO et al., 2013), and interestingly, the J2 strain in the United States was also isolated in 2008 from unvaccinated gamecocks, leading to speculation that the VFAR-043 strain may have been introduced to Peru via unvaccinated gamecocks originating from the United States (RUIZ et al., 2018b). In a global context, the similarity between the strains from Santa Catarina and those from other countries, despite their geographical distance, raises questions that complicate the search for hypotheses to explain their common origin. However, the hypothesis of cockfighting practices cannot be dismissed, as despite being illegal in Brazil, it is legal in Peru, a country with the longest tradition of this practice (ORTEGA et al., 2014). This hypothesis was also reported by Chacón & Ferreira (2009), suggesting that the virus may have been introduced into both Brazil and Peru through the illegal entry of birds imported from other countries.

This evidence demonstrates that the ILT virus sequences isolated in Santa Catarina in studies 1 and 2 originate from field strains (LONCOMAN et al., 2017; RUIZ et al., 2018a). However, phylogenetic analysis shows that the sequences from Santa Catarina have a greater evolutionary distance compared to field strains previously reported in Brazil. This distance starts with sequences isolated from the first outbreaks in the Bastos region, São Paulo, in 2002, as well as samples from the same region in 2003 and 2008, corresponding to sequences FJ477352.1, FJ477353.1, FJ477356.1, FJ477357.1, FJ794467, and FJ794468 (CHACÓN & FERREIRA, 2009). Additionally, in São Paulo during the years 2015 and 2016, field strains were isolated from chickens showing clinical respiratory signs like those observed in the initial outbreaks in the region, corresponding to sequences MF678664.1, MF678665.1, and MF678667.1 (SANTANDER-PARRA et al., 2022), which are also evolutionarily distant from the sequences from Santa Catarina.

Regarding the outbreaks in Minas Gerais since 2010, which were also caused by field strains, an even greater evolutionary distance than that observed

in São Paulo was found. This was seen in the sequences isolated during the first ILT outbreaks in the region between 2011 and 2013, corresponding to sequences KF786292-96.1 (COUTO et al., 2015), as well as in sequences found years later in the same region, from active and passive surveillance in local poultry farms, corresponding to sequences MN689092-95.1 (SANTOS et al., 2022). The samples from studies 1 and 2 also diverge from sequences associated with the TCO vaccine, even though this vaccine was previously used in the country to control outbreaks in the Bastos region in 2002, and authorized for use until 2012 in São Paulo, after which it was replaced by recombinant vaccines (CHACÓN et al., 2015). Moreover, in this same region, sequences correlating with the TCO vaccine were recently isolated from chickens with clinical signs, corresponding to sequences MF678668-69.1 (SANTANDER-PARRA et al., 2022).

Although the sequences from Santa Catarina were characterized as field strains by phylogenetic analysis, it is important to note that they are evolutionarily closer to the CEO-like sequences than to the TCO vaccine or other field strains reported in the country, as mentioned above. The CEO vaccine was also previously used in the country, in the Bastos region of São Paulo, to control the initial ILT outbreaks, along with the TCO vaccine. Years later, between 2010 and 2011, CEO-like strains (FJ477372-79.1 and KJ028222 to KJ028226) were isolated from clinically healthy laying hens, both in the Bastos region and in poultry farms about 350 km from the area where the CEO and TCO vaccines were allowed years earlier (CHACÓN et al., 2015). This phylogenetic proximity between the Santa Catarina sequences and those of CEO origin can be explained by the shared guanine to adenine changes at positions 438bp, 456bp, and 598bp of the ICP4 gene, as well as the cytosine to thymine change at position 3905bp of the same gene. However, the major divergence between the strains in this study and the CEO and CEO-like vaccine strains is the absence of the deletion area at position 272-283bp, which is characteristic of this type of sequence (CHACÓN & FERREIRA, 2009; COUTO et al., 2015). The nucleotide changes resulted in a modification of the translated amino acid at positions 200 and 1,304, with the production of methionine and leucine in the sequences from Santa Catarina and those originating from the CEO vaccine, producing methionine and leucine at these positions, respectively, while sequences from

other origins produced valine and proline. Further studies are needed to understand the effect that this amino acid alteration may cause.

According to the sequencing and phylogeny of the UL23 gene, which encodes the TK protein, the Santa Catarina samples did not show the threonine-to-methionine alteration at position 252 of the translated gene (T252M), which in the 1990's has been associated with virulent and non-virulent strains (HAN & KIM, 2001). Once again, the sequences from studies 1 and 2 demonstrated 100% identity with the VFAR-043 and J2 strains, all of which are non-virulent strains, and the 1874C5, also from United States. Studies have already demonstrated the close phylogenetic relationship among these three strains, grouping them into the same cluster, consistent with the genotype VI of the virus, containing field strains (RUIZ et al., 2018b; SPATZ et al., 2019), along with the strains identified in Santa Catarina. Strains within genotype VI cause acute disease between 4-7 dpi and, experimentally, show severe dyspnea, moderate to severe conjunctivitis, and severe depression during this period, and exhibit greater resilience in becoming asymptomatic in hosts after the clinical course of the disease (OLDONI et al., 2009). A recent study suggests classifying ILTV strains isolated in Brazil into five subclusters within genotype VI: subcluster VI-1 from the Bastos region, SP, in 2002; subcluster VI-2 from the Guatapará region, SP, in 2009; the Itanhandu subcluster, MG, in 2010; and subcluster VI-4, which includes strains from Santa Catarina along with recently isolated strains from the state of Paraná and other countries (CHACÓN et al., 2025), suggesting a common ancestor among these strains.

In the phylogenetic analysis, it was possible to observe that the strains with the T252M mutation, characterized as virulent, formed a specific cluster, evolutionarily distant from the non-virulent strains. In Brazil, to date, virulent field strains have only been detected in the state of São Paulo (MF678655-59.1, FJ444833-34.1, FJ444836.1, FJ444838-45.1, and DQ786401.1), all of them during the initial disease outbreaks in 2002, 2003, and 2004, as well as in 2015, isolated from chickens with respiratory clinical signs, facial edema, swollen and stretched heads, and sneezing (SANTANDER-PARRA et al., 2022). The virulent CL9 strain from Australia also clustered within the virulent strain cluster (JN804827.1), being the most circulating ILT strain in Australia, emerging from recombinant effects and leading to severe ILT outbreaks, characterized by severe

conjunctivitis, severe dyspnea, and high mortality (THILAKARATHNE et al., 2020). Although the partial sequencing of the UL23 gene, which encodes the TK protein, showed that the samples isolated in Santa Catarina cluster with other non-virulent strains from Brazil and other countries, it is important to emphasize that there are many other determinants in its genome necessary to correlate the genotype with the virus phenotype, linking it to the type of clinical presentation observed in the field (MENENDEZ et al., 2014), therefore, this study is not able to determine if the Santa Catarina strains are highly virulent or not. To achieve this, it is essential to conduct further whole genome studies to fully understand the virus's behavior in Santa Catarina.

CONCLUSION

The study demonstrates the occurrence of the first ILT outbreak in the state of Santa Catarina, affecting a region with a high density of layer farms. The outbreak was characterized by mild to moderate clinical signs, with the observation of classic microscopic lesions of the disease. Through active surveillance in 2020 and 2021 in the state, it was possible to perform partial sequencing of the ICP4 gene, characterizing the strains as field-origin, yet evolutionarily very close to CEO-like strains. This raises the hypothesis of virus entry from Peru and/or the United States, given their 100% identity with the VFAR-043 and J2 strains. The partial sequencing of the TK gene showed an absence of T252M mutation, and further genome analysis is needed, as TK gene sequencing alone is not sufficient to determine virulence. Whole-genome sequencing studies are recommended for a better understanding of the entry of these strains in Santa Catarina.

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5. ARTIGO 2

Two-year surveillance of infectious laryngotracheitis virus in layer farms from Southern Brazil: a seroepidemiological, molecular, and pathological approach

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ABSTRACT

Infectious Laryngotracheitis (ILT), caused by Gallid Herpesvirus 1 (GaHV-1), is a contagious and notifiable disease. In Santa Catarina, southern Brazil, it was exotic until 2020, when it was diagnosed in a commercial layer farm in São Ludgero/SC. This study consists of two phases: the first assesses ILT prevalence in municipalities near São Ludgero in 2020, while the second examines the entire state of Santa Catarina in 2021. A total of 44 farms were sampled in 2020 and 49 in 2021, including commercial layer and rearing farms. Twenty chickens per farm were sampled for serology, and 10 were euthanized for necropsy, respiratory organ collection and histopathological evaluation. Conjunctiva, larynx, trachea, and trigeminal ganglion samples were subjected to qPCR for ILT virus. Fisher's exact test and multivariate logistic regression (α=0.05) were applied to identify risk factors. In 2020, 95.4% of farms were seropositive, and 88.1% of seropositive farms tested qPCR-positive. In 2021, these rates were 65.3% and 21.8%, respectively. Syncytial cells and intranuclear inclusion bodies were observed in three farms (7.1%) in 2020 but were absent in 2021. Multivariate analysis confirmed flock replacement with older chickens as a significant risk factor for ILT in 2021 ($p<0.05$). These findings underscore the widespread ILT prevalence in São Ludgero and its dissemination across Santa Catarina, emphasizing the importance of biosecurity measures in preventing outbreaks in commercial layer farms.

KEYWORDS: layer chickens, histopathology, qPCR, GaHV-1, biosecurity measures.

INTRODUCTION

Infectious Laryngotracheitis (ILT) is a highly contagious respiratory disease in chickens that must be reported to the official veterinary authorities in Brazil (BRAZIL, 2021). The causative agent is Gallid Herpesvirus 1 (GaHV-1), classified under the genus *Iltovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* (GUY & GARCÍA, 2008; DAVISON et al., 2009). This virus establishes latency within the host and can periodically reactivate in response to stress or immunosuppression (DUFOUR-ZAVALA, 2008).

The disease can be present in two forms: the mild form, which is more common (GARCÍA et al., 2013), typically shows a morbidity rate of around 5% and mortality ranging from 0.1% to 2.0%. Clinical signs include lethargy, nasal discharge, conjunctivitis, and a reduction in egg production (SELLERS et al., 2004; GUY & GARCÍA, 2008; MORENO et al., 2010; PARRA et al., 2016). In contrast, the severe form can exhibit morbidity rates as high as 100%, with mortality varying from 5% to 80%, depending on the viral strain (KIRKPATRICK et al., 2006). Clinical signs of the severe form include pronounced dyspnea, rales, expectoration of blood-tinged mucus, coughing, and death occurring within two to three days (MORENO et al., 2010; GARCÍA et al., 2013).

