UNIVERSIDADE FEDERAL DE UBERLÂNDIA

THAIS FERNANDA MARTINS DOS REIS

PRIMEIRO ISOLAMENTO DE *ERYSIPELOTRIX* SP. STRAIN 2 EM PERUS COM SEPTICEMIA NO BRASIL: EPIDEMIOLOGIA E MORFOMETRIA CELULAR

UBERLÂNDIA

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Dissertação apresentada à Faculdade de Medicina Veterinária-UFU, como parte das exigências para a obtenção do título de Mestre em Ciências Veterinárias

Área de Concentração: Saúde Animal

Orientadora: Profª Drª Belchiolina Beatriz Fonseca

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PRIMEIRO ISOLAMENTO DE *ERYSIPELOTRIX* SP. STRAIN 2 EM PERUS COM SEPTICEMIA NO BRASIL: EPIDEMIOLOGIA E MORFOMETRIA CELULAR

Dissertação aprovada para a obtenção do título de Mestre no Programa de Pós Graduação em Ciências Veterinárias, da Universidade Federal de Uberlândia, pela banca examinadora formada por:

Uberlândia, 20 de março de 2019

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DADOS CURRICULARES DA AUTORA

Thais Fernanda Martins dos Reis - Nascida em Coromandel, Estado de Minas Gerais, filha de Juarez Pereira dos Reis e Edna Maria Martins Dos Reis. Médica Veterinária graduada em fevereiro de 2015, pela Universidade Antônio Carlos de Uberlândia. Em 2015 a 2017 cursou Residência em Medicina Veterinária Preventiva na Universidade Federal de Uberlândia. No ano de 2017 iniciou o Programa de Pós- Graduação em Ciências Veterinárias na Universidade Federal de Uberlândia, área de concentração em Saúde animal, na qual foi bolsista pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), de março de 2018 a fevereiro de 2019.

Dedico esta dissertação a minha avó Helena Joana dos Reis. Por ter me ensinado valores como paciência, simplicidade e amor, características necessária para a conclusão deste trabalho.

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Eu sou forte por dentro, mas não por inteiro. Eu nunca fui perfeito, mas você também não.

Chester Bennington

SUMÁRIO

LISTA DE FIGURAS

Capítulo 3

LISTA DE TABELAS

Capítulo 2

Capítulo 3

RESUMO

Pertencem ao gênero *Erysipelothrix* seis (6) espécies: *E. rhusiopathiae, E. tonsilarium, E. inopinata*, *Erysipelotrix* sp. strain 1, *Erysipelotrix* sp. strain 2 e *E. larvae* sp. nov. Entre as espécies que estão inseridas neste gênero *E. rhusiopathiae* é cosmopolita e é considerada agente causador da erisipela em aves, suínos e humanos. No entanto, este estudo apresenta a associação de um surto em perus com outra espécie muito pouco relatada: *Erysipelotrix* sp. strain 2. Os dados usados para a realização desse trabalho são provenientes de amostras de planteis de perus machos e de matrizes de uma grande empresa localizada no estado de Minas Gerais, Brasil. Num primeiro momento, descrito no artigo 1, amostras de órgãos do fígado, baço, pulmões, traqueia, rins, intestino e articulações de perus provenientes de 118 granjas associadas à empresa foram submetidas a análise bacteriológica para *Erysipelothrix* spp. e outras bacterioses que apresentam semelhante sintomatologia. As lesões macroscópicas revelavam uma septicemia generalizada, os animais apresentavam fraqueza e alta mortalidade. A PCR em tempo real identificou como positivas para *Erysipelotrix* sp. strain 2, 15,5% das amostras. Desta amostragem de positivos, 6 (seis) isolados foram enviados para o sequenciamento da região 16S rRNA. Foi identificada alta similaridade com as sequências de *E. tonsillarum* e *E. rhusiopathiae.* Pela realização do teste de sensibilidade a antimicrobianos constatou-se alta resistência à neomicina, apramicina, fosfomicina e ao sulfametoxazol/trimetoprim. Resistência intermediária à tetraciclina e sensibilidade a norfloxacina, amoxicilina, lincomicina/ espectinomicina. Em um outro momento, descrito no segundo artigo, um outro surto foi investigado. Um total de 92 amostras de 31 lotes, sendo 30 amostras de 8 lotes oriundas de perus com septicemia e alta mortalidade foram avaliados. As 30 amostras dos lotes positivos foram identificadas como positivas para *Erysipelotrix* sp. strain 2 e nenhuma outra espécie de *Erysipelothrix* spp., foi identificada. Foi realizada microscopia eletrônica de transmissão, e visualizadas as estruturas da espécie *Erysipelotrix* sp. strain 2 que em cortes longitudinais apresentaram formato de bastão e em cortes transversais estrutura circular. Foi também observada à membrana plasmática e a parede bacteriana. Para a avaliação dos dados epidemiológicos, a análise da correlação foi positiva para os parâmetro de mortalidade final, idade dos animais durante o surto e a proximidade com a criação de suínos (até 7 Km). A realização do PFGE em 19 amostras, para a genotipagem elucidou a presença de 2 clones provenientes das mesmas granjas, porém de diferentes aves e 2 *clusters*. No entanto, destaca-se a observação de uma alta variabilidade genética entre os isolados avaliados. Para visualizar a capacidade das alterações celulares, dois isolados de *Erysipelotrix*

sp. strain 2 foram inoculadas em cultura de células Vero para avaliação da morfometria celular. E assim pode se perceber que a bactéria foi capaz de aumentar a área e o perímetro da célula, do núcleo e do nucléolo. Este estudo é ínclito, pois informa a espécie *Erysipelotrix* sp. strain 2 como agente causador da erisipela em perus e pela primeira vez apresenta características da *Erysipelotrix* sp. strain 2 ainda não publicadas na literatura científica.

Palavras-chave: Erisipelas, Microscopia Eletrônica de Transmissão, Mortalidade, Variabilidade Genética, Sequenciamento da Região 16S rRNA, Suceptibilidade a Antimicrobianos

ABSTRACT

The genus *Erysipelothrix* compises by six species: *E. rhusiopathiae*, *E. tonsilarium*, *E. inopinata*, *Erysipelotrix* sp. strain 1, *Erysipelotrix* sp. strain 2 and *Erysipelotrix larvae* sp. Among then, *E. rhusiopathiae* is cosmopolitan and the main agent which causes the Erysipelas in poultry, swine and human. However, this study shows the association of an outbreak in turkeys with another very little reported species *Erysipelotrix* sp. strain 2. The data used to perform this work came from samples of commercial and breeder turkeys from a large company in the state of Minas Gerais, Brazil. First, as described in article 1, samples of turkeys' organs (liver, spleen, lungs, trachea, kidneys, intestine and joints) from 118 farms associated to the company were submitted to bacteriological analysis for *Erysipelothrix* spp. and other bacterial diseases that cause similar clinical signs. Macroscopic lesions revealed a generalized septicemia, the animals presented weakness and high mortality. Real-time PCR identified as positive for *Erysipelotrix* sp. strain 2 in 15.5% of the samples. From these positive samples, 6 (six) isolates were sent for the 16S rRNA region sequencing. We identified high similarity with *E. tonsillarum* and *E. rhusiopathiae*. Antimicrobial susceptibility testing showed high resistance to neomycin, apramicyn, fosfomycin and sulfametoxazol / trimethoprim and intermediate resistance to tetracycline and sensitivity to norfloxacin, amoxicillin, lincomycin / spectinomycin. In the second chapter, we investigaled another outbreak. We evaluated a total of 92 samples from 31 flocks and from these, 30 samples were derived from 8 flocks where turkeys had sepsis and high mortality. All 30 samples from the positive flocks were positive for *Erysipelotrix* sp. strain 2 and we have not found any other species of *Erysipelothrix* spp. Transmission electron microscopy presented the structures of *Erysipelotrix* sp. strain 2, which in a longitudinal section had a rod format, and in a cross section, circular structure, and we also observed the plasma membrane and bacterial wall. For epidemiological data evaluation, the correlation analysis was positive for the parameters of final mortality, animals' age during the outbreak and proximity to commercial swine system production (up to 7 km). The performance of PFGE in 19 samples for genotyping elucidated the presence of 2 clones from the same farms but from different turkeys and 2 clusters. However, we highlight the high genetic variability of the studied strains. To visualize the cellular alteration, we inoculated two *Erysipelotrix* sp. strain 2 isolates in Vero cell culture for evaluation of cell morphometry. Therefore, we can observe that the bacterium was able to increase the cell, nucleus and nucleolus area and perimeter. This study is very important, because it reveals the specie *Erysipelotrix* sp. strain 2 as the

causative agent of Erysipelas in turkeys and for the first time shows characteristics of *Erysipelotrix* sp. strain 2 not published in the literature so far.

Keywords: Erisipelas, Transmission Electron Microscopy, Mortality, Genetic Variability, 16S rRNA Region Sequencing, Antimicrobial Susceptibility

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Reuniu-se na Sala 2D54, Campus Umuarama, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências Veterinárias, assim composta: Professores Doutores: **ANA LAURA GRAZZIOTIN** - UNIVERSIDADE FEDERAL DE UBERLÂNDIA; **DAISE APARECIDA ROSSI** - UNIVERSIDADE FEDERAL DE UBERLÂNDIA; **MARCELO SEBASTIÃO REZENDE** - EMPRESA BRF S.A.; e **BELCHIOLINA BEATRIZ FONSECA** orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Belchiolina Beatriz Fonseca, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

Aprovado(a).

Esta defesa faz parte dos requisitos necessários à obtenção do título de Mestre.

O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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CAPÍTULO 1
CONSIDERAÇÕES GERAIS

1 INTRODUÇÃO

Estudos epidemiológicos associados ao arranjo molecular desempenham fator fundamental na descrição filogenética bacteriana, evidenciando a diversidade genômica do microrganismo o que possibilita a análise de sua diversidade (CROUCHER et al., 2013). A estrutura epidemiológica embasada em aspectos como hospedeiros e localizações geográficas são úteis na atribuição das identificações das espécies (MUELLNER et al., 2013).

As bactérias do gênero *Erysipelothrix* têm formato de bastonetes gram-positivos (BROOKE; RILEY, 1999) e são integrantes do filo Firmicutes, da classe Erysipelotrichia com sua ordem única que contém 10 gêneros (VERBARG et al.,2014). Deste gênero são descritas 6 espécies: *E. rhusiopathiae, E. tonsillarum, E. inopinata*, *Erysipelothrix* sp. strain 1 e *Erysipelothrix* sp. strain 2 (TAKAHASHI et al., 1992; VERBARG et al., 2004) assim como o último identificado *E. larvae* sp. nov. (BANG et al., 2016).

A erisipela é considerada uma doença cosmopolita, que acomete animais domésticos e selvagens, inclusive humanos (KANG et al., 2017). *E. rhusiopathiae*, é o principal agente causador da doença e provoca perdas econômicas na produção de suínos e aves (BROOKE; RILEY, 1999). Estas perdas quando associadas aos perus causam uma infecção aguda e fulminante, septicemia, diminuição de rendimento e condenação de carcaça, mortalidade em surtos (BRICKER; SAIF, 2013).

Erysipelothrix sp. strain 2 tem escassas descrições, anteriormente era classificada como sorotipo 18 da espécie *E. rhusiopathiae*. A patogenicidade foi demostrada em teste experimental com 23 sorotipos de *E. rhusiopathiae.* O sorotipo 18 induziu lesões como urticária local em suínos e foi altamente virulenta para camundongos (TAKAHASHI et al., 1992).

Em relação aos aspectos econômicos, a produção de carne de peru tem posição importante no mercado internacional. O Brasil produziu um total de 390,48 mil toneladas desta proteína animal no ano de 2017, volume que aumentou em relação ao ano anterior. Cerca de 28% da produção é destinada à exportação. O estado de Minas Gerais também é destaque ocupando a posição de quarto maior exportador no ranking nacional, liderado respectivamente por Santa Catarina, Paraná e Rio grande do Sul (ABPA, 2018).

Casos de erisipela em aves são considerados reemergentes (JANßEN et al., 2015). E na estrutura avícola de produção Européia, estudos relacionam o aumento de casos com os novos sistemas de habitação (ERIKSSON et al., 2013). Os surtos de erisipela em perus descritos atualmente estão associados à espécie *E. rhusiopathiae*. Então, há uma compreensão

limitada em relação a doença causada pela *Erysipelothrix* sp. strain 2 em escala produtiva global. Poucos dados estão disponíveis, como os que são apresentados neste estudo.

Os fatos apresentados acima sustentam a importância de se avaliar a epidemiologia da doença causada por espécies do gênero *Erysipelothrix*. Baseado nisso, o objetivo desse trabalho foi relatar dois surtos de erisipela em perus provocado pela espécie *Erysipelothrix* sp. strain 2 e descrever suas caraterísticas epidemiológicas.

1.1 OBJETIVOS

1.1.1 GERAL

Caracterizar a espécie *Erysipelotrix* sp. strain 2 isoladas de perus em surto, analisar epidemiologicamente os fatores de risco associados à infecção, assim como realizar à genotipagem dos isolados, análises moleculares e sequenciamento genético para confirmar o agente etiológico referido.

1.1.2 ESPECÍFICOS

- Realização de técnica molecular PCR em tempo real e sequenciamento da região 16S para a diferenciação entre as espécies *E. tonsillarum* e *E. rhusiopathiae* e o agente diagnósticamente confirmado *Erysipelotrix* sp. strain 2;

- Comparar a sintomatologia clínica e lesões macroscópicas causadas por *Erysipelothrix* sp. strain 2 e outra espécie, *E. rhusiopathiae,* que é o principal agente causador da erisipela em perus;

- Determinar o perfil de resistência dos isolados frente aos antimicrobianos;

- Caracterizar por microscopia eletrônica de transmissão as características estruturais da bactéria isolada com cerne nos cortes longitudinais e transversais e estruturas como membrana plasmática e parede celular;

- Averiguar a epidemiologia da doença fundamentada em analises estatísticas, correlacionando a aspectos de mortalidade final, idade dos animais durante o surto e conversão alimentar;

- Realização da técnica molecular de Eletroforese em campo pulsado (PFGE) para caracterização genotípica dos isolados para investigar as possíveis fontes de infecção;

-Mapeamento e estatística das proximidades entre as granjas de perus e suínos alerta;

- Execução da morfometria celular em cultura de células Vero com o intuito de examinar alterações nas estruturas celulares

O conjunto de todos os resultados obtidos por este trabalho é significativo para o conhecimento das características fundamentais deste importante microrganismo na produção avícola bem como na saúde pública.

2 REFERENCIAL TEÓRICO

2.1 Particularidades Gerais

Delineamentos recentes alicerçados em sequenciamento do genoma completo possibilitam a classificação taxonômica do gênero *Erysipelothrix* (KWOK et al., 2014), integrante do filo Firmicutes, da classe Erysipelotrichia com sua ordem única que contém 10 gêneros (VERBARG et al.,2014). A espécie mais conhecida é *E. rhusiopathiae* que compreende diversos sorotipos como os classificados em 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19 e 21 e tipo N, seguida por *E. tonsillarum* com os respectivos sorovares 3, 7, 10, 14, 20, 22 e 25, *Erysipelothrix* sp. strain 1 o sorotipo 13 e *Erysipelothrix* sp. strain 2 o sorotipo 18 (TAKAHASHI et al., 1992) assim como o último identificado *E. larvae* sp. nov. (BANG et al., 2016).

Apoiado pelo sequenciamento do gene 16S rRNA a filogenia aborda que *E. inopinata* divergiu antes da divisão entre a *E. rhusiopathiae* e *E. tonsilarum* (VERBARG et al., 2004). Estas espécies são descritas como distintas, pois apresentam diferenciações em suas características fenotípicas, patogenicidade e grupos de sorotipos. Esta análise fundamenta-se por estudos de hibridização por DNA-DNA que também verificou que os sorovares 13 e 18 exibem níveis de hibridização (6 a 47%) com as cepas mencionadas (TAKAHASHI et al., 1992).

Durante um surto de erisipela em suínos vacinados no centro oeste dos Estados Unidos foram avaliadas as espécies *E. rhusiopathiae*, *E. tonsillarum*, *Erysipelothrix* sp. strain 1 e *Erysipelothrix* sp. strain 2 em amostras dos animais e do ambiente. Todos os isolados obtidos dos suínos foram da espécie *E. rhusiopathiae* e em amostras ambientais foram isoladas *E. rhusiopathiae* e *Erysipelothrix sp*. strain 1. No entanto, isolados de *E. rhusiopathiae* apresentou cepas diferentes em relação aos animais e o ambiente (BENDER et al., 2010).

Foi demostrado por Forde et al. (2016) por sequenciamento genético, que a espécie *Erysipelotrix* sp. strain 2 é filogeneticamente distinta às espécies *E. rhusiopathiae* e *E. tonsillarum.* Fator que é apoiado pelo nível de similaridade correspondendo a uma sequência insuficiente para o mapeamento de leituras de sequências completas do genoma de uma *E. rhusiopathiae* Fujisawa ou SY1027 e para o alinhamento pelo Parsnp que solicita uma média ≥ 97% identidade de nucleotídeos. Com base na estrutura protéica conservada pode concluir que *E. rhusiopathiae e a Erysipelothrix* sp. strain 2 aparentam ter sua evolução distante em relação a espécies menos patogênica, *E. tonsillarum*. Estes autores inclusive salientam que

investigações subsequentes para a diferenciação genética que separam estas espécies podem fornecer dados esclarecedores dos motivos delas terem adquirido uma maior patogenicidade do que a *E. tonsillarum*.

Estudo realizado com o intuito de comparar a patogenicidade da *E. rhusiopathiae* e *E. tonsillarum* em galinhas de criação confirmou que as aves acometidas com a primeira apresentavam sinais clínicos da doença com lesões características, bacteremia e em algumas havia a recuperação da doença. Nas aves que foram inoculadas com as cepas da *E. tonsillarum* não houve lesões e sinais clínicos. Levando os autores a acreditar que *E. tonsillarum* não é patogênicas, para galinhas (TAKAHASHI et al., 1994).

2.2 Etiologia e Morfologia

Para o crescimento bacteriano em laboratório, as espécies do gênero *Erysipelothrix* tem a necessidade do uso do ágar columbia (CA) ou ágar sangue (AS) enriquecido com sangue de carneiro a 5% para a sua caracterização morfológica. Em sua maioria apresentam colônias claras, circulares, pequeno diâmetro e podendo ou não ocorrer a formação de alfa hemólise (BROOKE; RILEY, 1999).

O gênero *Erysipelothrix* é Gram-positivo, mesófilo, anaeróbico facultativo, não forma esporos e tem formato de bastonete. A catalase, oxidase e ureia são negativas, não ocorre motilidade, não hidrolisa esculina e não produz H_2S em sua linha de inoculação no TSI e seu crescimento é favorecido com uma incubação com 5% a 10% de CO_2 em temperatura ideal de 37ºC (BROOKE; RILEY, 1999). Em relação às provas bioquímicas a *E. tonsillarum* se diferencia da *E. rhusiopathiae* pelo fato de não fermentar sacarose (TAKAHASHI et al., 1994).

Na análise morfológica existem duas estruturas descritas, a primeira com colônias lisas, com possibilidade de beta hemólise e, em coloração de Gram formam bastonetes com ligeira curvatura. A outra forma são colônias irregulares sem a formação de hemólise e na coloração de Gram apresenta forma filamentosa (REBOLI; FARRAR,1989).

Uma característica importante das espécies do gênero *Erysipelothrix*, é a presença de uma parede celular do tipo B, baseado na formação das pontes peptídicas entre aminoácidos na posição 2 e 4 das cadeias laterais, considerando que na maioria das bactéria a ponte ocorre nas posições 3 e 4 (WILLEMS et al., 1997).

2.3 Aspectos econômicos

Quando acometidos por erisipela os perus não só morrem como também pode ocorrer a perda de fertilidade nos machos, desclassificação e condenação de carcaça resultante das alterações *post-mortem* provocadas pela septicemia (BRICKER; SAIF, 2013).

Deve ser considerado que a erisipela é uma doença que está ressurgindo nas produções avícolas europeias, hipoteticamente em associação com alterações no sistema de alojamento após a mudança de gaiolas convencionais para sistema de alojamento em piso (JANBEN et al., 2015; ERIKSSON et al., 2013)

Em suínos, a erisipela é capaz de gerar intensas perdas econômicas na produção devido às lesões agudas e crônicas tendo potencial para provocar grandes surtos (OPRIESSNIG et al., 2012). Outro possível prejuízo é a condenação de carcaça; esta doença é o principal fator propício a esse dano em suínos dos Estados Unidos (BENDER et al., 2011). A incidência desta patologia tem aumentado consideravelmente no Japão, China e Estados Unidos (BENDER et al., 2011; TO et al., 2012).

2.4 Importância em Saúde Pública

A infecção humana por *E. rhusiopathiae* gera três tipos de lesões, as de pele eritrematosas (erisipelóide), a forma cutânea generalizada e a forma septicêmica frequentemente associadas a endocardite (BROOKE; RILEY, 1999). Estão relacionadas com a forma ocupacional infectando essencialmente trabalhadores que lidam diretamente com a produção animal como é o caso de funcionários de frigoríficos, açougueiros e pescadores (VERALDI et al., 2009).

Estudo realizado descreveu um caso clínico em que um paciente que teve um caso grave de septicemia causada por *Erysipelothrix* spp. O paciente relatou um edema doloroso na mão esquerda proveniente de uma lesão que tinha sucedido a 10 dias. Por meio de uma hemocultura o agente causador da alteração foi identificado como o referido acima e com posterior sequenciamento a espécie *E. rhusiopathiae* foi confirmada. Aspecto importante a ser revelado é que não ocorrem lesões ou sequelas cardíacas (VOLARD et al., 2016).

Pesquisas recentes relatam que a família Erysipelotrichaceae está associada com alterações a nível intestinal com inflamações do trato gastrointestinal e câncer colorretal. Alterações na quantidade de bactérias dessa família foram observadas em pacientes

diagnosticados com câncer colorretal e disfunções inflamatórias, o que também foi verificado em modelos experimentais (KAAKOUSH, 2015; NAGAO-KITAMOTO et al., 2016).

2.5 Hospedeiros

A erisipela é uma doença cosmopolita, que acomete vários animais domésticos, selvagens e humanos (KANG et al., 2017). A espécie *E. rhusiopathiae* é o agente causador de perdas na produção avícola e na suinocultura (BRICKER; SAIF,2013). A doença quando remete aos suínos causa septicemia, coagulação intravascular, necrose vascular e infartos cutâneos, já na forma crônica provoca quadros de artrite e endocardite (BROOKE; RILEY, 1999). Em perus a sintomatologia baseia-se em uma septicemia aguda, com a formação de edema e hemorragia nos pulmões, coração e necrose disseminada (BRICKER; SAIF, 2013). Esta doença foi relatada em outros tipos de aves, como em galinhas poedeiras em gaiolas (MUTALIB; KING; MCDONOUGH, 1993), patos (DHILLON et al., 1980), codornas (MUTALIB; KEIRS; AUSTIN, 1995) e faisões de pescoço anelado (HENNIG et al., 2002).