Poultry production characteristics, combined with factors such as biosecurity lapses in poultry houses, co-occurring diseases, prolonged transportation, sudden temperature fluctuations, and inadequate feed or water management, contribute to the disease occurrence in flocks, triggering the reactivation of its latent form (DUFOUR-ZAVALA, 2008). In addition to the economic losses from chicken mortality, there are also losses from reduced egg production and increased vulnerability to secondary respiratory infections (GARCÍA et al., 2013). This results in additional costs for medications and vaccines, as well as restrictions on the movement of animals and their products (JOHNSON et al., 2004).

The virus was first identified in Brazil in 1974, in the state of Rio de Janeiro (HIPÓLITO et al., 1974). Significant outbreaks in Brazil occurred in São Paulo, in the Bastos region, in 2002, leading to high morbidity and mortality in an area with a dense concentration of layer farms (CHACÓN et al., 2007; CHACÓN & FERREIRA, 2009), and in Minas Gerais, in 2010, affecting a major egg

production region in the Mantiqueira Highlands, impacting a population of over eight million laying hens (PREIS et al., 2013; PREIS et al., 2014). In Santa Catarina, southern Brazil, ILT was considered exotic to the poultry population until August 2020, when an outbreak was detected in a laying farm in the municipality of São Ludgero in September of the same year (SANTA CATARINA, 2020). The São Ludgero County encompasses the municipalities of Braço do Norte, Orleans, Pedras Grandes, São Ludgero, Tubarão, and Urussanga, with a total population of over 2.9 million laying hens (IBGE, 2024).

In previous outbreaks in Brazil, epidemiological surveys played a critical role in effectively controlling and containing the disease, thereby preventing the emergence of new severe cases. These surveys were conducted in collaboration with the Official Veterinary Service of the affected states, such as São Paulo and Minas Gerais, and with the cooperation of poultry farmers, who implemented improvements in biosecurity measures on their egg farms (BUCHALA et al., 2011; HERGOT et al., 2021). To evaluate the situation of the disease in the São Ludgero County and in all regions of the state of Santa Catarina, this study aims to determine the serological, molecular, and pathological status of ILT through two studies: Study 1, conducted in 2020 in the São Ludgero County, and Study 2, conducted in 2021, covering all regions of the state of Santa Catarina. The study also sought to identify potential epidemiological factors associated with the occurrence of the disease in seropositive farms and/or those with molecular detection of the virus.

MATERIAL AND METHODS

SAMPLE SIZE

STUDY 1

For this cross-sectional epidemiological study, the primary sampling unit was defined as the number of commercial layer farms and rearing farms in the São Ludgero region. The sample size calculation for the primary unit was performed using the Epitools® (2020) tool (SERGEANT, 2018), based on the equation $n = [z^2 * P * (1 - P)] / e^2$. The four parameters used in the formula were: the estimated prevalence (P = design prevalence) of 50%, considering the lack of prior data on ILT occurrence in the São Ludgero region; the desired precision

(e = fixed at 10%); and the desired confidence level (z = 1.96, corresponding to a 95% confidence level).

After determining the sample size (n), an adjusted sample size for the region's population was calculated using the equation $n(adj) = (N * n) / (N + n)$, where n(adj) is the population-adjusted sample size; n is the sample size calculated from the previous equation; and N is the total population in the region, corresponding to 80 commercial layer and rearing farms registered in the official database of the Integrated Agricultural Development Company of Santa Catarina (SIGEN+/CIDASC).

The result of the adjusted sample size calculation for the São Ludgero County, based on the parameters of the formulas, was 44 farms. The farms were then randomly selected to participate in the survey and were visited during October and November 2020. Inspections were carried out by CIDASC, with the presentation of an informed consent form to the poultry farmer to confirm their authorization.

STUDY 2

In 2021, the sample size was determined using the same methodology as in Study 1, with some adjustments: the design prevalence (P) was set at 70% due to the high prevalence of the disease found in the previous year through Study 1; and N, corresponding to the total population, was 275 commercial layer and rearing farms across the entire state of Santa Catarina, also registered in SIGEN+/CIDASC. The result was a sample size of 63 farms to be surveyed in the state. The farms were randomly selected according to the SIGEN+ registry. Of the 63 farms, 14 were in the São Ludgero County and had already been sampled in Study 1. Therefore, Study 2 focused on the remaining 49 farms. The property visits occurred in February and March 2021, and were carried out using the same methodology as in Study 1.

SAMPLING PER FARM

In both studies, the hens on the farms were considered the secondary sampling unit. Using the Epitools® (2020) tool (SERGEANT, 2018), the equation indicated a minimum of 18 layer hens per farm to be selected for serological

analysis; however, whenever possible, blood samples were collected from 20 hens per farm. Subsequently, 10 of these sampled hens were euthanized via cervical dislocation, followed by necropsy and the collection of organs for histopathological examination to confirm the diagnosis of ILT and real-time polymerase chain reaction (qPCR) to confirm the infection.

As a criterion for selecting barns within the farm, barns housing chickens showing clinical signs compatible with ILT were prioritized, including lethargy, eyelid edema, tearing, coughing, dyspnea, comb cyanosis, and/or expectoration of mucus or blood-tinged secretions. If no chickens exhibited such signs, barns with the oldest hens were given priority. In the absence of clinical signs, chickens for sample collection were selected randomly, covering the entire barn to ensure representation of the entire flock housed within it.

SAMPLE COLLECTION

The sampled hens on each farm were selected from within the barns, taken to an outdoor area, and restrained by immobilizing the wings and pelvic limbs. Blood was then collected via jugular or ulnar vein puncture, with a volume of 3 to 5 mL. The obtained serum was transferred to labeled microtubes and stored at -20°C until the serological test was performed. For the 10 hens sampled for histopathological and molecular analysis, fragments of the conjunctiva, nasal sinuses, larynx, lungs, and proximal and distal trachea were collected into a pooled sample, fixed in 10% buffered formalin for histological processing. For molecular analysis, fragments of the conjunctiva, larynx, medial trachea, and trigeminal ganglion were pooled and stored in Falcon tubes at -20°C until qPCR was performed (HERGOT et al., 2021).

SEROLOGICAL ANALYSIS

To detect exposure to the ILT virus, the serum samples were thawed, kept at room temperature for approximately 30 minutes, and then centrifuged. They were individually subjected to enzyme-linked immunosorbent assay (ELISA) using the ILT ELISA® commercial kit (BioCheck) according to the manufacturer's instructions. The results were interpreted such that S/P ratios below 5% were considered negative, while S/P ratios above 5% were considered positive.

HISTOPATHOLOGICAL ANALYSIS

Tissue fragments pooled from each organ per farm, fixed in 10% buffered formalin, were routinely processed for histopathology, embedded in paraffin blocks, and stained with hematoxylin and eosin for examination under a light microscope. The lesions found in each organ pool were then characterized.

MOLECULAR ANALYSIS

Fragments pooled from the conjunctiva, larynx, medial trachea, and trigeminal ganglion were thawed at room temperature for approximately 30 minutes. Each pool was homogenized for subsequent DNA extraction. Total DNA from the samples was extracted using the IndiMag 48 automatic extractor (Indical Bioscience GmbH, Leipzig, Germany) with the IndiMag Pathogen Kit® (Indical Bioscience GmbH, Leipzig, Germany), following the manufacturer's instructions.

The glycoprotein C (gC) gene of the ILT virus was amplified using the primers ILTVgCU771 (5'-CCTTGCGTTTGAATTTTTCTGT-3'), ILTVgCL873 (5'-TTCGTGGGTTAGAGGTCTGT-3'), and the Taqman probe ILTVprobe817 (5'-FAM-CAGCTCGGTGACCCCATTCTA-BHQ1-3'), according to Callison et al. (2007). The reaction was performed using the GoTaq Probe qPCR Master Mix protocol (Promega, Madison, USA) in a QuantiStudio 6 thermocycler (ThermoFisher, Waltham, USA), with the following program: 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds, and 60°C for 32 seconds. A sample from the ILT AGID (Agar Gel Immunodiffusion Assay) antigen was used as a positive control, and autoclaved ultrapure water was used as a negative control.

EPIDEMIOLOGICAL DATA

An epidemiological survey was also conducted with the producer, using a questionnaire covering 50 multiple-choice questions that addressed general farm characteristics, production, health, biosecurity, bird and litter management, and previously reported diseases. Prioritizing fully completed questionnaires, 38 out of the 44 farms from Study 1 were used for the farm characterization and evaluation of factors associated with infection, while all the 49 farms were used for epidemiological analysis from Study 2.

The epidemiological survey covered various topics related to general characteristics and biosecurity measures on the farms, such as the type of production, housing, barn structure, replacement chickens, water quality, previously reported diseases, management challenges, proper waste disposal, farm isolation, biosecurity practices, and animal circulation.

STATISTICAL ANALYSIS

The data obtained was compiled into spreadsheets for analysis using descriptive and inferential statistics with Excel® Software (2023). For interpretation purposes, farms were considered positive if at least one hen was seropositive or tested positive by qPCR. Descriptive statistics were conducted to characterize the study based on the obtained values. To test the hypothesis of positivity by serology and qPCR for the ILT virus in the farms and the variables obtained from the epidemiological survey, a binomial logistic regression model was performed using IBM® SPSS® Statistics 25 Software. Initially, the independent variables obtained from the questionnaire were organized into binary tables (yes/no), then distributed into contingency tables and subjected to Fisher's Exact Test using Sigma Plot 14.0 Software. Variables with $p < 0.20$ were then considered for multivariate analysis. All variables were tested for collinearity, and those showing collinearity were removed from the multivariate analysis. The interaction between variables in the final model was assessed at a significance level of $\alpha = 0.05$. Questionnaires with 100% responses were prioritized, and variables with large amounts of missing data (>10%) or limited variability (<20%) were not included in the analysis.

ETHICS COMMITTEE

The study was approved by the Animal Ethics Committee (CEUA) of the Universidade do Estado de Santa Catarina (UDESC) under approval number 2793260221.

RESULTS

SEROLOGICAL ANALYSIS

Regarding the percentage of seropositive farms, the result from Study 1 was approximately 1.46 times higher than that observed in Study 2, indicating significant exposure of the layers to the virus in 2020 in São Ludgero County (Table 6). In both studies, most layers exhibited titers up to 1000-3000, with a decreasing number of layers corresponding to higher titers (Figure 5).

Table 6. Infectious Laryngotracheitis in commercial layers from the Studies 1 and 2 in Santa Catarina state, Brazil. Characterization of titers found in seropositive farms.

Parameter	Study 1	Study 2
Total seropositive farms	95.4% (42/44)	65.3% (32/49)
Average seropositive layers per farm	15.8	3.8
Total seropositive layers	70.3% (619/880)	13.9% (124/886)
Mean titer	5728.1 (S/P ratio = 2.3)	3595.7 (S/P ratio = 1.9)
Minimum titer	1071 (S/P ratio = 0.5)	1074 (S/P ratio = 0.5)
Maximum titer	18729 (S/P ratio = 6.7)	11687 (S/P ratio = 4.4)

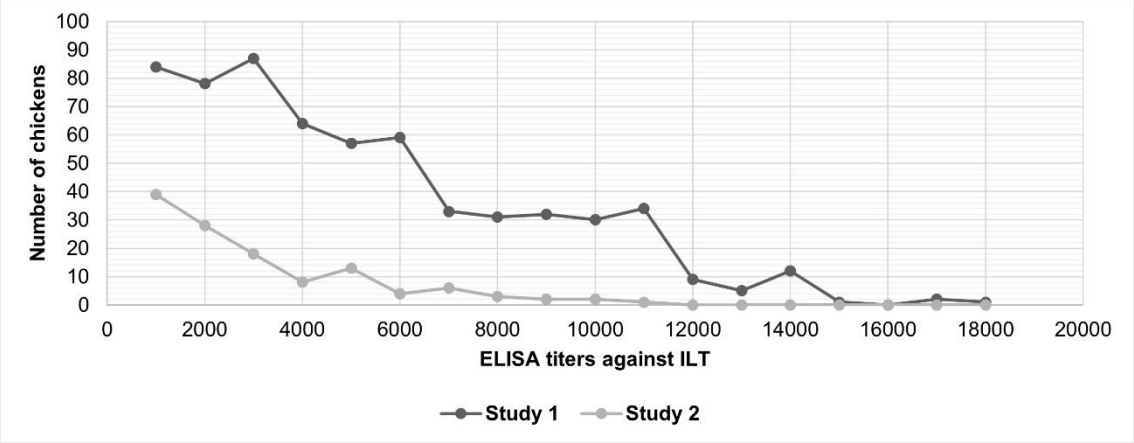

Figure 5. Antibody titers against Infectious Laryngotracheitis Virus (ILTV) detected by ELISA in chickens from different commercial layer and rearing farms. The dark gray line represents the titers from Study 1, while the light gray corresponds to Study 2.

MOLECULAR ANALYSIS

STUDY 1

In qPCR, positivity for the ILT virus glycoprotein C gene was detected in at least one organ pool in 88.1% (37/42) of the seropositive farms. The organ pools with the highest frequency of viral DNA detection were the larynx (89.2% - 33/37) and medial trachea (89.2% - 33/37), followed by conjunctiva (83.8% - 31/37) and trigeminal ganglion (81.1% - 30/37). Of the five farms negative for the virus in qPCR (11.9% - 5/42), three tested seropositive for the ILT virus, with titers ranging

from 1367 (S/P ratio = 0.62) to 6834 (mean S/P ratio = 2.64). The two seronegative farms were also negative for molecular detection of the ILT virus.

STUDY 2

Detection of the ILT virus glycoprotein C (gC) by qPCR was observed in seven seropositive farms (21.9% - 7/32), of which five (15.6% - 5/32) were positive in all collected pools. The farms positive by qPCR were distributed across different regions of the state, with 57.1% (4/7) in the South region, 28.5% (2/7) in the West region, and 14.3% (1/7) in the East region of the state. No seronegative farms tested positive by qPCR.

The qPCR results, along with the histopathological and serological findings for each farm in Studies 1 and 2, are presented in Table 7.

Table 7. Infectious Laryngotracheitis in commercial layers from the Studies 1 and 2 in Santa Catarina state, Brazil. Farms with virus positivity by qPCR associated with histopathology and serology.

Study	Farm	qPCR				Histopathology	Serology	
		L	T	C	TG	Classic lesions of ILT	Mean antibody titer	S/P ratio
1	1	+	+	+	+	-	9156,3	3,49
1	2	+	-	+	+	-	3866,7	1,57
1	3	+	+	+	-	-	4720,7	1,89
1	4	+	+	+	+	-	3108,4	1,31
1	5	+	+	+	+	-	4975,9	2,01
1	6	+	+	+	+	-	5441,3	2,18
1	7	+	+	+	+	-	5718,9	2,25
1	8	+	+	+	+	-	10282,5	3,89
1	9	+	+	+	+	-	6877,1	2,68
1	10	+	+	-	-	-	1778,3	0,78
1	11	+	+	+	+	+	8349,4	3,19
1	12	+	+	+	+	-	2204,2	0,95
1	13	+	+	+	+	-	6375,8	2,51
1	14	+	+	+	+	-	6371,6	2,48
1	15	+	+	-	-	-	1193,3	0,55
1	16	+	+	+	+	-	5471,6	2,18
1	17	+	+	+	+	-	4850,5	1,96
1	18	+	+	+	+	-	5273,6	2,11
1	19	+	+	+	+	-	4729,3	1,91
1	20	+	+	+	+	-	4864,2	1,96
1	21	+	+	+	+	-	6675,8	2,62
1	22	+	+	+	+	-	6979,4	2,71
1	23	+	+	+	+	-	5753,2	2,28
1	24	-	-	-	+	-	3960,7	1,61
1	25	+	+	-	+	-	6239,7	2,45
1	26	-	-	-	+	-	3506,7	1,45
1	27	+	+	+	+	+	3412,2	1,41
1	28	+	+	+	+	-	10388,5	3,91
1	29	+	+	+	-	-	8350,2	3,19
1	30	+	+	+	+	-	8829,9	3,38
1	31	+	+	+	-	-	5634,6	2,22
1	32	+	+	+	+	-	8865,8	3,39
1	33	-	+	-	-	-	1772	0,78
1	34	+	+	+	+	-	7849,5	3,03
1	35	+	+	+	+	+	2711,2	1,15
1	36	+	+	+	+	-	4590,1	1,86
1	37	-	-	+	-	-	2169,8	0,94
2	1	+	+	+	+	-	3254,12	1.618
2	2	+	+	+	+	-	2523,5	1.231
2	3	+	+	-	-	-	3338	1.405
2	4	-	+	+	-	-	3000,66	1.396
2	5	+	+	+	+	-	2762,88	1.659
2	6	+	+	+	+	-	6710,55	2.638
2	7	+	+	+	+	-	4916,31	2.125

+: Positive for ILT virus by qPCR/presence of histological lesions of ILT; -: Negative for ILT virus by qPCR/absence of histological lesions of ILT; L: Larynx; T: Trachea; C: Conjunctiva; GT: Trigeminal ganglion.

CLINICAL SIGNS AND ANATOMOPATHOLOGICAL ANALYSIS

STUDY 1

In five seropositive farms (11.9% - 5/42), suspected ILT was reported at the time of the survey, with clinical signs including dyspnea, eyelid edema, and expectoration of catarrhal secretions. Two of these farms also showed increased flock mortality rates, with percentages of 3.8% and 12.4%, respectively. Macroscopic evaluation of carcasses revealed air sac opacity in only one

seropositive farm, observed in 2/10 sampled hens, and mucus accumulation in the tracheal lumen in 1/10 hens. The remaining hens showed no macroscopic lesions. The mean antibody titer for ILT among farms reporting respiratory clinical signs ranged from 3318.4 (mean S/P ratio = 1.38) to 8350.2 (mean S/P ratio = 3.19), with the virus being detected by qPCR in 80% (4/5) of these farms. Despite serological and molecular detection, no characteristic microscopic lesions of the disease were found to confirm an ILT diagnosis.

In seropositive farms with characteristic microscopic ILT lesions (7.1% - 3/42), the organs predominantly affected by histopathological changes were the proximal trachea and larynx, where multifocal mild to moderate lymphoplasmacytic tracheitis was observed, associated with syncytial cells containing basophilic intranuclear inclusion bodies in 7.1% (3/42) of the cases (Figure 6A). Similarly, multifocal mild to severe lymphoplasmacytic and fibrinous laryngitis was found, also associated with syncytial cells containing basophilic intranuclear inclusion bodies, in 7.1% (3/42) of the farms (Figures 6B and 6C). The same tracheitis pattern was observed in distal trachea pools in 4.7% (2/42) of the farms. In the lungs and nasal turbinates, multifocal moderate lymphoplasmacytic bronchitis and rhinitis were visualized, respectively, both associated with syncytial cells containing basophilic intranuclear inclusion bodies, each with one case (2.4%).