Um estudo recente comprova que Erysipelothrix *rhusiopathiae* causou uma septicemia fatal em dois golfinhos do Atlântico (*Stenella frontalis* e *Tursiops truncatus*) (DÍAZ-DELGADO et al., 2015). Estudos realizados por Boerner et al. (2004) relataram o primeiro caso de um diagnóstico *post-mortem* de septicemia por *Erysipelothrix* spp. em um grupo de Pinguins azuis adultos (*Eudyptula minor*) criados em cativeiro, baseados em estudos histopatológicos, bacteriológicos e moleculares. A erisipela em galinhas poedeira é incomum, porém tem evoluído como uma doença emergente e deve ser considerada a mortalidade em lotes mais velhos, especialmente com infestação intensa de ácaros vermelhos (*Dermanyssus gallinae*) que atuam como vetores da doença (SCHMITT et al., 2014). Pesquisa realizada identificou oito sorotipos de *E. rhusiopathiae*, em 39 surtos de erisipelas em galinhas de criação, sendo que os sorotipos 1, 5, 6, 9 foram os mais relevantes (BISGAARD; NORRUNG; TORNOE, 1980).

2.6 Patogenicidade

Proteínas imunogênicas definidas como antígenos de proteção de superfície (Spa), são usadas para a distinção das cepas de suas respectivas espécies. As proteínas são classificadas em três SpaA, SpaB e SpaC. A SpaA foi identificada nos sorotipos de *E. rhusiopathiae* 1a, 1b, 2, 5, 8, 9, 12, 15,16, 17 e N, as SpaB foram identificadas nos sorotipos 4, 6, 11, 19 e 21 e

a proteína SpaC foi identificada no sorotipo 18 classificada com *Erysipelothrix* sp. strain 2 (TO; NAGAI, 2007).

O mecanismo de patogenicidade do gênero é um processo que ainda não foi elucidado totalmente nem mesmo para espécie mais conhecida (WOOD; HENDERSO, 2006; Zhu et al. 2017). O que foi demontrado é que *E. rhusiopathiae* tem a capacidade de recrutar plasminogênio do hospedeiro via Spa e para que isso ocorra tem a necessidade da presença de lisina, realizando a adesão da bactéria as células hospedeiras e inibindo a capacidade de destruição mediada pelo complemento (HARADA et al., 2014). Mas ainda não se sabe se a Spa tem outros tipos de virulência (ZHU et al., 2017).

2.7 Mortalidade e Morbidade

Tem-se uma alta mortalidade e morbidade dos animais infectados pela *E. rhusiopathiae* considerando animais que não foram imunizados, ou não tiveram tratamento precoce administrado, sendo que a maioria morre com o processo patológico. O padrão de mortalidade pode variar, em aves com boas condições pode ocorrer uma queda repentina do animal e morrem de 24 a 48 horas. A mortalidade pode variar muito dentro de determinado grupo (BRICKER; SAIF,2013).

2.8 Epidemiologia

A porta de entrada e a patogênese de *E. rhusiopathiae* e outas espécies do gênero em aves ainda não foi devidamente estabelecida, embora sabe-se que materiais contaminados são fontes de infecção assim como lesões na pele e membranas mucosas (GRENCI, 1943; MURASE, SUZUKI, NAKAHARA, 1959).

Aves silvestres, mamíferos, solo contaminado, esterco, alimentos e água podem ser fonte de infecção por *E. rhusiopathiae* (SHUMAN, 1971). Cousque (2005) relatou um surto de erisipela em pombos após a ingestão de resíduos de compostagem. Canibalismo e luta entre as aves resultam na formação de portas de entrada para o agente. O fato de existirem carcaças de aves mortas infectadas nas instalações também aumenta a propagação da doença.

A erisipela é diagnosticada com maior frequência em perus machos, no entanto não há evidências de predileção entre os sexos. Por meio de observações a campo o que pode ser notado é que a principal porta de entrada para o patógeno são lesões de pele, estrutura mais violada no macho por seu temperamento (BRICKER; SAIF, 2013).

Um estudo retratou um surto de *E. rhusiopathiae* em galinhas poedeiras e isolou o microrganismo do esterco, poeira dos exaustores e *swabs* de bebedouros. Em relação ao isolamento do organismo da ave *E. rhusiopathiae* foi isolada no conteúdo do jejuno. Isso indica uma rota de transmissão oro-fecal vigente em aves de produção (ERIKSSON et al., 2014).

A ampla distribuição ecológica e geográfica do gênero é atribuída a sua capacidade de infectar um grande número de indivíduos hospedeiros que podem funcionar como portadores, permanecendo no ambiente e disseminando a doença (BROOKE; RILEY, 1999). Autores relataram o isolamento da espécie *Erysipelothrix rhusiopathiae* em porcos saudáveis (STEPHENSON e BERMAN, 1978; OKOLO, 1986) em cordeiros nos seus nódulos linfáticos (KAFERSTEIN; EKDAHL; ALMAND, 1972).

Surtos a campo ocorrem geralmente em perus mais velhos, porém pode ocorrer em aves de qualquer idade. Hollifield et al. (2000) relataram a infecção em perus com idade de 4 dias, a possibilidade é que a infecção ocorreu por meio do corte do dedo gerado ainda no incubatório.

A função dos vetores na transmissão da erisipela não é algo totalmente elucidado. Wellmann (1950) relatou que esse patógeno poderia ser transmitido mecanicamente por ratos, pombos, moscas, mosquitos e diretamente entre os hospedeiros definitivos. Relato de Eriksson et al. (2010) sugeriu que o (*Dermanyssus gallinae*) conhecido como ácaro vermelho é vetor da espécie *E. rhusiopathiae* entre aviários. Sua análise foi baseada em isolados da bactéria em 56 aves e nove ácaros. Pela eletroforese em gel de campo pulsado (PFGE) os autores confirmaram que uma única cepa foi responsável pelo surto, validando a proposta do ácaro como um meio de transmissão da doença.

Outra forma de transmissão que deve ser destacada é a capacidade de infecção pela *Erysipelothrix* spp., por meio do contato com produtos de origem animal contaminados. Durante o período de um ano 153 amostras para destino doméstico de carne de frango crua, de um frigorífico localizado em Nagano no Japão, foram coletadas e analisadas em relação à presença de *Erysipelothrix* spp., o resultado mostrou 67 isolados considerando que 65 eram *E. rhusiopathiae* e dois *E. tonsillarum*. Pode-se concluir com esse experimento que a carne de frango tem a possibilidade de ser contaminada por *E. rhusiopathiae* o que a torna uma possível fonte de infecção para humanos (NAKAZAWA,1998).

2.9 Sinais Clínicos

Na criação de perus, geralmente os surtos acontecem de forma repentina, com perda de um grande número de aves dos planteis. O que se observa são diversas aves caídas pelo galpão e outras com marcha instável. Algumas lesões cutâneas podem ser evidenciadas. Apresentam emagrecimento gradual, infertilidade no macho, fraqueza e sinais de anemia e em alguns casos endocardite e morte. Perus que foram anteriormente vacinados podem morrer de forma repentina e sem sinais cínicos aparentes, provavelmente como resultado de uma embolia (BRICKER; SAIF, 2013).

Em galinhas podem ocorrer perdas repentinas 4 a 5 dias após a inseminação artificial com alterações como peritonite, congestão perineal e descoloração de pele. Nas poedeiras pode ocorrer a diminuição na produção de ovos com uma queda na produção de 50 a 70% (KILIAN et al., 1958).

2.10 Patologia

Em surtos endêmicos, as lesões macroscópicas são sugestivas de septicemia generalizadas, as principais lesões observadas em perus são: degeneração de gordura na borda anterior da coxa, hemorragia na gordura pericárdica, petéquias na gordura abdominal, hemorragia do músculo cardíaco, fígado friável, aumento do baço e rins, exsudato fibrinopurulento nas articulações e saco pericárdico, placas de fibrina no músculo cardíaco, espessamento do proventrículo e ulcerações na parede da moela. Além de pequenos nódulos amarelos no ceco, enterite catarral ou sanguinolenta, endocardite, lesões cutâneas escuras e crostas (ROSENWALD; DICKINSOM, 1941).

Em relação às lesões microscópicas, as características histológicas da erisipela aguda em perus têm alterações esperadas comumente em infecções septicêmicas (BICKFORD; CORSTVET; ROSENWALD, 1978). Alterações vasculares dominam o padrão com o ingurgitamento generalizado dos vasos sanguíneos, agregações intravasculares causada pela bactéria com interação de fibrina em trombos são frequentes vistos em capilares e vênulas.

2.11 Período de Incubação

A inoculação experimental de perus por via subcutânea (SC) revelou que ocorre a mortalidade em 44 a 70 horas na maioria dos animais e em outros depois de 96 a 120 horas. Com a inoculação realizada de forma oral o parâmetro foi alterado para 2 a 3 dias podendo chegar a 2 a 3 semanas com uma menor taxa de mortalidade (BRICKER; SAIF,2013)

2.12 Diagnóstico

A sorotipagem que é o teste de aglutinação com anti-soro específicos usados para reconhecer antígenos de peptidoglicanos diferentes na parede celular (KUCSERA, 1973) é um teste que tem valor diagnóstico para as diferentes espécies do gênero *Erysipelothrix* por seu conjunto diverso de sorotipos (PAL; BENDER; OPRIESSNIG, 2010). No entanto, não é adequado para análise da evolução dentro da espécie porque pode ocorrer a troca horizontal entre genes específicos de cápsula em muitas bactérias (KING et al., 2002).

A PCR é uma técnica importante empregada para complementar os métodos de detecção (PAL; BENDER; OPRIESSNIG, 2010). O estudo realizado por Forde et al. (2016) confirmaram a espécie *E. sp. strain* 2 com primer específico alicerçado no gene 23S rRNA que já tinha sido utilizado por outros autores (TAKESHI et al., 1999).

A correlação genética entre os isolados e a epidemiologia das cepas e espécies pertencentes ao gênero *Erysipelothrix* pode ser realizado usando a técnica de eletroforese em campo pulsado-PFGE (WANG; CHANG; RILEY, 2010).

2.13 Diagnóstico Diferencial

De acordo com Bricker e Saif (2013) diversas patologias podem ser confundidas com a Erisipela em perus. Podemos citar: cólera aviária, infecções por *Escherichia coli*, salmonelose. A doença de Newcastle em estado agudo pode ser confundida pelo fato de causar mortalidade aguda. *Lactobacillus* SP. que são isolados do trato gastro intestinal ou fígado das aves são bioquimicamente semelhantes ao gênero *Erysipelothrix*, o que pode ser diferenciado é o uso do meio seletivo de Packer contendo azida de sódio e cristal violeta.

Os agentes relacionados ao diagnóstico diferencial são facilmente diferenciados em relação a *Erysipelothrix*, baseados em coloração de Gram, realização das provas bioquímicas, crescimento em meio Packer e o padrão típico de crescimento no meio TSI. O diagnóstico confirmatório é feito por metodologia molecular, como teste de PCR, teste de anticorpos fluorescentes ou teste de patogenicidade em animais (BRICKER; SAIF,2013).

2.14 Vacinação

Para a imunização de perus contra a Erisipela os produtos usados são o hidróxido de alumínio inativado com formalina e bacterinas de *E. rhusiopathiae* adsorvidas que podem conter células inteiras ou lisadas. Estas bacterinas foram desenvolvidas para o uso em suínos e mostra-se eficaz para o uso em perus (BRICKER; SAIF, 2013). Pesquisadores sugeriram o uso do ELISA específico para caracterização do antígeno (BECKMANN; GYRA; CUSSLER, 1996). Somente algumas cepas de *E. rhusiopathiae,* pertencente ao sorotipo 2, tem sido eficazes para o uso em bacterinas. Essas cepas são altamente imunogênicas por causa da produção de um produto solúvel em substância ionizante que é adsorvida e precipitada pelo hidróxido de alumínio, que também adsorve células bacterianas inteiras ou lisadas.

Um protocolo de vacinação para perus é sugerido para o controle de erisipela em regiões endêmicas, tanto para os de corte como para os produtores de ovos. A proposta é uma única dose subcutânea (SC) inoculada na superfície dorsal do pescoço atrás do atlas. Primeiramente, a dose foi aplicada por via intramuscular (IM), no entanto, ocorreu a formação de abscessos com a possibilidade de desclassificação no abate, então a forma mais indicada é SC. Para perus classificados como reprodutores, é indicado o uso de duas doses com intervalo de 4 semanas, as vacinas devem ser realizadas antes do início da produção. A primeira dose deve ser realizada com 16-20 semanas de idade e a segunda imediatamente ao início da postura (BRICKER; SAIF,2013).

Experimento realizado por Bisgaard, Norrung e Tornoe (1980) constatou mais de um sorotipo de *E. rhusiopathiae* em aves de uma mesma criação, eles discutiram a vacinação com possibilidade de considerar o uso de vacinas autógenas como uma alternativa de ação. Bender et al. (2010) avaliaram 11 surtos de *E. rhusiopathiae* em suínos com o intuito de comparar o sorotipo que causou a infecção nos animais e a usada na vacinação. Em seis propriedades o sorotipo vacinal era homólogo ao isolado dos animais e, em cinco granjas eram heterólogos. O autor ressaltou a reavaliação do uso da vacina e a utilização de cepas de campo para a produção de vacinas autógenas.

2.15 Tratamento

O tratamento ideal para perus, assim que o diagnóstico para erisipela for confirmado, é o uso de antibioticoterapia. A penicilina sódica é o fármaco de primeira escolha a ser administrada IM 10.000 U/1b do peso corporal em animais individualmente. No entanto, em criações de perus de corte, a captura e manuseio de cada ave é muitas vezes prejudicial, gerando abscessos e lesões que podem surgir pelas administrações IM. Então, o controle pode

ser feito com a administração da penicilina (1.000.000 U/gal) em água de bebida para todo o plantel com diagnóstico presuntivo, considerando que todas as aves devem ser tratadas. Existem algumas indicações para o uso de penicilina procaína ou outros derivados de longa duração, em determinados casos seu uso é satisfatório, mas em surtos ocorre a necessidade de antibióticos de ação rápida, por isso usar a penicilina sódica (BRICKER; SAIF, 2013). Estudos comparativos são necessários para testar a eficácia de outros antibióticos no controle da *E. rhusiopathiae* em populações aviárias. Testes demonstraram que a Eritromicina e alguns antibióticos de amplo espectro foram eficientes, no entanto foi encontrada resistência à neomicina (FUZI, 1963); e sulfonamidas e oxitetraciclina não são tratamentos satisfatórios. A maioria dos isolados da *E. rhusiopathiae* são resistentes aos aminoglicosídeos, tetraciclina, rifampicina e trimetoprim-sulfametoxazol (VOLARD et al., 2016).

2.16 Estratégias de prevenção

Bricker e Saif (2013) descrevem diversos fatores ambientais associados a surtos por *E. rhusiopathiae* em espécies aviárias. Infecções a campo podem ser desencadeadas com o começo do tempo chuvoso, frio, que coincide com a maturidade sexual. A fonte de infecção pode ser alimentos contaminados, solo ou matéria orgânica em decomposição, aves portadores inclusas no plantel ou roedores. Sendo associado a desinfecção inadequada e períodos de vazio sanitário insuficientes.

Sugestões gerais incluem o uso de equipamentos limpos e desinfetados, produção em locais distantes de plantas previamente contaminadas. Uso de desinfetantes, como soluções de hidróxido de sódio 1-2%, fenóis, cresóis são eficazes contra *E. rhusiopathiae*, e assim, importantes para o controle da doença (BRICKER; SAIF, 2013).

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CAPÍTULO 2

FIRST REPORT OF *ERYSIPELOTRIX* SP. STRAIN 2 IN TURKEYS OF BRAZIL WITH HIGH HOMOLOGY WITH OTHER SPECIES

Artigo a ser publicado no periódico

Scientific Reports

First report of *Erysipelothrix species* strain 2 in turkeys of Brazil with high homology with other species

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ABSTRACT

Erysipelothrix rhusiopathiae was thought to be the only species from the genus to cause erysipelas outbreaks in poultry. In the present study we performed the diagnosis of *Erysipelothrix* sp. strain 2 (ES2) in organ samples from flocks of male turkey located in poultry-producing farms, in Brazil. We evaluated 118 farms for bacteriological analysis and TaqMan real-time PCR, having 18 (15.25%) identified as ES2 positive flocks, without any other species from the same genus being found. After analysing organ samples, such as liver, spleen, heart, lungs, kidneys, trachea, joints and intestine, we found liver as the organ of choice for the isolation of the ES2. The results obtained by sequencing the 16S rRNA region of ES2 identified high homology with as *E. tonsillarum* and *E. rhusiopathiae*, suggesting that it is not the best suited target to identify this species. We found a positive association between isolation of the bacteria in organs and flocks' mortality. Positive flocks had a mortality mean rate of 6.87%, while in the negative flocks, this index was 3.76%. Ill turkeys had gross lesions of generalized septicaemia, a common clinical sign of *E. rhusiopathiae*. The bacterial isolates showed high resistance to fosfomycin and trimethoprim/sulfamethoxazole, intermediate resistance to tetracycline and sensibility to norfloxacin, amoxicillin and lincomycin/spectinomycin. Also, all strains of ES2 showed to be resistant to neomycin and apramycin, which suggests an intrinsic resistance to this class of antimicrobials. This was the first study in the world that addressed ES2 as the causative agent of erysipelas in turkey.

Keywords: poultry, erysipela, septicaemia, sequencing the 16S rRNA region, antimicrobial resistance, organs

1. INTRODUCTION

Erysipelothrix is a genus of bacteria that until very recently was thought to comprise only the *Erysipelothrix rhusiopathiae* species, shown to be untrue due to DNA–DNA hybridization advancements [1]. Multilocus enzyme electrophoresis and restriction fragment length polymorphism [2, 3] methods were important to separate *Erysipelothrix* into six different species, *E. rhusiopathiae*, *E. tonsillarum*, *Erysipelothrix* sp. strain 1 (ES1), *Erysipelothrix* sp. strain 2 (ES2), *E. inopinata* and *E. larvae* [1, 4, 5, 6]. The infection prevalence of *Erysipelothrix* occurs worldwide and affects a wide variety of invertebrate and vertebrate groups, such as humans. *E. rhusiopathiae* is an occupational pathogen mostly prevalent among people working close to animals as well as animal products [7, 8]. In addition, *Erysipelothrix rhusiopathiae* is the etiological agent of turkey erysipelas, being characterized by an acute and fulminating infection, which in turns may affect the fertility of the male turkey. Therefore, animal production companies are directly interested in controlling the *Erysipelothrix rhusiopathiae* uprisings due to its effect on the turkey meat production industry. Correctly identifying the disease is then a key factor for controlling erysipelas to avoid having downgrading caused by septicaemia lesions [8].

Brazil has an important position in the world's turkey meat industry, being the third largest producer and the second most exporter. The State of Minas Gerais, south-eastern Brazil, was responsible for 13.75% of Brazil's turkey meat exportations in 2015 [9]. Knowledge and pathogen control are essential tasks to avoid detrimental effects to the economy, especially in poultry-producing sites throughout the country. Needless to say, Brazil as being a large poultry producer, researchers should, therefore, perform detailed researches in order to make new approaches to diseases and /or microorganisms more feasible.

In Brazil, there has been an outbreak in 70-day-old broiler male turkeys, characterized by septicaemia and high mortality. Preliminary data led us to believe that this was an outbreak caused by a different *Erysipelothrix* species, other than *E. rhusiopathiae*. After previous evaluations, we performed real time PCR and confirmed the presence of ES2. The aim of this study was to report the first isolation of ES2 associated with clinical signs of diseases as well as mortality in turkeys, as well as to identify the antimicrobial resistance of the isolates.

2. MATERIAL AND METHODS

A disease outbreak in broiler turkeys over 70 days old was recorded from January 2014 to February 2015, being recognized by the presence of septcemia and high mortality, located in poultry producing farms associated with a large regional company, in Minas Gerais state, Brazil. Initially we collected and analyzed organ samples from 147 flocks, of male Nicholas lineage over 70 days old. From the 147 flocks, we selected for this study 118 flocks that had similar conditions of management, poultry house structure and feed type. Additionally, all individuals were older than 70 days and no isolation of pathogens that could cause septicemia or sudden death (Avian Pathogenic *Escherichia coli* (APEC)*, Mycoplasma Galliepticum, Mycoplasma sinoviae, Pasteurella multocida,*, and *Salmonella* spp). For study, we necropsied 2-3 birds per flock up to one hour after death, the coelomic cavity was accessed with clean scissors, having the following organs sampled: trachea, lungs, heart, kidneys, spleen, liver, joints and intestines. The same organs from different birds within the same flock were pooled together in proper packing (Nasco®), totaling 960 samples collected in the 118 flocks. We collected organ samples once in 116 flocks and twice in two flocks. The samples were kept refrigerated with ice packs until analysis in the laboratory of Animal Health. The exterior surface from each organ was heat-sterilized, and the internal part of the

organ was inoculated on sheep blood and MacConkey agar (Oxoid®) and then incubated aerobically at 37°C from 18 to 24 hours. After incubation, colonies were routinely analysed for the following bacterial agents: *Pasteurella multocida,* Avian Pathogenic *Escherichia coli, Salmonella* spp., *Streptococcus* spp., *Staphylococcus* spp. and *Aspergillus* spp [10]. To identify *E. coli* as APEC we performed Taqman PCR using a commercial kit (Simbios Biotecnologia,). Isolates positive for four or five of the following virulence-associated genes (VAG) were considered APEC: aerobactin (iutA) avian haemolysin (hlyF), increased serum survival (iss), salmochelin (iron) and outer membrane protease (ompT) , following the protocol described by Ikuta et al. [11]. *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were analyzed with a commercial real-time PCR (Simbios®), following the protocol described by the manufacture. *Erysipelothrix* spp. colonies isolated on sheep blood agar were selected to Taqman real-time PCR. Colonies had a clear appearance with or without partial hemolysis, having circular shape and from 0.1 to 0.5 mm in diameter. The colonies had also biochemically-tested non-motile Gram-positive rods, and were negative for catalase, oxidase and indol, producing H_2S in triple sugar agar [12, 13].

We performed TaqMan real-time PCR for differentiation between *E. rhusiopathiae, E. tonsillarum* and ES2 as previously described by Pal et al [14] with few modifications. Ten typical colonies of each isolate, grown for 24 hours on blood agar plates, were harvested and suspended in ultrapure water (Invitrogen®) by vigorous stirring. The suspension was then used for DNA extraction using the Blood and Tissue kit (Qiagen®); following the manufacturer's instructions, the extracted liquid was used for amplification and identification of the DNA. For each test, we used positive controls of *E. rhusiopathiae* and *E. tonsillarum* isolated from swine, kindly provided by Professor Dr. David Emílio Santos Neves de Barcellos. The amplification and identification were performed in three reactions of TaqMan real-time PCR, one reaction for each species. The reactions had a total volume of 25

μL containing 12.5 μL of the commercial master mix (TaqMan® Universal PCR master mix, Applied Biosystems®), 5 μL of DNA, 0.4 μmol 1^{-1} of each primer and 0.2 μmol 1^{-1} of each probe. The reactions were carried out in a StepOne Real-Time PCR system (Applied Biosystems®). The PCR cycling parameters were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 30 seconds at 55°C and 50 seconds at 60°C. The results were analysed with StepOne Plus (version 2.1) software. The cycle number at which the reporter dye fluorescence was detectable was used to determine the Ct (i.e., the PCR cycle number at which the fluorescent IppS signal crossed the cycle threshold). We considered samples to be positive when the Ct value was ≤ 35, and negative when the Ct was > 35 or when no fluorescent IppS signal crossed the cycle threshold.