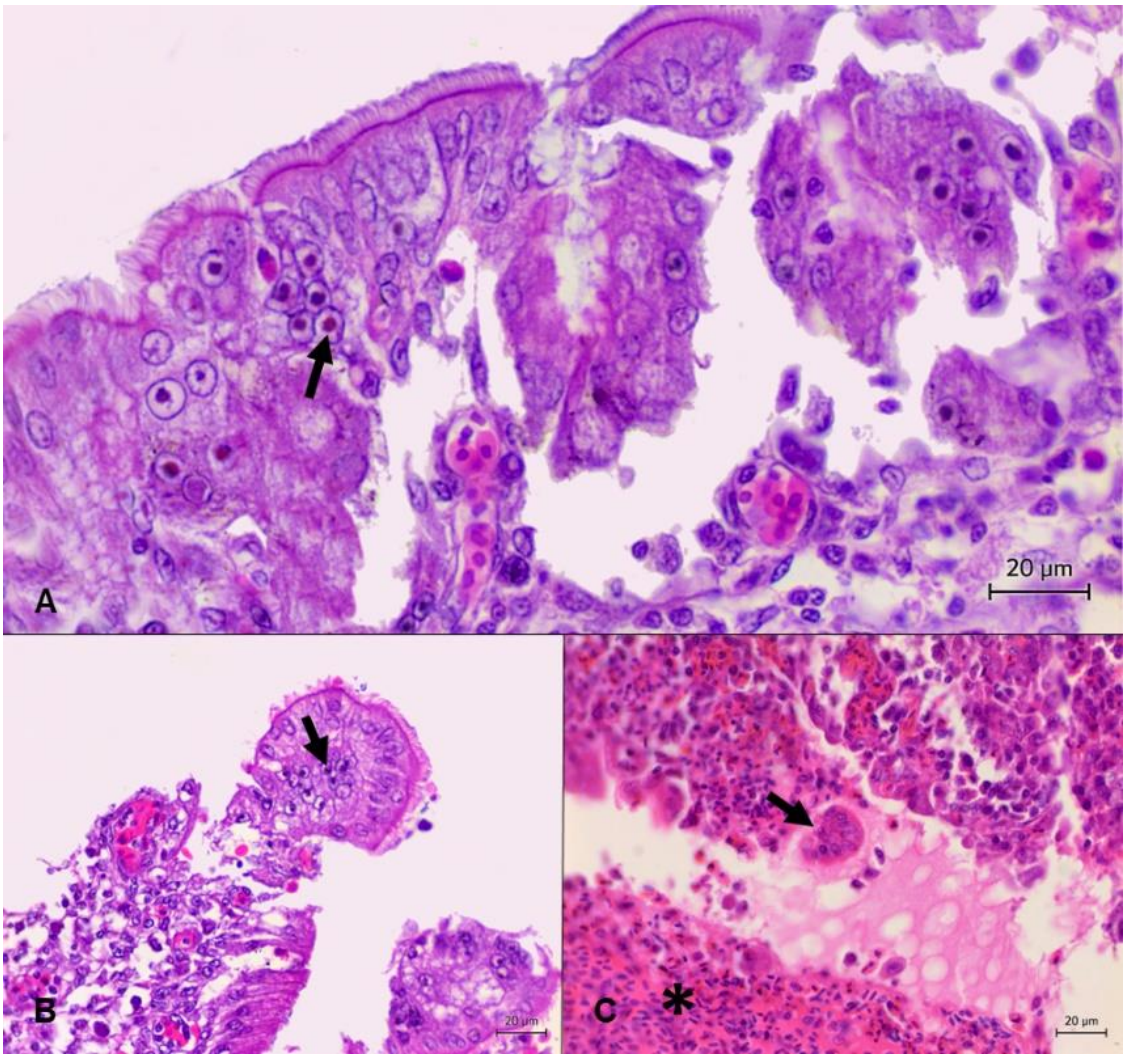

Figure 6. Infectious Laryngotracheitis in commercial layers from the Study 1 in the São Ludgero region from Santa Catarina state, Brazil. **A)** Trachea: Mild multifocal lymphoplasmacytic tracheitis with basophilic intranuclear inclusion bodies in epithelial cells (arrow). H&E, Obj: 40x. **B)** Larynx: Mild multifocal lymphoplasmacytic laryngitis with syncytial cells containing basophilic intranuclear inclusion bodies (arrow). H&E, Obj: 40x. **C)** Larynx: Severe diffuse lymphoplasmacytic laryngitis associated with syncytial cells (arrow), fibrinous exudate, and red blood cells in the lumen (asterisk). H&E, Obj: 40x.

STUDY 2

No clinical signs or macroscopic lesions were found in the sampled hens at the time of the survey. However, a significant proportion of seropositive properties exhibited some form of histopathological alteration in the respiratory tract, with varying intensities and distributions (90.6% - 29/32). Nevertheless, the observed changes were nonspecific and common to various infectious or non-infectious processes affecting the avian respiratory tract. Therefore, no classic ILT lesions were found.

EPIDEMIOLOGICAL SURVEY

STUDY 1

Among the 44 sampled farms, the average age of the housed chickens was 69.5 weeks, ranging from 6 to 286 weeks, with a median of 64.5 weeks and a standard deviation of 43.3 weeks. The number of hens per barn varied widely, with a mean density of 11,886.3 chickens (range: 280 to 56,300), a median of 6,830, and a standard deviation of 12,692.7. Regarding infrastructure, the number of barns per farm averaged 1.5, ranging from 1 to 5 barns, with a median of 1.0 and a sample standard deviation of 0.9.

The 38 farms consisted of 36 seropositive farms (94.74%) and two seronegative farms (5.26%). The sampled farms were predominantly located in the municipality of São Ludgero (52.63% - 20/38), followed by Braço do Norte (26.31% - 10/38), Orleans (10.53% - 4/38), and Tubarão (5.26% - 2/38). The municipalities of Urussanga and Pedras Grandes each had one farm sampled (2.63% - 1/38). In the municipality of Tubarão, the two sampled farms were the seronegative ones in the epidemiological survey (Figure 7A and 7B).

All poultry farmers declared that they exclusively acquired chickens of known origin to compose their flock, and the vaccination program in the region predominantly included immunization against Fowlpox, *Mycoplasma* sp., Infectious Bronchitis, Pneumovirus, Infectious Coryza, and *Salmonella* sp. For statistical interpretation, the independent variables were tested only for qPCR positivity, due to the low number of seronegative farms, and their univariate and multivariate analyses are presented in Table 8.

Table 8. Infectious Laryngotracheitis in commercial layers from the Study 1 in the São Ludgero County, from Santa Catarina state, Brazil. Odds ratios and p-values for univariate and multivariate analyses of epidemiological variables in layer farms and their association with qPCR positivity for the virus.

Characteristic		Group	Frequency	Univariate for +qPCR		Multivariate for +qPCR	
				p-value	OR (CI 95%)	*p*-value	OR (CI 95%)
Production system	Commercial layers	Yes	73.7% (28/38)	1.0	0.5 (0.05-5)	-	-
	Rearing + layers	Yes	23.7% (9/38)	1.0	1.7 (0.2-16.5)	-	-
	Only rearing	Yes	2.6% (1/38)	-	-	-	-
Layer housing	Collective cages	Yes	84.2% (32/38)	0.234	3.5 (0.5-25.7)	-	-
	Floor with litter	Yes	15.8% (6/38)	0.234	0.3 (0.04-2.1)	-	-
Barn type	Automated	Yes	57.9% (22/38)	0.682	1.4 (0.2-8.4)	-	-
	Californian	Yes	26.3% (10/38)	1.0	1.9 (0.2-19.1)	-	-
	In paddocks	Yes	15.8% (6/38)	0.234	0.3 (0.03-2.1)	-	-
Replacement hens	Chicks (1 day old)	No	60.5% (23/38)	**0.027***	11 (1.1-106.8)	0.997	-
	Reared hens (90 days)	Yes	71% (27/38)	**0.047***	7.1 (1.07-47.4)	0.998	-
	Reared hens (20 weeks)	Yes	10.5% (4/38)	0.513	0.5 (0.04-6)	-	-
History of diseases	Infectious bronchitis	Yes	23.7% (9/38)	0.3	-	-	-
	Infectious coryza	Yes	10.5% (4/38)	-	-	-	-
	Gumboro disease	Yes	2.6% (1/38)	-	-	-	-
	Mycoplasmosis	Yes	10.5% (4/38)	-	-	-	-
	Total	Yes	36.8% (14/38)	0.067	-	0.997	-
Management issues	Excessive heat	Yes	78.9% (30/38)	0.094	5.4 (0.8-34.8)	-	-
	Inability to purchase supplements	Yes	7.9% (3/38)	0.412	0.3 (0.02-4.4)	-	-
	Absence of problems	Yes	21.1% (8/38)	0.094	0.2 (0.03-1.2)	0.997	-
Proper waste disposal	Culling birds	No	5.3% (2/38)	0.294	0.1 (0.01-3)	-	-
	Manure	No	34.2% (13/38)	0.392	0.4 (0.07-2.6)	-	-
	Trash	No	7.9% (3/38)	0.412	0.3 (0.02-4.4)	-	-
Farm isolation	Fence	No	2.6% (1/38)	-	-	-	-
	Natural barrier	No	52.6% (20/38)	1.0	1 (0.2-6.5)	-	-
Biosecurity measures	Vehicle disinfection	No	5.3% (2/38)	0.294	0.1 (0.01-3)	-	-
	Third-party restriction	No	13.2% (5/38)	0.570	-	-	-
	Use of PPE by technicians	No	47.3% (18/38)	0.083	0.13 (0.01-1.3)	0.979	1 (0.2-46.2)
	Technicians' own equipment	No	18.4% (7/38)	1.0	1.1 (0.1-11.8)	-	-
	Hygiene and disinfection of tools	No	13.2% (5/38)	0.169	0.2 (0.03-1.6)	0.875	0.7 (0.2-29.8)
	Shower on entry/exit	No	78.9% (30/38)	0.3	-	-	-
	Insect control	No	26.3% (10/38)	1.0	1.9 (0.2-19.1)	-	-
	Treated water	No	13.2% (5/38)	1.0	0.7 (0.06-7.8)	-	-
Animal movement	Rodents	Yes	36.8% (14/38)	0.167	0.2 (0.03-1.4)	0.438	0.18 (0.002-14.1)
	Others (domestic or wild animals)	Yes	21.1% (8/38)	1.0	1.4 (0.14-14)	-	-

OR: odds ratio; CI 95%: 95% confidence interval; *: significant p-value (p<0.05); PPE: personal protective equipment.

STUDY 2

The sampled farms housed laying hens with an average age of 75.1 weeks (range: 15 to 208 weeks), a median of 62.0 weeks, and a standard deviation of 39.9 weeks. In terms of infrastructure, farms had an average of 1.4 barns, with most facilities consisting of a single barn (77.5% - 38/49), and a maximum of six barns per farm (2.1% - 1/49). The median number of barns per farm was 1.0, with a standard deviation of 1.04. The average layer density per barn was 8,519.6 birds, ranging from as few as 16 to as many as 150,051 laying hens, with a median of 950 and a standard deviation of 23,142. The 49 farms sampled were spread across 42 municipalities, located in all mesoregions of the state of Santa Catarina (Figure 3B). In most municipalities, only one farm was sampled (83.3% - 35/42), while in six municipalities, two farms were sampled (14.3%) and in one municipality, three farms were sampled (2.4%).

The vast majority of poultry farmers had stocking densities below 1,000 hens per barn (53.1% - 26/49), followed by farms with between 1,000 and 5,000 hens (22.4% - 11/49) and 5,001 to 12,000 hens (12.2% - 6/49). The remaining six farms had stocking densities above 12,000 hens per barn, reaching up to 150,000 (12.2% - 6/49). Similar to Study 1, all poultry farmers reported exclusively acquiring hens from known sources to compose their flocks. The main vaccination protocols used in the state were also the same as those in Study 1. The questionnaire variables associated with serology (ELISA) and qPCR positivity in the univariate and multivariate analyses are presented in Table 9.