We performed an epidemiological analysis by association analyses selecting 18 positive flocks, two collected twice during the life time (160 samples) and 10 negative control flocks (80 samples) we recorded the weekly mortality of the selected lots from 70 weeks of age. Besides being negative for pathogens that cause sudden death or septicemia, the selected flocks for association analysis were also negative for *Streptococcus* app, *Staphylococcus* spp. and *Aspergillus* spp.

Among the studied isolates, six strains identified as ES2 by the TaqMan real-time PCR technique were randomly selected and their total genomic DNA samples were sent to Macrogen (Korea), so that the 16S rRNA gene could be amplified using the primers 27F (5'-AGAGTT TGATCMTGGCTCAG-3) and 1492R (5'-CGGTTACCTTGTTACGACTT-3). The amplified product was then used to sequence reactions using primers 785F (5'-GGATTAGATACCCTGGTA-3) and 907R (5'-CCGTCAATTCCTTTRAGTTT-3). The DNA sequence was compared with data stored in the GenBank database, available on the National Center for Biotechnology Information website - NCBI (www.ncbi.nlm.nih.gov).

This analysis was performed to compare sequences with the characterization from the TaqMan real-time PCR technique.

We analysed the antimicrobial susceptibility from the 28 isolates using the disc diffusion test, following the protocol described by the Clinical and Laboratory Standards Institute guidelines, using *Staphylococcus aureus* (ATCC 2923) as a control [15]. The tested antimicrobial agents were as follows: norfloxacin (10 µg), amoxicillin (10 µg), enrofloxacin (5 µg), ceftiofur (30 µg), tetracycline (30 µg), neomycin (30 µg), trimethoprim/sulfamethoxazole (1.25 µg / 23.75 µg), lincomycin-spectinomycin (109 µg), fosfomycin (50 µg), apramycin (15 µg) and oxytetracycline (30 µg). All used discs for the diffusion test analysis belonged to the Oxoid®'s brand. We selected such antimicrobial agents due to their common usage in the poultry industry either as growth promoters or as disease prevention treatments. As for the neomycin and apramycin, we used them to characterize the bacteria. We defined isolates as multidrug resistant when they showed resistance to ≥ 3 classes of antimicrobial agents [15].

We performed an epidemiological analysis by measuring the association between the average number of mortality and positivity for ES2 using the Fisher's exact test followed by an analysis of differences between proportions ($p < 0.05$) and the odds ratio calculation OR. The prevalence and antimicrobial resistance of ES2 were analysed with descriptive statistics. We used GraphPad Prism (version 7.04).

3. RESULTS

We isolated and confirmed through real-time PCR the presence of ES2 in 15.25% (18/118) of the flocks that had samples sent to the laboratory. In the 18 positive flocks with 160 samples, there were 58 ES2 isolations. ES2, as shown in Figure 1, was isolated from

176 samples of liver (88.9%, 16/18), spleen (83.3%, 15/18), heart (55.55%, 10/18), lung (38.9%,
177 7/18), kidney (27.77%, 5/18), trachea (22.2%, 4/18) and joint (5.5%, 1/18). Birds in ES2-
178 positive flocks were 111 days old, on average, being the most frequent reported clinical signs
179 by the veterinarian in the affected flocks, weakness and increased mortality. The mainly
180 observed gross lesions were friable, enlarged and in some cases mottled liver, spleen and
181 kidneys. In some flocks, turkeys also had slight catarrhal enteritis.

182 We compared the mortality mean from the 18 ES2-positive flocks with 10 assigned
183 flocks, which stated negative for the same pathogens. We found a positive association
184 between mortality rate and ES2 incidence. The mortality mean rates for positive and negative
185 flocks were 6.87% and 3.76%, respectively (Table 1). The OR results showed that there are
186 1,1.826 times more chance of positive flocks to die than negative flocks.

187 **Table 1.** ES2 isolates and positive association with bird's mean mortality.

	Dead turkeys	Alive turkeys
Pos turkeys	694	10,105
Abs turkeys	397	10,553

Pos: ES2-positive individuals; Abs: ES2-negative individuals. $p<0.0001$.

188 The sequences of 16S rRNA from the six strains of ES2 are shown in Table 2. Studied
189 isolates had similarity from 99 to 100% with those strains already deposited in the GenBank
190 database.

191 **Table 2**. ES2 strains and its 16S-rRNA sequence along with the comparison with GenBank
192 database.

Strain	Identification	Size (bp)	Accession number of the homologous strain in GenBank	% Homology*
1	F: *Erysipelothrix tonsillarum*	1138	NR_113036.1	99%
	R: *Erysipelothrix rhusiopathiae*	905	KP063151.1	99%
2	F: *Erysipelothrix rhusiopathiae*	1263	CP005079.1	99%
	R: *Erysipelothrix rhusiopathiae*	920	KP063151.1	99%
3	F: *Erysipelothrix rhusiopathiae*	785	CP005079.1	99%
	R: *Erysipelothrix rhusiopathiae*	911	KP063151.1	100%
4	F: *Erysipelothrix rhusiopathiae*	1167	CP005079.1	99%
	R: *Erysipelothrix rhusiopathiae*	914	KP063151.1	100%

5	F: *Erysipelothrix tonsillarum*	1207	NR_113036.1	99%
	R: *Erysipelothrix rhusiopathiae*	1247	KP063151.1	99%
6	F: *Erysipelothrix tonsillarum*	1185	NR_113036.1	99%
	R: *Erysipelothrix rhusiopathiae*	922	CP005079.1	99%

F: Forward; R: Reverse

* Criteria for species identification: minimum > 99% sequence similarity (ideal: >99.5%).[29]

193 Twenty-eight isolates of ES2 were analysed for antimicrobial resistance. The tested
194 isolates showed complete resistance to antimicrobial agents from the aminoglycoside group
195 (e.g., neomycin and apramycin); a high degree of resistance for trimethoprim-
196 sulfamethoxazole (82.2%) and fosfomycin (60.7%); and intermediate or low resistance for
197 tetracycline (25%), ceftiofur (10.7%), enrofloxacin (7.1%), oxytetracycline (3.6%),
198 amoxicillin (3.6%), norfloxacin (3.6%) and lincomycin-spectinomycin (3.6%).

199 The analysis of antibiotic action sites showed that 67.85% of the isolates were resistant
200 to at least one of the cell-wall-acting antimicrobials (ceftiofur, amoxicillin and fosfomycin),
201 decreasing to 10.71% if fosfomycin was excluded. When considering the antimicrobials that
202 inhibited nucleic acid synthesis, 7.14% of the isolates were resistant to at least one of the
203 antimicrobials (enrofloxacin and norfloxacin). As for the antimicrobials that block protein
204 synthesis, 28.57% were resistant to at least one of the antimicrobials (tetracycline,
205 oxytetracycline and lincomycin-spectinomycin), excluding apramycin and neomycin, which
206 failed to inhibit bacterial growth in all cases. 82.1% were resistant to trimethoprim-
207 sulfamethoxazole, which targets folate synthesis.

208 All isolates were resistant to at least one antimicrobial agent, and 18 isolates
209 (64.28%) were resistant to 3 or more classes of antimicrobial agents and were thus considered
210 multidrug resistant. Eight isolates (28.57%) were only resistant to two antimicrobial classes.
211
212
213

4. DISCUSSION

Erysipelas in poultry leads to septicemia and is characterized by an acute and fulminating infection. The disease is of great importance in turkeys, even though having recent outbreaks in chickens only occurring in the European continent [16, 17, 18]. Clinical signs and gross lesions found in the present study for ES2-positive flocks are in accordance with the reported outbreaks of *E. rhusiopathiae* in turkeys. Erysipelas can cause sudden increase in the mortality rate of turkey flocks, increasing in the following days to the rest of the individuals of the same flock. Even though the mortality rate for outbreaks of *E. rhusiopathiae* in poultry has already been previously reported [8], this is the first study of mortality caused by ES2. Nevertheless, the mortality rate showed in this study (6.87%) could be higher. To guarantee the animal welfare and prevent economic losses, several flocks received antibiotic treatment in drinking water after the onset of the mortality. Therefore, the mortality reported by this study did not represent the natural course of the disease.

Although *E. rhusiopathiae* has been considered pathogenic for turkeys at any age [8, 19] in our study, the epidemiological analysis showed that ES2 was only clinically diagnosed after 70 days of bird's age. On average, the birds were 111 days old at the onset of the disease. Our results showed a positive association between mortality rate and ES2 incidence after 70 days of age (Table 1). Other studies have shown that *E.rhusiopathiae* prevalence and mortality are higher in older birds. Nevertheless, experimental inoculation of a field isolate of *E.rhusiopathiae* in specific pathogen- free (SPF) laying hens found no mortality in 119 days old birds and 100% mortality in birds 259 days old [20, 21].

We have primarily isolated ES2 from samples from the following organs: liver, spleen, heart, lungs, kidneys, trachea and joints (Figure 1). Such organs are important sites of bacteria isolation in septicemia outbreaks in poultry such as caused by *Pasteurella multocida*, *Salmonella* Gallinarum, avian pathogenic *Escherichia coli* and *E. rhusiopathiae*. In case of

239 future outbreaks veterinarians must collect samples of the cited organs to succeed the
240 laboratory ES2 isolation.

241 A previous research, utilizing selective culture, isolated *E. rhusiopathiae* from
242 jejunum contents during an outbreak of erysipelas in organic laying hens. Besides, *E.*
243 *rhusiopathiae* was isolated from manure, dust and swabs from water nipples [17]. Therefore,
244 isolation from manure and intestinal contents indicates a faecal-oral transmission route in
245 poultry during the disease caused by *E. rhusiopathiae*. We hypothesize that the faecal-oral
246 route of ES2 contamination plays an important role in the disease epidemiology.

247 Nevertheless, we have not found ES2 in intestine samples, even though catarrhal
248 enteritis occurred in some flocks. Since we did not use any selective agar to isolate ES2 from
249 intestine and other bacteria could have overgrown it on non-selective agar media, it is hard to
250 be sure whether the ES2 was present or not in intestine in the outbreaks studied. For future
251 outbreaks, selective agar should be used in intestine samples. Besides, in the studied
252 outbreaks, catarrhal enteritis could be caused by intestinal pathogens, since the overall
253 immune system of the birds can be affect by septicemia.

254 Even though in the present study the source of infection was not investigated, ES2 was
255 found positive in tracheas in 22.2 % of the flocks. Despite the lack of information about the
256 long-term survival of the bacteria in dust, this source of contamination inside the flock area -
257 and the possibility that contaminated dust can be spread throughout different places - must be
258 considered. In the present study, only two properties showed recurrence of erysipelas in the
259 succeeding flocks. In such cases, it is possible that insufficient down time periods and
260 improper disinfection have occurred [8], which in turns have led to ES2 recontamination.

261 High resistance to the aminoglycoside group was already expected, since other
262 researches have showed that *E. rhusiopathiae* was resistant to neomycin [22, 23] and
263 apramycin [22]. Neomycin is commonly included in broth and agar to inhibit other

264 contaminating bacteria and further *E. rhusiopathiae* isolation [24, 25, 26]. Thus, our findings
265 show that ES2 can be characterized by intrinsic resistance to apramycin and neomycin, and
266 the use of neomycin as a contaminant inhibitor may be used for ES2 isolation then.