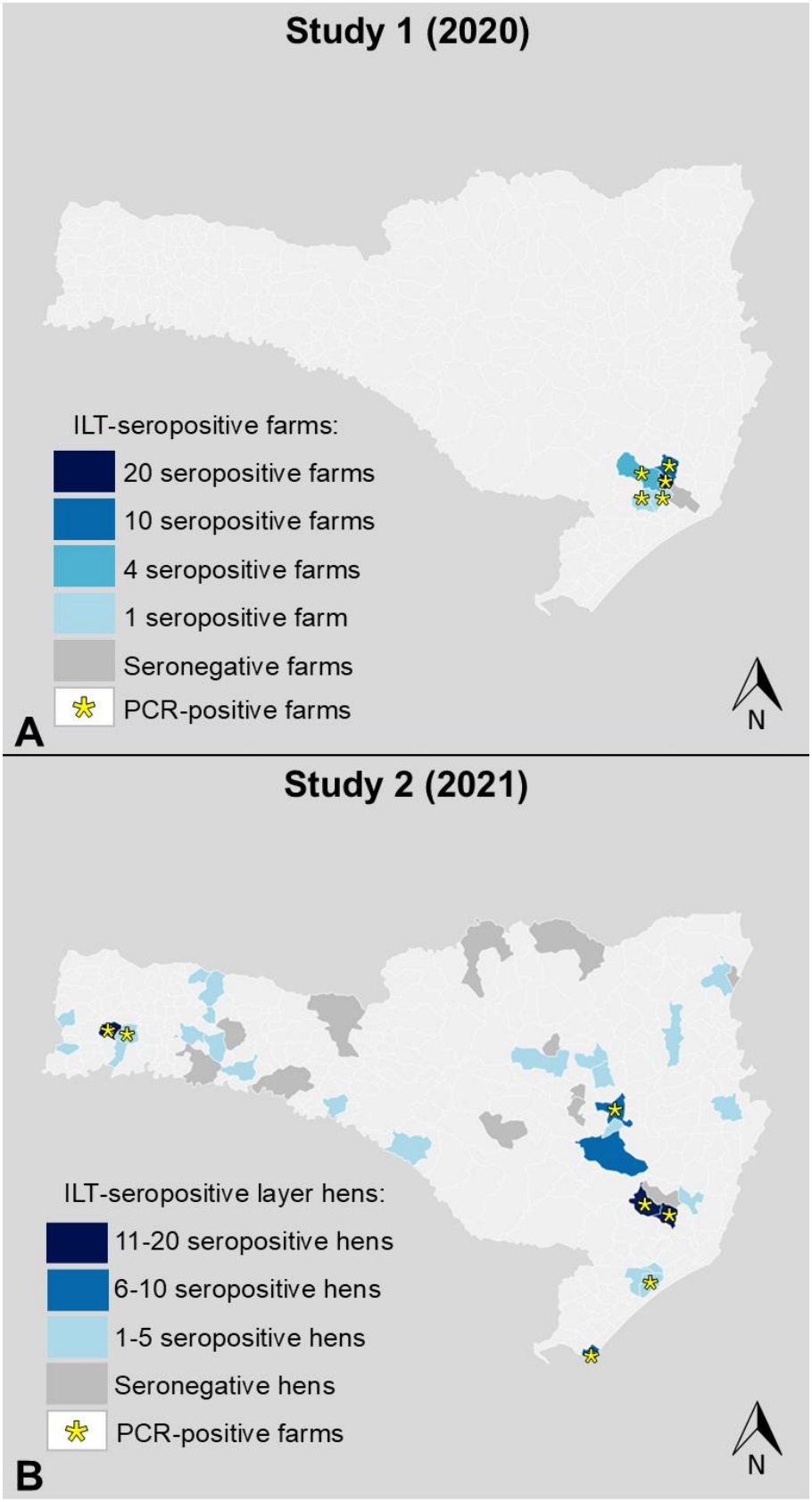

Figure 7. Frequency of seropositive commercial layer and pullet-rearing farms for Infectious Laryngotracheitis virus by municipality. Territory of the state of Santa Catarina, southern Brazil. **A)** Emphasis on the municipalities sampled in Study 1. **B)** Emphasis on the municipalities sampled in Study 2.

Table 9. Infectious Laryngotracheitis in commercial layers from the Study 2 in the Santa Catarina state, Brazil. Odds ratios and p-values for univariate and multivariate analyses of epidemiological variables in layer farms and their association with ELISA and qPCR positivity for the virus.

Characteristic		Group	Frequency	Univariate for +ELISA		Multivariate for +ELISA		Univariate for +qPCR		Multivariate for +qPCR	
				p-value	OR (CI 95%)	*p*-value	OR (CI 95%)	*p*-value	OR (CI 95%)	*p*-value	OR (CI 95%)
Production system	Commercial layers	Yes	83.7% (41/49)	1.0	1.16 (0.24-5.56)	-	-	1.0	1.2 (0.12-11.6)	-	-
	Rearing + Layers	Yes	16.3% (8/49)	1.0	0.86 (0.18-4.15)	-	-	1.0	0.83 (0.09-8.1)	-	-
	Only rearing	Yes	0	-	-	-	-	-	-	-	-
Layer housing	Collective cages	Yes	32.6% (16/49)	0.354	0.56 (0.16-1.92)	-	-	0.4	0.3 (0.04-2.73)	-	-
	Floor with litter	Yes	67.3% (33/49)	0.354	1.79 (0.52-6.15)	-	-	0.4	3.3 (0.37-30.35)	-	-
Barn type	Automated	Yes	8.2% (4/49)	0.114	0.15 (0.01-1.58)	0.312	0.21 (0.01-4.36)	-	-	-	-
	Californian	Yes	28.6% (14/49)	1.0	0.94 (0.26-3.44)	-	-	1.0	1.0 (0.17-5.9)	-	-
	In paddocks	Yes	63.3% (31/49)	0.275	1.96 (0.58-6.56)	-	-	1.0	1.54 (0.27-8.9)	-	-
Replacement hens	Chicks (1 day old)	No	55.1% (27/49)	**<0.001***	14 (3.18-61.59)	-	-	0.436	2.2 (0.4-13.05)	-	-
	Reared hens (90 dias)	Yes	51% (25/49)	**<0.001***	10.3 (2.4-43.9)	**0.032***	13.58 (1.2-147.1)	0.417	2.75 (0.5-15.8)	-	-
	Reared hens (20 weeks)	Yes	4.1% (2/49)	0.537	-	-	-	-	-	-	-
History of diseases	Infectious bronchitis	Yes	10.2% (5/49)	0.326	0.31 (0.05-2.1)	-	-	0.554	1.5 (0.15-16.67)	-	-
	Infectious coryza	Yes	12.2% (6/49)	0.08	-	-	-	**0.002***	26.7 (3.4-209.9)	0.999	-
	Gumboro disease	Yes	0	-	-	-	-	-	-	-	-
	Mycoplasmosis	Yes	0	-	-	-	-	-	-	-	-
	Total	Yes	20.4% (10/49)	1.0	1.31 (0.3-5.9)	-	-	**0.025***	8.0 (1.42-45.1)	0.999	-
Management issues	Excessive heat	Yes	24.5% (12/49)	0.729	0.67 (0.18-2.6)	-	-	1.0	1.28 (0.21-7.64)	-	-
	Inability to purchase supplements	Yes	6.12% (3/49)	0.542	-	-	-	**0.05***	16.4 (1.2-215.1)	0.119	14.7 (0.5-429.7)
	Absence of problems	Yes	69.4% (34/49)	0.894	0.92 (0.25-3.31)	-	-	0.179	0.27 (0.05-1.4)	-	-
Proper waste disposal	Culling birds	No	22.4% (11/49)	0.071	7.2 (0.8-62.7)	0.654	2.23 (0.06-74.13)	1.0	0.5 (0.05-4.97)	-	-
	Manure	No	53.1% (26/49)	**0.016***	4.5 (1.28-16.36)	0.99	0.98 (0.1-9.61)	0.424	2.5 (0.43-14.35)	-	-
	Trash	No	10.2% (44/49)	0.149	-	0.99	-	-	-	-	-
Farm isolation	Fence	No	18.3% (9/49)	0.136	5.3 (0.6-46.8)	0.414	6.3 (0.07-537.12)	0.598	2 (0.32-12.46)	-	-
	Natural barrier	No	51.1% (25/49)	0.315	1.8 (0.55-6.1)	-	-	1.0	1.3 (0.26-6.7)	-	-
Biosecurity measures	Vehicle disinfection	No	55.1% (27/49)	0.153	2.3 (0.71-7.92)	0.957	0.94 (0.11-7.81)	0.685	0.5 (0.11-2.83)	-	-
	Third-party restriction	No	30.6% (15/49)	**0.037***	5.1 (1-26.33)	0.284	3.61 (0.3-37.92)	0.66	1.8 (0.36-9.66)	-	-
	Use of PPE by technicians	No	59.2% (29/49)	**0.013***	4.6 (1.33-16.48)	0.204	0.12 (0.005-3.1)	1.0	0.9 (0.18-4.57)	-	-
	Technicians' own equipment	No	75.5% (37/49)	0.729	1.5 (0.4-5.67)	-	-	0.665	2.1 (0.23-19.72)	-	-
	Hygiene and disinfection of tools	No	36.7% (18/49)	**0.008***	7.5 (1.47-38.28)	0.232	6.9 (0.29-163.3)	0.398	2.66 (0.52-13.6)	-	-
	Shower on entry/exit	No	87.7% (43/49)	1.0	0.9 (0.15-5.7)	-	-	1.0	0.8 (0.08-8.2)	-	-
	Insect control	No	28.6% (14/49)	**0.018***	10.9 (1.28-93.03)	0.2	8.8 (0.31-251.1)	0.656	0.37 (0.04-3.41)	-	-
	Treated water	No	24.5% (12/49)	0.175	3.4 (0.65-17.82)	0.371	0.24 (0.01-5.35)	1.0	1.3 (0.21-7.64)	-	-
Animal movement	Rodents	Yes	26.5% (13/49)	1.0	1.27 (0.33-4.9)	-	-	0.658	0.42 (0.04-3.9)	-	-
	Others (domestic or wild animals)	Yes	32.6% (16/49)	**0.023***	5.83 (1.14-29.8)	0.571	2 (0.18-22.25)	0.668	1.67 (0.32-8.57)	-	-

OR: odds ratio; CI 95%: 95% confidence interval; *: significant p-value (p<0.05); PPE: personal protective equipment.

DISCUSSION

As observed in previous epidemiological surveys of the disease in Brazil (TOCANTINS, 2014; HERGOT et al., 2021), the seropositivity found in the São Ludgero County in Study 1 was alarmingly high, particularly for a disease newly diagnosed in the local poultry flocks. In the State of Minas Gerais, a seroprevalence of anti-ILT antibodies was reported in 55.6% of layer hens, with observed titers ranging from 1,071 (S/P ratio = 0.5) to 10,547 (S/P ratio = 3.9) (SANTOS et al., 2022), which were similar to the titers observed in Study 1. Likewise, in Minas Gerais, all farms sampled in a survey conducted after outbreaks in the Mantiqueira Highlands region were found to be seropositive (HERGOT et al., 2021). In the Brazilian Federal District, among non-technified flocks, a seroprevalence of 48.26% (111/230) for ILT virus was found, with 82% of the sampled properties testing positive (TOCANTINS, 2014).

The first ILT outbreak in the São Ludgero region was diagnosed in September 2020, and the epidemiological survey from Study 1 was conducted in October and November of the same year. The serological results confirm the circulation and exposure to the virus within the region poultry flocks, aligning with the expected seroconversion period. As ILT was an exotic disease to the poultry industry in Santa Catarina, recombinant vaccines were not permitted in the state until the first cases emerged (SANTA CATARINA, 2020). Furthermore, recombinant vaccines do not elicit a strong immune response that results in detectable titers through commercial ELISA kits (COPPO et al., 2013). These factors related to recombinant vaccines reinforce that the presence of anti-ILT antibodies in the São Ludgero region is associated with the circulation of virulent field strains or vaccine-derived strains from live attenuated vaccines that underwent virulence reversion (GARCÍA et al., 2013).

ILT virus is endemic worldwide and affects chickens housed in various production systems, particularly in areas with high poultry farm density. It has been detected in North America, Europe, Asia, Oceania, Africa, and South America (HIDALGO, 2003; PARRA et al., 2016). When compared to other countries, the seroprevalence found in layer hens in Studies 1 and 2 from Santa Catarina, Brazil, was higher than in other production systems in South America, such as in Uruguay, where a seropositivity of 31.5% was found in unvaccinated broilers (TRENCHI et al., 2012). This high prevalence persists when the present results are compared to those from other continents, such as in the United States, where a study reported a 45% ILT

seropositivity rate in extensively raised birds (DERKSEN et al., 2018), in Turkey, where 64% of sampled commercial poultry farms were seropositive (ARAS et al., 2018), in Ethiopia, where a study showed that 13.3% of commercial flocks and 34.4% of backyard birds tested positive for ILT (TESFAYE et al., 2019), and in Belgium, where 30% of sera from recreationally raised poultry tested positive for ILT (HAESENDONCK et al., 2014).

The seropositivity for ILT in layer hens sampled in the state of Santa Catarina in Study 2 was lower than that found in the São Ludgero region in Study 1, and this difference may be attributed to the broader geographic area covered in Study 2 and the magnitude of the outbreak reported in São Ludgero, the first municipality to report the occurrence of the disease in the state. This outbreak likely facilitated the spread of the virus, consequently increasing ILT exposure in layer hens from neighboring municipalities. Viral exposure was detected in all mesoregions of Santa Catarina, and since this is considered a newly introduced disease in the region, the statewide epidemiological survey is crucial to guide containment measures that should be implemented by the official veterinary service to prevent severe outbreaks and associated economic losses (SANTA CATARINA, 2020).

Also in Study 2, the average antibody titer found in the sampled hens was considered lower compared to the titer of birds from the São Ludgero region. This difference can be explained by the chronology of viral exposure, as studies show a peak in antibody concentration at 21 dpi (days post-infection), with detection starting around 5 to 7 dpi and levels gradually declining after a few months (JORDAN et al., 1981). While Study 1 was carried out between October and November 2020, the broad survey in Santa Catarina was conducted from February to March 2021, covering a total span of five months from the beginning of the surveys in October until their conclusion in March.

The seropositive farms with the highest antibody titers in Study 2 were located in the southern part of the state, specifically in the municipalities of Braço do Norte (6,710.55; average S/P ratio = 2.638), and Grão Pará (4,916.31; average S/P ratio = 2.125), suggesting a consistent viral exposure source in the region, even after the first study. The straight-line distances to São Ludgero are 5.5 km and 25 km, respectively, indicating geographic proximity. The southern region of the state accounts for the majority of egg production, with nearly 3 million housed layer hens (IBGE, 2024). However, poultry farmers in this region tend to be independent, with a predominance

of small to medium-sized farms, which can pose challenges in implementing biosecurity measures to prevent disease transmission (GEWEHR et al., 2010; CARDOZO et al., 2015; MACIEL et al., 2016), a situation known to favor the spread of ILT (GARCÍA et al., 2013).

In Brazil, no epidemiological studies covering the entire territory of a state had been conducted for ILT until now. Instead, previous studies focused on specific regions that experienced ILT outbreaks or on farms at higher risk for ILT detection, such as in the Petrópolis region of Rio de Janeiro, where chickens around 10 months old suffered mortality and a drop in egg production (ARAÚJO et al., 1982). This was followed by the Bastos region in São Paulo, which experienced a severe ILT outbreak with high mortality in commercial layers (CHACÓN et al., 2007), and more recently in the Mantiqueira Highlands region of Minas Gerais, where morbidity reached up to 90% in an area housing approximately 8 million layer hens (PREIS et al., 2013; HERGOT et al., 2021; SANTOS et al., 2022). Poultry farms in some areas of Rio Grande do Sul have also been found to be seropositive for the virus, although no outbreaks have been detected (VARGAS, 2000).

Clinical signs generally appear around 7dpi under natural conditions; however, in severe cases, chickens may die as early as 2 to 3dpi (JORDAN, 1966; GUY et al., 2013). In non-fatal cases, recovery occurs between 10 and 14dpi, although signs can sometimes persist for several weeks (JORDAN, 1966; OU et al., 2012). The occurrence of mild clinical signs during Study 1, combined with the absence of clinical signs during Study 2, suggests that there was no acute disease at the time, but rather viral exposure followed by clinical recovery. This conclusion is further supported by the mild macroscopic lesions observed during Study 1 and the absence of macroscopic lesions in the necropsied layer hens from Study 2. When lesions were present in early stages, they included varying degrees of conjunctivitis, rhinitis, laryngitis, and tracheitis with mucus accumulation in the lumen, progressing to severe inflammation with necrosis, hemorrhage, and diphtheritic plaques in the most severe cases (HIDALGO, 2003; PARRA et al., 2016).

After the clinical phase of the disease, the virus stops replicating in tissues and, via retrograde axonal transport, reaches the trigeminal ganglion, where it remains latent, as with other *Herpesviruses* (GAMA & CANAL, 2009). During this latency period, there is no viral transmission or clinical manifestation since no viral proteins are expressed, keeping the virus inactive (BRANDÃO & CHACÓN, 2009). This latent

phase may also explain the absence of clinical signs during the epidemiological survey from Study 2, as well as the mild lesions observed in Study 1. Once infected, the chicken remains a lifelong carrier, with periodic reactivations leading to viral replication in respiratory tissues, especially after stress or immunosuppression (DUFOUR-ZAVALA, 2008). Therefore, future clinical signs of ILT could appear in the sampled farms, even though they were clinically healthy during the survey.

When clinical signs of ILT do appear, they are often nonspecific and can resemble those of other infectious respiratory diseases such as avian influenza, Newcastle disease, infectious bronchitis, infectious coryza, and mycoplasmosis (PARRA et al., 2016). This highlights the importance of combining multiple laboratory diagnostic methods (GARCÍA et al., 2013). Histopathology is considered the most reliable method for confirming ILT, especially between 1 and 5dpi, when syncytial cells and intranuclear inclusion bodies are observed in key sites of viral replication (predominantly the larynx, trachea, nasal turbinates, and conjunctiva) — these are classic diagnostic features of the disease (RODRÍGUEZ-ÁVILA et al., 2008; GARCÍA et al., 2013).

Due to the viral latency process and the varying clinical manifestations, ranging from mild to severe, molecular detection of viral DNA or positive titers alone are insufficient to confirm the disease. Histopathological analysis is considered the primary method for diagnosing ILT and differentiating it from carrier animals (PREIS et al., 2014). The lesions observed in the Study 1, which included syncytial cells and intranuclear inclusion bodies, are considered classic for ILT (RODRÍGUEZ-ÁVILA et al., 2008). When present, these lesions confirm the disease; however, they are not always visible due to their short period of occurrence, typically appearing between 1 to 5dpi and disappearing shortly thereafter due to necrosis and epithelial desquamation, becoming part of the exudate in the airways (SELLERS et al., 2004; VILLANUEVA, 2005; GARCÍA et al., 2013; GUY et al., 2013).

Histological changes most frequently occur in the larynx and trachea due to the higher intensity of viral replication in these tissues (BRANDÃO & CHACÓN, 2009), as well as in the nasal turbinates, bronchi, and conjunctiva (PREIS et al., 2014). In addition to syncytial cells and inclusion bodies, changes such as necrosis and hemorrhage of the respiratory epithelium, along with exudates composed of cellular debris, fibrin, heterophils, and free red blood cells in the lumen, can also be observed (TIMURKAAN et al., 2003). The frequency of classic lesions in the Study 1 was lower

than those seen in outbreaks elsewhere, such as in the United States, where 83% of the tissues examined exhibited characteristic ILT lesions, with syncytial cells present in nasal turbinates, conjunctiva, larynx, trachea, lungs, and air sacs (CRESPO et al., 2007). It is known that the severity of histological lesions varies according to the stage of the disease (GARCÍA et al., 2013), and the lower occurrence of classic ILT lesions in the São Ludgero region can be explained by the subacute to chronic nature of the disease at the time of the epidemiological survey, with epithelial desquamation and the persistence of nonspecific changes (PREIS et al., 2014). The timeline of the disease, as well as the progression of microscopic changes such as epithelial desquamation, are key factors that help explain the absence of classic lesions during Study 2. In cases of nonspecific changes or the absence of histological lesions, as observed in Study 2, it is important to combine histopathology with molecular analyses, such as qPCR, due to its high specificity and sensitivity in detecting the ILT virus (BELTRÃO et al., 2003; PARRA et al., 2016; CALLISON et al., 2017).

The epidemiological survey from Study 1, in addition to the high seropositivity, also demonstrated a high frequency of viral infection through molecular detection by qPCR. The use of pooled samples from various organs for qPCR detection, including the larynx, medial trachea, conjunctiva, and trigeminal ganglion, has been previously applied during past outbreaks in Brazil (PREIS et al., 2013). In contrast, field studies in California, United States, have predominantly used molecular detection from the larynx and proximal trachea (CRESPO et al., 2007). In ILT outbreaks in Egypt, lung samples were also used alongside larynx and trachea for molecular analysis (BAYOUMI et al., 2020). The selection of these tissues for qPCR is based on the intense viral replication that occurs in the epithelium of the larynx and trachea (BRANDÃO & CHACÓN, 2009), which showed the highest positivity for ILTV DNA detection in these tissues in the present study as well. Regarding viral tropism, after experimental inoculation of ILT virus in birds in China, a high viral load of GaHV-1 was detected by qPCR in the larynx and trachea, as well as in the lungs and cecum. Although tissues like the thymus, esophagus, pancreas, and kidneys also tested positive, the viral loads were lower (WANG et al., 2013), confirming that the virus replicates in multiple organs and is not restricted to the respiratory tract. Its DNA can be amplified from a wide range of samples (TRAN et al., 2021).

The two farms from Study 1 that showed positivity exclusively in trigeminal ganglion pools may indicate the virus's latent state during the epidemiological survey,

raising concerns about potential future reactivations following stress or immunosuppression. In Study 2, among all the farms that tested positive by qPCR, the organ pool with the highest positivity rate was the trachea, followed by the larynx and conjunctiva. The trachea can remain positive for the detection of GaHV-1 DNA even in chickens that do not exhibit clinical signs of ILT, suggesting a small latency rate in ganglia located within the trachea (WILLIAMS et al., 1992; BELTRÃO et al., 2003), as indicated in this study. Previous studies have not demonstrated the same sequence of positivity intensity in the collected organ pools, typically showing a predominance of the conjunctiva, followed by the larynx/trachea, nasal turbinates, and lungs (OU & GIAMBRONE, 2012; PREIS et al., 2014). However, in Studies 1 and 2, the conjunctiva and larynx exhibited positivity rates very close to those of the tracheal pools, indicating a high distribution of the virus across different tissues. In Study 2, two farms tested negative for virus detection in the trigeminal ganglion. This observation may suggest that the infection in these two farms was still in the early stages, particularly between 3 to 5dpi, when the viral load is still low for detection by qPCR and the distribution of viral DNA among tissues remains limited (WANG et al., 2013).