267 The indiscriminate use of antimicrobials in the poultry industry could have led to the
268 high resistance to trimethoprim-sulfamethoxazole and fosfomycin found in this study.
269 Surprisingly, we found low resistance to norfloxacin, enrofloxacin, amoxicillin and
270 oxytetracycline. We did not expect such results, as these antimicrobial agents have been used
271 in recent years as growth promotors and are currently being used to treat several diseases in
272 poultry-producing farms. The resistance to oxytetracycline and enrofloxacin are in accordance
273 with a study of antimicrobial susceptibility performed with *E. rhusiopathiae*, which found
274 2.7% and 12.8% resistance, respectively [27].

275 A low resistance to ceftiofur was expected, as its use is not common in turkey-
276 producing farms**.** Our findings showed 25% of resistance to tetracycline, a considerable
277 resistance, even though previous studies with *E. rhusiopathiae* showing that the high
278 susceptibility to tetracycline used to treat swine erysipelas had changed. The *tet* (M) gene in
279 naturally occurring *E. rhusiopathiae* is believed to be tetracycline-resistant, and this gene was
280 considered necessary for the expression of tetracycline resistance in *Escherichia coli* [28, 29].

281 Since the classification of the *Erysipelothrix* genus into six separated species*,* ES2 had
282 not been isolated from outbreaks of erysipelas in poultry [26, 16, 18]. Before the classification
283 of ES2 as a novel species, it has been classified as *E. rhusiopathiae* serotype 18. Its
284 pathogenicity was demonstrated in an experimental test with all twenty-three serotypes of *E.*
285 *rhusiopathiae*, with *E. rhusiopathiae* serotype 18 inducing local urticarial lesions in swine and
286 being highly virulent to mice [1].

287 *E. rhusiopathiae* infection in man occurs as an occupational disease. Human infection
288 can take three forms, as follows: a mild cutaneous infection known as erysipeloid, a diffuse

289 cutaneous form and a serious but rare complication with septicaemia and endocarditis [7].
290 Since this study was the first in naturally occurring outbreaks caused by ES2 in farmed
291 animals, the pathogenicity of ES2 for humans is still not clear. ES2 in turkeys causes *E.*
292 *rhusiopathiae*-like, which led us to speculate about the possible zoonotic potential of ES2 and
293 its importance for the public health. We suggest future studies to analyse such possibility.

294 The use of molecular tools based on 16S rRNA gene sequencing is recognized by the
295 fact that the 16S region is highly conserved among bacterial species, which is more easily
296 sequenced and is considered a powerful method for microbial taxonomic identification [30].
297 In addition, it is possible to compare the identified sequences to the GenBank dataset,
298 allowing for new sequences identification. Although 16S rRNA gene sequencing is widely
299 used for bacterial identification, the technique has already been described as having low
300 phylogenetic power at the species level and low discriminatory power for some genus [31].
301 The difficulty to engage in phylogenetically distinct species and/or genus relies on the fact
302 that there are still a low number of sequences deposited in 16S rRNA sequences database.
303 Therefore, more sequences are needed to reduce ambiguity and increase phylogenetic power
304 of ES2 sequences.

305 Hence, the use of the 16S rRNA region sequencing for microbial identification should
306 be applied with caution, and it should also bring together a harmonious set of guidelines for
307 the sequence interpretation, so that the results of a study could be compared with precision
308 with other ones [32]. Therefore, DNA-sequencing of bacteria is necessary to provide solutions
309 to taxonomic problems, as is the case of the use of real-time PCR, which has been previously
310 described as being simple, rapid, reliable, specific and highly sensitive for identification and
311 discrimination, mainly of species of the genus *Erysipelothrix* [14]. Future studies should be
312 performed using whole genome sequencing of ES2 to verify whether further evaluation of
313 other regions is required to discriminate from different species.

We have not isolated ES2 after the outbreaks that took place in 2015, we believe that the downsizing of the turkey production in the area culminating with the complete ending recently, rather than the ES2 elimination was responsible for the absence of new outbreaks.

So far most of the studies have stated that only *E. rhusiopathiae* causes erysipelas in poultry, but in the present study we isolated ES2 associated with septicemia and increased mortality. Therefore, it might be possible that ES2 dissemination has occurred before this report, but it was not correctly identified, due to misleading biochemical analysis. In conclusion, the results of the present study demonstrated for the first time ES2 causing disease and mortality in turkeys and high index of isolated antimicrobial resistance. Besides we found ES2 16S rRNA sequence to be very similar from those of *E. rhusiopathiae* and *E. tonsillarum*. We suggest that other studies to be conducted to evaluate the possible public health issue due to ES2 incidence in turkey-producing farms.

DATA AVAILABILITY STATEMENT

The authors declare that the data presented are available for the journal.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

PH and TF associated the outbreak with the *Erysipelothrix* strain 2, isolated and identified the bacterium and conducted the revision of the English language. PH and BF have developed and planned the project, analysed and interpreted the data and wrote the manuscript. TR, DR and EM wrote the manuscript, isolated and kept the bacterium. PK, JZ carried out the epidemiological date of the field and assisted in the analysis of the data. EV and BB interpreted the sequencing data and reviewed the rules for the journal.

CAPÍTULO 3

FIRST REPORT OF THE EPIDEMIOLOGY AND GENETIC VARIABILITY OF *ERYSIPELOTRIX* SP. STRAIN 2 IN TURKEYS THAT CAUSES MORPHOMETRIC ALTERATIONS IN VERO CELLS

Artigo a ser publicado no periódico

Frontiers in Cellular and Infection Microbiology

FIRST REPORT OF THE EPIDEMIOLOGY AND GENETIC VARIABILITY OF *ERYSIPELOTRIX* SP. STRAIN 2 IN TURKEYS THAT CAUSES MORPHOMETRIC ALTERATIONS IN VERO CELLS

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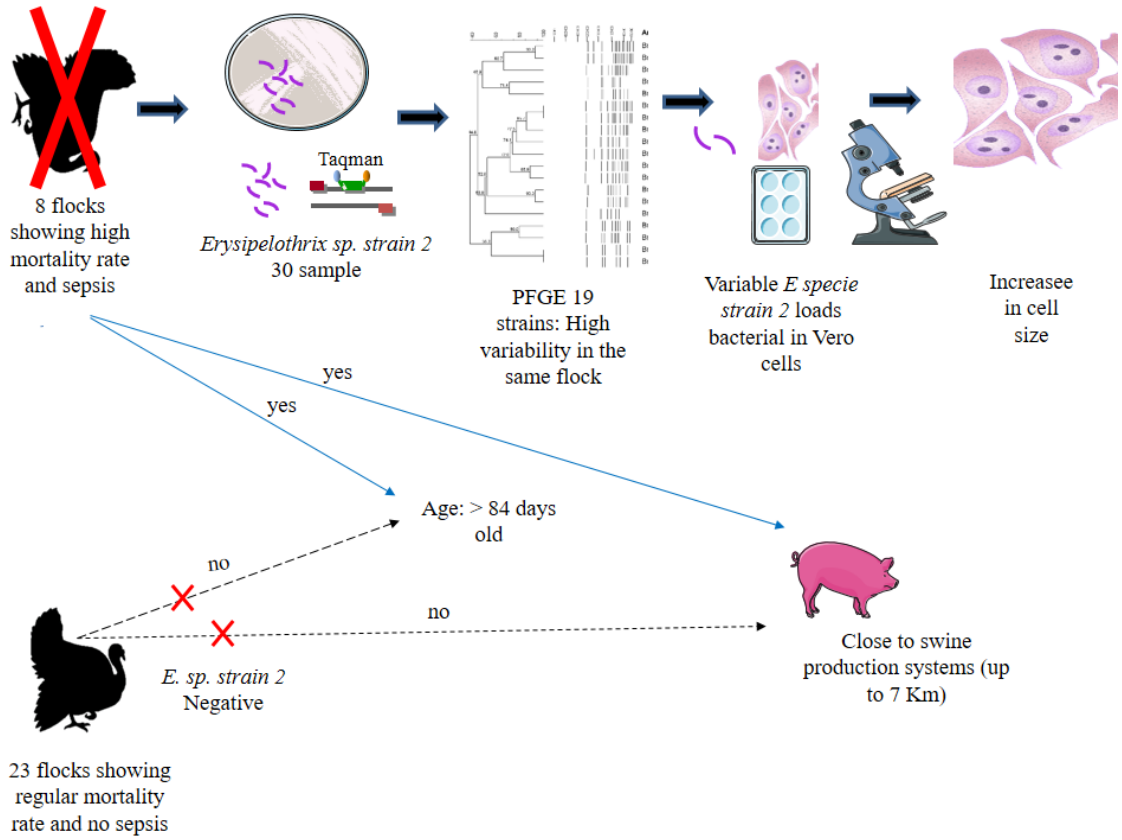
Abstract

Erysipelas is an illness caused by species of the *Erysipelothrix* genus whose main specie is *E. rhusiopathiae*. In this study, we carried out diagnoses and epidemiological analyses of commercial and breeder turkeys in Brazil with high mortality and sepsis. From 18 analyzed flocks (182 samples), 8 flocks (84 samples) presented clinical symptoms and high mortality. All samples from sick flocks were positive for *Erysipelotrix sp.* strain 2, and no sample was positive for other species of *Erysipelothrix* genus. Through epidemiological analysis, we have found a positive correlation between *Erysipelotrix sp*. strain 2 positive flocks and the parameters of final mortality and age of the animals during the outbreak and positive association with proximity with commercial swine plants (up to 7 km). The PFGE analyses of 19 samples presented 2 clones, both from the same flocks in different animals and 2 clusters, both from the same flocks. However, there were 15 samples with high genetic variability from the same flocks or/and same birds. We checked the morphometry of Vero cells infected by different amounts of *Erysipelotrix sp*. strain 2 and we verified a nucleus and nucleolus increase. We saw that the infected cell had higher apoptotic and necrotic cells and the bacterium multiplies in the extracellular environment and survives inside the cells. For the first time a study presents important data regarding the genetic variability of *Erysipelotrix sp*. strain 2 isolated from turkeys as well as the cellular alteration caused by it.

Keywords: Erysipelas, Injuries, Transmission Electron Microscopy, PFGE, Similarity



Graphical abstract: Turkey flocks showing high mortality rate and sepsis were positive for *Erysipelothrix sp. strain 2*. We found an association between positive flocks and proximity to swine production systems (up to 7 Km) and a correlation with age (>84 days). Isolated strains showed high genetic variability within and between flocks. *Erysipelothrix sp. strain 2*-infected cells showed increased area and perimeter as well abnormal nucleus and nucleolus size.

1. Introduction

Erysipelas is considered a cosmopolitan illness, which occurs in several types of vertebrates, domestic and wild animals and human as well (WOOD, 1999). *Erysipelothrix rhusiopathiae* is the main agent which causes the illness and increased cases of erysipelas in swine and chickens have been observed in many countries recently (HOLLIFIELD; COOPER; CHARLTON, 2000; TO et al., 2012). In turkeys, *Erysipelothrix* spp. causes an acute infection, septicemia, feed intake reduction, carcass condemnation, mortality and male infertility (Bricker and Saif, 2013).

Bacterias from the genus *Erysipelothrix* are gram-positive and rod-shaped (Brooke and Riley, 1999). They belong to the phylum Firmicutes, class Erysipelotrichia, order Erysipelotrichales and family Erysipelotrichaceae (Verbarg, 2004). From this genus, the known species are *E. rhusiopathiae*, *E. tonsillarum*, *E. inopinata, Erysipelothrix* sp.strain 1 and *Erysipelothrix* sp. strain 2 (Takahashi, et al. 1992; Verbarg, et al. 2004) as well as the last identified *Erysipelothrix* larvae sp. nov. (Bang,et al., 2016).