In the epidemiological survey from Studies 1 and 2, the age range among the different sampled aviaries was quite broad, demonstrating that, as described in the ILT literature, chickens of any age can be susceptible to infection, and the importance of including a wide age range in the study lies in the characteristic of ILT, which can affect susceptible chickens at any point in their lives, typically after four weeks of age. (DUFOUR-ZAVALA et al., 2008). The higher stocking density of birds per shed, as observed in some of the aviaries sampled in Study 1, is also an important factor that may contribute to the occurrence of the disease. Sheds with high population density, especially when different ages are mixed, are highly prone to virus spread (GOWTHAMAN et al., 2020), while in Study 2 the vast majority of poultry farmers operated with fewer than 1,000 birds per shed, indicating a predominance of small-scale operations. This type of farming is generally independent, with no ties to the agro-industry, and is common in the state of Santa Catarina (GEWEHR et al., 2010). Such operations may exhibit vulnerabilities and characteristics related to biosecurity failures, which are critical for the emergence of ILT outbreaks in farms that can quickly escalate into larger outbreaks (DUFOUR-ZAVALA, 2008).

When evaluating the results of the univariate analysis, the type of hen selected for the replacement of flocks, consisting of chickens reared for approximately 90 days,

showed a significant association with the detection of the virus in both studies. Although infection by GaHV-1 can occur at any stage of the chicken's life, ILT outbreaks primarily occur in chickens from four weeks of age, with reports of younger chickens as early as three weeks old (DUFOUR-ZAVALA et al., 2008), increasing the likelihood that chickens at 90 days have already been exposed to the virus and, conversely, in older birds, susceptibility to the disease decreases (FAHEY et al., 1983). The study suggests, therefore, that birds at 90 days represent an intermediate point of vulnerability to seroconversion and molecular detection for ILT among the three replacement hen categories, being more susceptible than 1-day-old chicks or older chickens (close to the start of production at 120 days). The finding that replenishing the flock with birds at 90 days of age was associated with seropositivity in Study 2, and was further supported by multivariate analysis, where this variable was the only one that demonstrated significance, indicating 13.5 times higher odds of seropositivity for LTI compared to the other categories of replacement birds.

This finding also reduces the chances of day-old chicks acting as carriers and transmitters of the virus to flocks, as GaHV-1 has not been shown to be transmitted vertically or through the egg (GOWTHAMAN et al., 2020). This characteristic was also observed in both studies during the univariate analysis, with farms that do not replenish their flocks with day-old chicks having greater chances of being qPCR positive for ILT in Study 1 and seropositive for ILT in Study 2.

Regarding respiratory diseases previously reported by producers, infectious coryza was the most frequently mentioned in Study 2. This bacterial disease affects the upper respiratory tract of chickens and, like ILT, is associated with immunosuppression, stress, and management errors. It can be a significant co-infection in cases of infectious bronchitis, mycoplasmosis, pasteurellosis, and ILT, increasing the mortality rates associated with these pathogens (BLACKALL & VARGAS, 2020). In the univariate analysis, it emerged as an important factor linked to ILT occurrence in Study 2. Moreover, reports of any previous diseases increased the likelihood of molecular ILT detection in the univariate analysis, underscoring their association with immunosuppression, which may be triggered by certain diseases.

Climatic factors, such as excessive heat, listed as the main challenge in farm management in Study 1, are important triggers for ILT outbreaks, particularly by reactivating latent infections following sudden temperature changes, however, these factors were not statistically significant in this epidemiological survey. Excessive heat

leads to thermal discomfort, stress, and immunosuppression in chickens (DUFOUR-ZAVALA et al., 2008). As a result, ILT outbreaks in higher temperatures tend to be more severe (BERCHIERI, 2004). This factor has also been previously reported as a key cause for ILT outbreaks in the Bastos region of São Paulo, Brazil (BUCHALA et al., 2011). In Study 2, although the most commonly reported issue was the absence of management problems, reflecting an improvement in animal welfare and consequently reducing episodes of immunosuppression linked to respiratory diseases like ILT (DUFOUR-ZAVALA, 2008), there was a significant association between the factor "inability to purchase supplements" and the detection of ILT by qPCR in univariate analysis. This may reflect financial difficulties faced by independent producers, preventing them from acquiring not only supplements but also other important factors necessary for the flock's welfare.

The improper disposal of manure on the farms from Study 2 was a significant factor in the univariate analysis. Among the reported types of improper disposal was the direct application of poultry manure on the farm itself, which has previously been associated with virus transmission when the material is contaminated and not properly treated (DUFOUR-ZAVALA, 2008). More recently, however, it has been demonstrated that although high loads of ILTV DNA can be detected in chicken excreta, this DNA is not infectious and is not associated with virus transmission, suggesting possible inactivation of the virus in the chicken’s gastrointestinal tract (YEGORAW et al., 2021). Various biosecurity failures were associated with ILT seropositivity in Study 2 during the univariate analysis, such as the failure to restrict the entry of third parties into the farm. This practice was identified as a critical biosecurity lapse, as visitors can carry viral particles from one shed to another or from one farm to another via their clothing, footwear, and/or transport vehicles (VOLKOVA et al., 2012). The absence of the practice of using PPE by technicians visiting farms was another significant factor, as clothing acts as fomites in the indirect transmission of the virus to susceptible birds, especially after individuals come into contact with waste and/or contaminated biological materials (DUFOUR-ZAVALA, 2008). The habit of not using protective clothing and footwear when visiting poultry houses has already shown a significant association with ILT seropositivity in other parts of the world, confirming that in poultry farm routines, clothing is a critical vehicle for virus transmission (BIRHAN et al., 2022).

GaHV-1 can be inactivated on equipment and in the environment after proper cleaning followed by the use of certain disinfectants, particularly those based on

sodium hypochlorite, quaternary ammonium, phenolic compounds, and iodophores (GARCÍA et al., 2013). This explains the association between the lack of cleaning and disinfection practices of utensils after use and seropositivity for ILT in the univariate analysis. Previous studies have also identified the absence of poultry house disinfection as a risk factor for ILT virus seropositivity (BIRHAN et al., 2022). The difficulty in insect control was a common sanitary challenge reported in Study 2. Although previous studies did not show an association between the effectiveness of fly control and the introduction of the ILT virus on the property (VOLKOVA et al., 2012), it is known that insects, such as beetles, in both their adult and larval stages, can carry viral particles and act as significant mechanical vectors for this virus, and beetles have also had GaHV-1 detected through PCR (OU et al., 2012).

The circulation of domestic or wild animals on the farm increased the chances of ILT seropositivity in the univariate analysis from Study 2. Dogs, due to their carnivorous habits, may feed on carcasses of contaminated birds, transporting them from one poultry house to another or carrying viral particles, thereby facilitating the spread of the agent (DUFOUR-ZAVALA, 2008). This observation contrasts with previous studies that did not find a significant association between ILT seropositivity and the presence of dogs and cats on the premises of farms (VOLKOVA et al., 2012). Study 1 could not perform univariate analysis for serology due to the high rates of seropositivity, which led to a limited number of negative controls among the sampled farms. There was also a high number of qPCR-positive farms, which resulted in limited univariate analysis results for qPCR in Study 1. Due to the same reason, it was not possible to establish statistically significant logistic regression models in the multivariate analysis of Study 1.

Numerous factors were associated with seropositivity to ILT in the univariate analysis from Study 2, however, while useful for an initial screening of potential associations, does not account for the interdependence between variables. Therefore, incorporating multiple factors into the multivariate model allows for the identification of variables that truly influence the ILT outcome, preventing biased interpretations (GIMENO & SOUZA, 1995). Accordingly, the findings of this study highlight the relevance of the only factor that remained significant after statistical adjustment, the restocking of the flock with 90-day-old chicks, suggesting its independent role in the epidemiological dynamics of ILT seropositivity.

CONCLUSION

This study revealed a high level of exposure to the virus among layer hens in the São Ludgero County, with widespread dissemination in the area confirmed by qPCR. Although the disease caused histological lesions only in São Ludgero region in 2020, the virus was detected across the entire state of Santa Catarina in 2021, demonstrating rapid geographic spread. The statistical findings underscore the critical importance of implementing robust biosecurity measures in commercial layer farms throughout the state, with particular attention to the type of replacement chickens introduced in the flocks, with the introduction of 90-day-old chickens significantly increasing the likelihood of seropositivity for ILT, with an odds ratio of 13.5.

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6. CONSIDERAÇÕES FINAIS

As investigações conduzidas permitiram a caracterização do primeiro surto de laringotraqueíte infecciosa (LTI) no estado de Santa Catarina, evidenciando a presença do vírus em uma região de alta densidade de granjas de postura. O surto foi caracterizado por sinais clínicos leves a moderados, acompanhados de lesões microscópicas clássicas da doença. Além disso, este estudo revelou um elevado nível de exposição ao vírus entre as galinhas poedeiras no Bolsão de São Ludgero, com ampla disseminação confirmada por ELISA e qPCR. Embora as lesões histológicas tenham sido observadas apenas na região de São Ludgero em 2020, a detecção viral em todas as regiões do estado de Santa Catarina em 2021 demonstra uma rápida expansão geográfica do vírus.

Os achados estatísticos ressaltam a importância da implementação de medidas robustas de biosseguridade em granjas comerciais de postura em todo o estado, com atenção especial ao tipo de aves introduzidas nos lotes. A introdução de aves com 90 dias de idade aumentou em 13,5 vezes a probabilidade de soropositividade para LTI.

Por meio da vigilância ativa realizada entre 2020 e 2021, também foi possível realizar o sequenciamento parcial do gene ICP4, identificando estirpes do genótipo VI, com origem de campo, mas evolutivamente muito próximas das estirpes derivadas da vacina CEO. A identidade de 100% com as estirpes VFAR-043 e J2 reforça a hipótese de introdução do vírus a partir do Peru e/ou dos Estados Unidos. O sequenciamento parcial do gene TK demonstrou a ausência da mutação T252M, sugerindo baixa virulência, no entanto, necessitando de avaliações genômicas mais abrangentes para melhor compreensão da virulência das estirpes identificadas. Nesse sentido, estudos de sequenciamento completo do genoma são recomendados para elucidar a dinâmica de introdução e dispersão dessas variantes em Santa Catarina.