Initially, only *E. rhusiopathiae* was considered within the genus *Erysipelothrix.* However, studies using DNA-DNA hybridization (Takahashi, et al. 1992), multilocus enzyme electrophoresis (Chooromoney, et al. 1994) and restriction fragment length polymorphisms (Ahrné, et al. 1995) revealed distinct species*, E. rhusiopathiae*, *E. tonsillarum*, *Erysipelothrix sp*. strain 1 and *Erysipelothrix sp.* strain 2 (Takahashi, et al. 1992; Takeshi, et al. 1999). Previously to these studies, *Erysipelothrix sp.* strain 2 was identified as *E. rhusiopathiae* serotype 18. All 18 serotypes of *E. rhusiopatiae* were shown as highly virulent for mice and pathogenic for swine, causing localized urticaria (Takahashi, et al. 1992).

Erysipelas is considered an emerging disease in poultry production (Janßen, et al. 2015). Previous outbreaks in turkeys were caused by *E. rhusiopathiae*. Thus, there is a limited understanding of the disease caused by *Erysipelothrix sp.* strain 2 on a global scale. In addition, *Erysipelothrix* species (*E. rhusiopathiae* and *Erysipelothrix sp.* strain 2) may be misidentified during outbreaks due to great biochemical and phenotypic similarities in addition to highly similar infection symptoms between the two species.

During an event of the disease outbreak, it is important to identify genetic differences among strains to better understand the epidemiology of the disease (Forde, et al. 2016). Pulsed-field gel electrophoresis (PFGE) plays a key role in identifying the outbreak-associated isolates and their genomic variability and thus, PFGE provides an excellent epidemiological tool. Considering that, the hosts geographic classification and localizations are useful in the attribution of the species epidemiological identification (Muellner, et al. 2013), the assessment of PFGE associated with geographical locations enriches the epidemiological assessment.

The evaluation of cellular cytotoxicity by a new type or a little studied specie is an important information prior to its inoculation in animals. One of the ways to measure the cell alteration caused by a pathogen is to study its cell morphometry. This tool offers a better evaluation when comparing only the cells morphological changes.

To develop a better understanding of the pathogen, the aim of this study was to report the occurrence of sepsis and mortality related to outbreaks of *Erysipelothrix sp.* strain 2 in turkeys, and for the first time, to evaluate the epidemiology and genetic variability of the isolated strains and finally, to study the morphometric alterations of this pathogen in Vero cells.

2.Materials and Methods

2.1 Samples

From December 2014 to March 2015, there was an outbreak causing sepsis and mortality in commercial turkeys from a large Brazilian poultry company integrator. A high mortality rate was observed in flocks that had not received the antimicrobial treatment during the onset of clinical signs, such as weakness and increased mortality. During necropsy, the lesions observed were suggestive of generalized septicemia, mainly friable, enlarged and, in some cases, mottled liver, spleen and kidneys, and some turkeys also had slight catarrhal enteritis. In another research (data not yet published) we observed association between the mortality and *Erysipelothrix sp.* strain 2.

In the study presented in this manuscript, we aimed to select some strains of *Erysipelothrix sp*. strain 2 for our work. Initially, we worked with 45 commercial turkey flocks with similar house structure, food and water management, being 44 Nicholas line male broiler flocks (from 36 days old until the age of slaughter) and one Nicholas line turkey breeders flock (which had clinical disease or not). We necropsied turkeys right after death and we collected samples of liver, lung, spleen, kidneys, trachea, intestine and heart, which were sent to the laboratory for cultivation and identification. From each flock we collected one sample from the same organ separately in pools of 2 to 3 freshly killed birds (up to one hour after death). Although we worked with freshly killed birds, we informed the Committee on Ethics in the Use of Animals (CEUA) of the Federal University of Uberlandia upon the advice number A004/19.

As previously mentioned, as there had been an outbreak of *Erysieplothrix* sp. strain 2 previously, we already suspected that this microrganism was the cause of high mortality. However, in order to select the flocks to be worked, we evaluated in the same tissues *Erysipelothrix* spp. microorganisms of veterinary interest that isolated or in co-infections can cause symptoms of high mortality and septicemia, such as *Pasteurella multocida*, Avian Pathogenic *Escherichia coli* (APEC), *Bordetella avium*, *Ornithobacterium rhinotracheale, Salmonella* spp., *Streptococcus* spp., *Staphylococcus* spp., *Aspergillus* spp., *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. Positive flocks to APEC, *B. avium, Salmonella* spp. *Ornithobacterium rhinotracheale, M. gallisepticum* and *M. synoviae* were not included in the study. These flocks were also monitored serologically by the ELISA method (IDEXX) for New Castle disease and turkey rhinotracheitis and none of them presented problems related to these diseases.

After analyses, we selected 18 flocks, being 8 with clinical signs and 10 without clinical sighs. We studied 182 samples, being 84 from positive flocks and 98 from negative flocks (supplementary material 1 and 2). Within the positive flocks, 7 were commercial

198 turkeys flocks and one, breeder turkeys. We performed all laboratory analyses, but the
199 company provided the final mortality data.

200
201 **2.2 Identification of *Erysipelothrix* spp.**

202 We analysed the samples in the animal health laboratory, inoculated on sheep Blood
203 (OXOID) and MacConkey agar (OXOID) and incubated aerobically at 37°C for 18 to 24
204 hours. We considered the following characteristics to select the colonies to *Erysipelothrix* spp
205 in sheep blood: circular, 0.1-0.5 mm in diameter, clear appearance, narrow zone of partial
206 hemolysis, non-motile gram-positive rods, negative for catalase, oxidase and indol, as well as
207 producing H_2S in triple sugar agar. We subcultured the colonies and performed the Taqman
208 Real Time PCR to differentiate among *E. rhusiopathiae, E. Tonsillarum* and *Erysipelotrix* sp.
209 strain 2.

210
211 **2.3 Real Time PCR**

212 The DNA primer set and three probes (Table 1) used were specific for the detection of
213 *E. rhusiopathiae, E. tonsillarum* and *Erysipelothrix sp.* strain 2. We used Blood and Tissue kit
214 (Qiagen®) for DNA extraction, following the manufacturer's instructions. For each test, we
215 used positive controls of *E. rhusiopathiae* and *E. tonsillarum.* The TaqMan real-time PCR
216 was performed as described (Pal et al., 2010) with few modifications. The amplification and
217 identification were performed in one reaction for each species. Each reaction consisted of a
218 total volume of 25 µl containing 12.5 µl of the commercial master mix (TaqMan® Universal
219 PCR master mix, Applied Biosystems®), 5 µl of DNA, 0.4 µmol of each primer and 0.2 µmol
220 of each probe. The reactions were carried out in a StepOne Real-Time PCR system (Applied
221 Biosystems®) in cycling parameters: 2 minutes at 50°C and 10 minutes at 95°C, followed by
222 50 cycles of 15 seconds at 95°C, 30 seconds at 55°C and 50 seconds at 60°C. We analyzed
223 the results with StepOne Plus (version 2.1) software. We considered samples to be positive
224 when the Ct value was ≤ 35, and negative when the Ct was > 35. For each test, we used
225 positive controls of E. rhusiopathiae and E. tonsillarum isolated from swines kindly provided
226 by Professor Dr. David Emílio Santos Neves de Barcellos.

227 **Table 1.** Specific primers and probe sequences used in this study.

Primer/Probe Identification	Sequence*
Ery4423 Forward	5'-ATTTCTCTAGCAGGTGATTGG-3'
Ery4587 Reverse	5'-ACCCTCTAATCGATATGCATCA-3'
E. rhusiopathiae probe	5'-FAM-AACGAAACGATTAGTAGTCCAACA-TAMRA-3'
E. tonsillarum probe	5'-FAM-AAATATTCATGAGACAATCAGCAGT-TAMRA-3'
Erysipelothrix sp. strain 2 probe	5'-FAM-CGAAGGGTTTAAATATTTCTGAGAC-TAMRA-3'

* Pal et al. (2010)

228

2.4 Transmission electronic microscopy

We used the Transmission Electron Microscopy (TEM) in order to test bacterial ultrastructure. The analyses were performed in Electron Microscopy Center in Federal University of Uberlandia. Strain 6 and 7 were fixed and washed with 0.1 M phosphate with buffer (pH 7.2), centrifuged at 2900 rpm for 20 minutes and placed on agar 1%. Resultant material was counted in cubes of approximately 3 mm^3 and placed in osmium tetroxide at 1% in phosphate buffer at 0.1 M (pH 7.2) for 1 hour with treatment of 1% of osmium tetroxide plus and 1.25% of potassium ferrocyanide for 30 minutes. The fragments were dehydrated in increasing concentrations of acetone, placed in epon resin and cut using ultramicrotome, so that we could obtain ultrathin cuts. Such cuts were used to make comparisons by uranyl acetate and lead nitrate on nickel small screens (Bozzola and Russell, 1998). Small screens were analyzed in a transmission electron microscope (Hitachi HT - 7700) with ESPRIT data acquisition and analysis software.

2.5 Pulsed field gel electrophoresis (PFGE)

We evaluated the genetic similarity among 19 isolates of *Erysipelothrix sp.* strain 2 using pulsed field gel electrophoresis following the protocol recommended by CDC (2013) with modifications. We worked with only 19 isolates because the others did not survive to our previous conservation methodology. The strains used were: Est02, Est03, Est04, Est06, Est07, Est08, Est09, Est11, Est12, Est14,Est16, Est17, Est18, Est23, Est24, Est26, Est28, Est29 and Est30.

Bacteria was cultivated in BHI (brain heart infusion) agar supplemented with 5% defibrinated lamb blood and incubated at 37ºC. We have treated the strains with SmaI restriction enzyme (40 U/uL) to continue with the plug modeling. In addition, the isolated fragments resulted from pulsed field electrophoresis. The electrophoresis conditions consisted of an initial switch time of 2.2 s and a final switch time of 64 s, with a gradient of 6 V/cm for 21 hours. The DNA fragments were separated on 1% agarose gel (SeaKem Gold) in 0.5X TBE buffer on CHEF-DR® III Pulsed Field Electrophoresis Systems (Bio-Rad) and buffer temperature at 14°C.

We stained the gels with ethidium bromide and photographed under UV light. A dendrogram was built using Bionumerics 7.6. The obtained patterns were analyzed using the Dice similarity coefficient with tolerance of 0.5% and the dendrogram modeling was performed by the UPGMA analysis method (Unweighted Pair Group Method with Arithmetic Mean).

2.6 Epidemiological and Statistical Analysis

Data obtained from the *Erysipelothrix sp.* strain 2 positive and negative flocks were compared. The analyzed parameters were the following: mortality rate, age at the outbreak and proximity to swine production (up to 7 km). All poultry houses had similar technology to control the environment inside the facilities such as fans, nebulizers and light control. The system to provide water and feed was also the same, as well as the source of feed and poultries. The largest distance among the farms was 110 km. Although the commercial

turkeys' farms had good biosafety standards, disinfection tools and mandatory shower for staff and visitors were practiced only in the breeder farm.

To evaluate the proximity to swine flocks, we considered a distance up to 7.0 km from the turkey farms. Only the presence of swine farms from the same company was considered because we did not have information about the proximity of poultry or swine farms from other companies or the proximity of backyard poultry or pigs. Distances among infected-turkey farms and swine farms from the same company were mapped using Google Maps.

Data were tested for normality assumptions using Kolmogorov Smirnov test. Spearman correlation coefficient was calculated among pairs of variables. Bacterial incidence (presence or absence) was used as the response variable, being correlated to the following variables on separate models: mortality rate and swine presence and age of the birds on the onset of the disease outbreak. We also used the odds ratio analysis to test for *Erysipelothrix sp.* strain 2 presence and swine proximity, the distance up to 7.0 km of swine plants from the turkey farms. Lastly, we performed ANOVA to test for differences in cell morphometric with regards to the bacterial incidence, with 95% of confidence level. All analyses were performed in GraphPad Prism 7.04.