Dessa forma, os resultados apresentados destacam a necessidade de ações efetivas de vigilância epidemiológica, controle sanitário e aprimoramento das estratégias de biosseguridade para mitigar os impactos da LTI na avicultura catarinense. Estudos adicionais são essenciais para aprofundar o entendimento sobre a dinâmica de transmissão e persistência do vírus na região, contribuindo para a formulação de políticas públicas e medidas preventivas mais eficazes.

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APÊNDICES

APÊNDICE 1

TERMO DE LIVRE CONSENTIMENTO

Declaração de Consentimento: Fui devidamente esclarecido(a) sobre todos os procedimentos deste estudo, seus riscos e benefícios ao(s) animal(is) pelo(s) qual(is) sou responsável. Fui também informado que posso retirar meu(s) animal(is) do estudo

a qualquer momento. Ao assinar este Termo de Consentimento, declaro que autorizo a participação do(s) meu(s) animal(is), identificado(s) / rebanho a seguir, neste projeto.

Este documento será assinado em duas vias, sendo que uma via ficará comigo e a outra com o pesquisador.

Lages – SC, _____ / _____/ _____

_____	_____
Nome do Responsável / RG	Nome do Pesquisador / RG
Assinatura do Responsável	Assinatura do Pesquisador

Identificação do animal (repetir tantas vezes quantos forem os animais):

Granja: _____ Número de identificação: _____

Espécie: _____ Raça: _____

APÊNDICE 2

QUESTIONÁRIO

Granja com suspeita de Laringotraqueíte?	
Sim	
Não	

1. Sistema de produção:	
Postura comercial	
Recria de Postura comercial - até 20 semanas	
Ambos na mesma propriedade	
2. As aves são criadas:	
Em gaiolas coletivas com Nº adequado de galinhas por gaiola	
Em gaiolas coletivas com Nº acima adequado de galinhas por gaiola	
Sobre piso com cama	
3. Nas criações em galpões, estes são do tipo:	
Californiano tradicional	
Californiano modificado	
Automatizado e/ou galpão climatizado	
Em piquetes	
Outros	
4. Compra aves de origem conhecida?	
Sim	
Não (de acordo com oferta de mercado)	
Nem sempre se compra de origem conhecida	
5. A água de bebida das aves é retirada de	
Poço comum	
Poço artesiano	
Cacimba	
Lago	
Riacho	
Nascente	
Outros	
6. A água de bebida das aves é clorada?	
Sim.	
Não	
7. A água de bebida das aves é armazenada?	
Em caixas de água com tampa	
Em caixas de água sem tampa	
Não armazena	
8. O maior desafio sanitário é	
Doença respiratória	
Diarreia	
Moscas e roedores	
Mortalidade por salmonela	
Doença respiratória, moscas e roedores	

Diarreia, moscas e roedores	
Todas as acima citadas	
Não possui desafio sanitário	
9. Quais são as doenças respiratórias mais frequentes? (pode marcar mais que uma opção)	
Bronquite	
Newcastle	
Coriza	
Gumboro	
Micoplasmose	
Laringotraqueíte	
Não tinha doença respiratória, mas só desta vez	
Nunca teve doença respiratória	
10. Neste ano, quais foram os fatores mais importantes que afetaram a sua criação?	
Calor excessivo	
Impossibilidade de comprar suplementos como vitaminas e minerais	
Doença respiratória de sempre (nada estranho)	
Muda forçada	
Não sei	
Não teve fatores importantes que afetaram a criação	
11. Durante a influência desses fatores indicados na pergunta anterior, a mortalidade:	
Aumentou	
Permaneceu sem alteração	
Diminuiu	
Não sabe informar	
Não se aplica	
12. Durante a influência desses fatores, a produção de ovos:	
Aumentou	
Permaneceu sem alteração	
Diminuiu	
Não sabe informar	
Não se aplica	
13. Depois que os fatores cessaram, a mortalidade e postura de ovos:	
Continuaram alterados	
Voltaram à normalidade	
Não sabe informar	
Não se aplica	
14. Na sua granja, você tem permitido realizar testes experimentais?	
Sim, só com medicamentos	

Sim, com qualquer produto ou vacina solicitado pelo interessado	
Não	
15. Diante de uma emergência, tem importado vacinas ou medicamentos? *verificar se é vacina autorizada	
Sim	
Não	
16. Pratica muda forçada?	
Sim	
Não	
Não se aplica (para recrias e novos estabelecimentos)	
17.Qual o destino das galinhas de descarte?	
Venda para abatedouros dentro do estado	
Venda para abatedouro fora do estado	
Venda para abatedouro às vezes dentro, às vezes fora do estado	
Venda para terceiros	
Venda para terceiros e para abatedouros	
Não se aplica (para recrias e novos estabelecimentos)	
18. A granja possui cerca ao seu redor?	
Sim e com portão	
Sim e sem portão	
Não tem cerca e nem portão	
19. A granja é cercada por barreira natural?	
Sim, de árvores não frutíferas	
Sim, de árvores frutíferas	
Não	
20. A desinfecção de veículos é feita quando:	
Entram na granja	
Entram e saem da granja	
Não faz desinfecção	
21. A desinfecção de veículos é feita:	
Em arco de desinfecção	
Com bomba costal	
Bomba sob pressão	
Não faz desinfecção	
22. Impede entrada de pessoas estranhas na granja?	
Sim, todas	
Sim, algumas	
Não	

23. Quando profissionais terceirizados e RT entram na granja	
Trocam de roupa ou vestem EPI sobre a roupa e calçam botas de circulação interna	
Sem troca de roupa e uso de EPI	
Entram com a roupa que estão vestindo	
Não tem profissionais terceirizados	
24. Quando entram profissionais terceirizados e RT	
Os equipamentos e instrumentos são desses profissionais terceirizados	
Os equipamentos e instrumentos são da granja	
Não tem profissional terceirizado	
25. Quando esses veículos entram, são desinfetados no portão de entrada?	
Sim	
Não	
Sim, só quando tem desinfetante	
Sim, só quando não falta água	
26. Higienização de objetos e utensílios de uso na granja é uma prática rotineira?	
Sim	
Não	
Sim, só quando tem tempo	
Sim, só quando não falta água	
27. Depois da lavagem aplica desinfetantes?	
Sim	
Não	
Sim, só quando tem tempo	
Sim, só quando não falta água	
Sim, só quando tem desinfetante	
28. Trabalhadores tomam banho, na granja comercial, antes de iniciar a rotina?	
Sim	
Não	
29. Funcionários da granja de cria de pintainhas e recria de frangas	
Tomam banho, trocam de roupa e calçam botas limpas para entrar no núcleo	
Tomam banho e trocam de roupa e calçam botas limpas para entrar só no núcleo de pintainhas	
Tomam banho e trocam de roupa e calçam botas limpas para entrar só no núcleo de frangas	
Só trocam de roupa e calçam botas limpas para entrar no núcleo de pintainhas e de frangas	
Não tomam banho, não trocam de roupa e nem de botas	
Não é o meu caso	

30. Descarta adequadamente resíduos e lixo?	
Sim	
Não	
31. Já visitou sua granja à noite?	
Sim	
Não	
32. Controla roedores e moscas?	
Sim	
Não	
Sim, roedores	
Sim, só moscas	
33. Vê ratos vivos durante o dia?	
Sim	
Não	
34. Sente cheiro de ratos?	
Sim	
Não	
35. Vê fezes de ratos quando limpa o piso dos galpões?	
Sim	
Não	
36. Como controla moscas?	
Aplicando Inseticidas	
Tomando cuidado com umidade de esterco, regulando bebedouro para não pingar água	
Cuidado com umidade de esterco e inseticidas	
Não faz controle	
37. Durante o dia são observados, na área de produção, presença de	
Animais de estimação próprios ou estranhos	
Galinhas soltas que não são da granja	
Pássaros que vêm comer ração	
Animais de estimação e pássaros que vêm comer ração	
Animais silvestres	
Todos mencionados acima	
Não são observados outros animais	
38. Descarta adequadamente esterco?	
Sim, depois de fermentado na granja	
Sim, sem fermentar na granja	
Não, envio direto para agricultura	

39. Manejo de esterco é realizado:	
Em composteira	
Em esterqueira	
Deixa sobre solo sem lona	
Deixa sobre solo com lona	
Vende esterco cru	
Usa na propriedade esterco cru	
Transfere para terreno que fica perto da granja	
40. Empresta objeto, utensílios ou equipamentos para outras granjas?	
Sim, sempre	
Sim, ocasionalmente	
Não	
41. Aves de descarte com sinais da nova doença, são enviadas?	
Abate	
São sacrificadas na granja e levadas para compostagem	
São sacrificadas na granja e enterradas na granja	
São sacrificadas na granja e destinadas a fossas sépticas	
São deixadas à céu aberto em terrenos baldios	
Não se aplica	
42. Há quanto tempo está tendo esta nova doença?	
Há 1 mês .	
Há menos de 3 meses.	
De 3 meses a 6 meses.	
Há mais de 6 meses.	
Não se aplica	
43. Os maiores prejuízos que está tendo com esta nova doença?	
Mortalidade de galinhas	
Mortalidade de pintinhos de recria	
Mortalidade de galinhas e de pintinhos de recria	
Diminuição da postura	
Aumento da quantidade de ovos com casca rugosa	
Não se aplica	
44. Desta vez, as aves apresentam?	
Dificuldade para respirar como se estivesse engasgada	
Conjuntivite com olhos inchados com aspecto de “olho chinês”	
Eliminam, pela boca, catarro amarelo ou sanguinolento	
Todos os sinais acima mencionados	
Nenhum dos sinais acima mencionados	
Não se aplica	
45. Com o passar dos dias, os sinais:	

Pioraram	
Melhoraram	
Não se alteraram	
Não se aplica	
46. Caso tenha havido melhora, qual foi a possível razão:	
Tratamento com antibióticos	
Espontânea	
Desinfecção das instalações e dos objetos de uso	
Vacinação contra (citar)	
Outras (citar)	
Não se aplica	
47. Foram acometidas:	
Todas as aves da granja	
Apenas alguns galpões	
Apenas parte de alguns galpões	
Não se aplica	
48. A doença ocorreu logo após:	
Intenso calor	
Teste de produto comercial e em todas as aves da granja	
Teste de produto comercial em parte das aves da granja	
Logo depois de aplicação de vacinas de rotina	
Logo após a aplicação de um medicamento ou produto novo até então não usado	
A entrada de pessoas que visitaram outras granjas já com problemas	
Não sei	
Não se aplica	
Outro	
Transporte de aves mortas de granjas que tiveram a doença previamente, pela estrada em frente à granja	
49. Na maioria das vezes, qual tipo de ave compra para reposição do plantel	
Pintos de 1 dia	
Aves recriadas (aproximadamente 90 dias)	
Aves recriadas (próximas ao início de produção)	

Assinaturas do documento

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