2.7 Cellular morphometry

To test the *Erysipelothrix* sp. strain 2 cells change capacity we used Vero cell morphometric evaluation with different quantities of the bacterial inoculum. Nakamura and collaborators in 1962 in Japan, designated the Vero lineage, originating from the kidney of African Green Monkey (Cercopithecus aethiops) as a model for laboratory experiments (Bretas, 2011).

We seeded Vero cells in DMEM (Dulbecco's Modified Eagle Medium) enriched with 5% bovine fetal serum in circular laminules with coverslips and placed in a 6-well plate at a density of 1.68x104 cells/well and incubated for 24 hours. We measured cell growth within each sample and inoculated in triplicate two *Erysipelothrix* sp. strain 2 isolated (Est06 and Est07), using the following bacterium inoculum: D0: >1 Log CFU.well; D2: 2 Log CFU.well; D6: 6 Log CFU.well and NC: Negative control treated with same diluent used for bacteria (PBS) but sterile. After 4 and 24 hours the coverslips were removed and fixed in formalin 10%, washed in PBS, stained with panoptic dye, and lastly placed on slides with Entellan (Merk). The visualization and photographic documentation were carried out under a camera-coupled (Olympus camera, 200) microscope, type Olympus BX 40 under 100x magnification, using the Data Translation 3153 software. The images editing, processing and analyses were performed through the image J program, version 1.51 2017. A total of 8 different fields per slide were evaluated, totaling 64 cells per slide. We measured the following parameters: Nucleus Area, Nucleus perimeter, Nucleolus area and nucleolus perimeter.

2.8 Apoptose

We seeded Vero cells in DMEM (Dulbecco's Modified Eagle Medium) enriched with 5% bovine fetal serum in circular laminules and placed in a 6-well plate at a density of $2x10^4$ cells/well and incubated for 48 hours at 37 ^{o}C and 5% CO2 atmosphere when the cells were confluent. In this moment, we inoculated in triplicate: (i) *Erysipelothrix* sp. strain 2 isolated Est06, (ii) *Erysipelothrix* sp. strain 2 isolated Est07 both using 3 log CFU/well, (iii) 3 log CFU/well *Lactobacillus* spp (LB) isolated from Probiotic milk beverage fermented (CALU) in MRS agar (deMan, Rogosa and Sharpe) (OXOID) and (iv) NC: Negative control treated with same diluent used for bacteria (PBS) but sterile.

After 4 and 24 hours the cells were three times washed with PBS, treated with Yo Pro-01 (YP) and propide iodate (PI) (1:1000 each) (Invitrogen) during 30 minutes at room temperature. For labeling of apoptotic cells, we used YP and to mark cells in necrosis, we used PI. After treatment of the cells with YP and PI, we washed the laminates for three times and fixed with 4% formalin for 10 minutes. Then we treated the cells with Hoechst (Sigma) to mark the DNA of the cell. The laminates were washed and mounted in anti-fading ProLong (Invitrogen). The counts and counts were evaluated under a fluorescence microscope. From each slide, five different fields were evaluated.

2.9 Invasion Test

After 24 hours, we made a bacterial invasion assay and we quantified the bacteria in the extracellular medium. For this, after 24 hours, 100uL of the supernatant was withdrawn and after serial dilutions, was counted in blood agar or MRS agar for *Erysipelothrix sp*. strain 2 and for *Lactobacilus* spp. respectively. After this, the cells were four times washed with PBS and in the fourth wash, there was another bacterial count. The cells were treated with trypsin for the removal of the plate's cells, then centrifuged (1500rpm/10min), treated with triazole for cell membrane disruption and release of intracellular bacteria and then washed four times (1500rpm/10min), and the bacteria counted again.

3. Results

3.1 Identification of *Erysipelothrix* sp. strain 2

Out of 182 analyzed samples from 18 flocks, 84 (46,15%) samples were positive for *Erysipelothrix* sp. strain 2, which represents 44,44% (8/18) of positive flocks. All positive samples were collected from 8 flocks (7 commercial turkey flocks and 1 breeder turkey flock). In the positive flocks, no other isolates belonged to another species of *Erysipelothrix* spp. other than *Erysipelotrix* sp. strain 2. All 62 samples from 23 flocks from turkeys without clinical symptoms were negative for *Erysipelotrix* spp. Some flocks had the samples collected at more than one age (supplementary table 2).

3.2 Electron microscopy of *Erysipelothrix* sp. Strain 2

The photographic documentation of the Erysipelothrix sp. strain 2 was carried out using TEM (figure 1). Photomicrograph of *Eryispelothrix sp*. strain 2. is showning two different cutting positions: longitudinal section in rod format (Fig. A and B) and cross section (Fig. A, B, C and D). The bacterial wall was visualized (Fig. A, C and D) as well as cytoplasmic membrane (Fig. B, C, and D). We measured the mean size of the bacteria in bacillary form and the value found was 2.16umX0.55um.

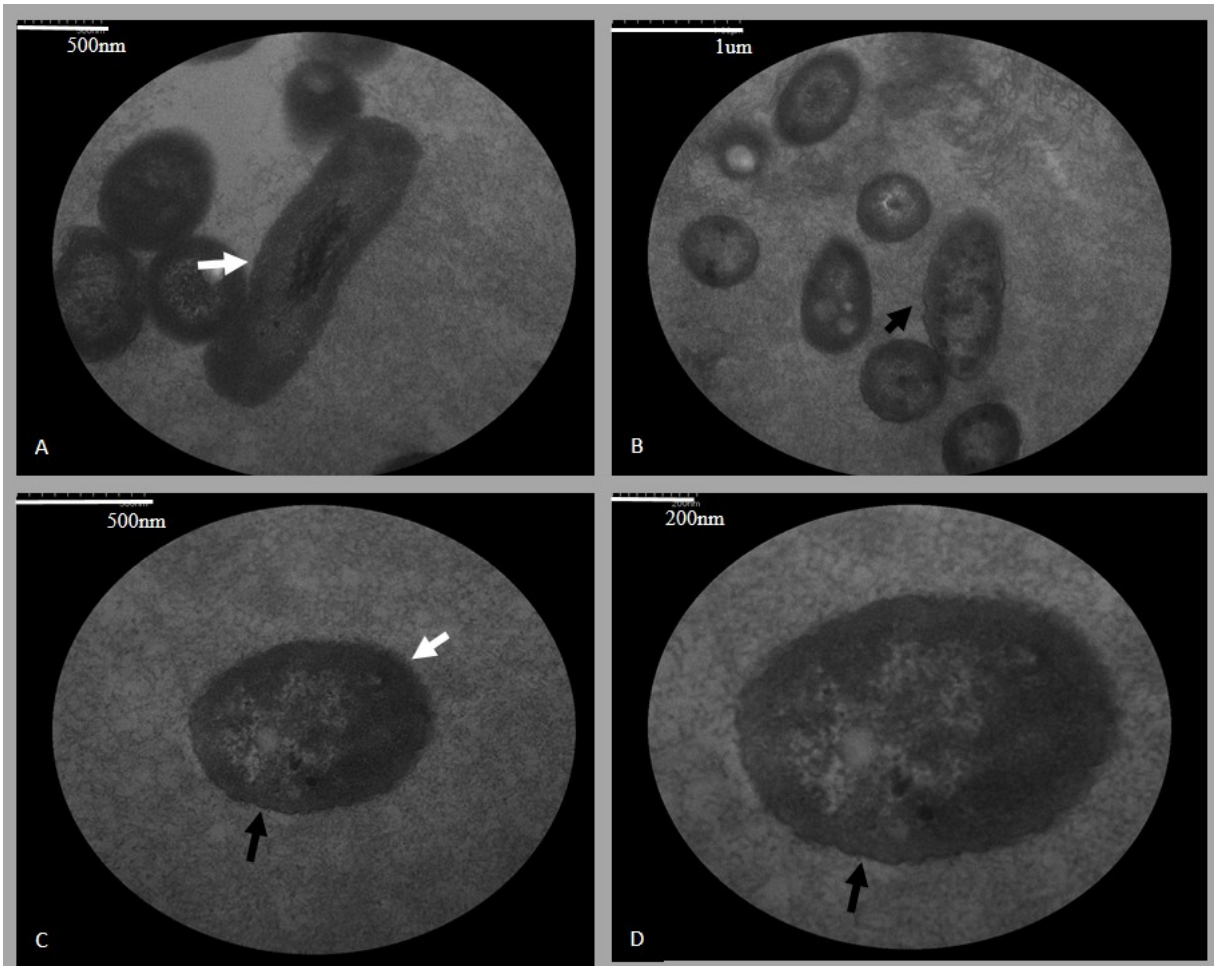


Figure 1. Photomicrograph *Erysipelotrix* sp. strain 2 in four cutting positions. In A an B the bacterium rod format (longitudinal section) and cross section. In A, C and D part of the bacterial wall can be visualized (white arrow). In B, C and D cytoplasmic membrane can be visualized (black arrow).

3.3 Epidemiologic analysis and PFGE

For epidemiologic analysis, we used those data kindly provided by the company. The parameters of final mortality rate, poultries age, final feed conversion and proximity to swine farms (up to 7 km) were evaluated.

We found positive correlation among animals age during the outbreak, final mortality rate and proximity to swine farms (table 2). There was no correlation with feed conversion.

Table 2. Correlation between presence of *Erysipelothrix* sp. strain 2 and mortality rate, proximity of swine farms, feed conversion and poultries birds' age during the outbreak

	Final Mortality Rate	**Age of animals**
Positive correlation	Yes	Yes

P	<0.0001	<0.0448
R	0.8027	0.3967

p: significance r: Correlation coefficient

The final mortality rate for the *Erysipelothrix sp*. strain 2 positive turkeys was on mean 13.26% ranging from 7.93% to 19.82%. For *Erysipelothrix* sp. Strain 2 negative turkeys, the mean final mortality rate was 6.48% ranging from 4.96 to 7.83%.

The mean age of turkeys in outbreak farms was 120.5 days old for commercial turkeys (only turkeys between 73 to 145 days old became ill on commercial turkeys' flocks). The outbreak in breeding turkeys occurred at 210 days old, when the birds are at the beginning of egg production. Flocks where the outbreak did not occur, the mean age was 94 days old.

There was association between the presence of *Erysipelothrix sp*. strain 2 in organ samples and proximity to swine farms, showing positive association with p <0.0001 and odds ratio = 9.286. Figure 2 shows a geographical scheme for the localization of turkeys and swine farms.

The turkey farms identified as D, E, G and H were close to swine farms, some of them were as close as 1.7 km. The PFGE of 19 samples showed high variability among the *Erysipelothrix sp*. strain 2 isolates. There were only 2 clones (100% similarity) and 2 clusters (>90% similarity). The PFGE of *Erysipelothrix sp*. strain 2 showed high genetic variability (Figure 2) and different banding patterns were observed among isolates (supplementary figure). Two sets of clones were found. Clones E02 and E03 were isolated in a flock from farm A, one from the heart and the other from the spleen of two distinct turkeys (Fig. 2). Clones E14 and E16 were isolated from farm E, one from the lung and the other from the heart of two distinct turkeys (Fig. 2). We found 2 clusters; isolates E04 and E07 were found in one of the clusters. E04 was isolated from the lung of a turkey in farm A whereas E07 was isolated from the kidney of a turkey in farm B. The other cluster grouped isolates E17 and E24. E17 was isolated from the liver of a turkey in farm C whereas E24 was isolated from the spleen of a turkey in farm H.

Although we identified clones and clusters, there was high variability among the isolated strains even from samples from the same flocks or same birds. The strains E08, E09 and E11 were from the same flock D with just 44.8% of similarity. E012 is different from the clones E014 and E016, which were in farm E, with 56.3% of similarity. Strains E24, E26, E28, E29 and E30 were in the same flock H with high variability (Fig.2). The strains E02 and E04 were isolated from the same turkeys but from different organs and showed high genetic variability. The same event occurred with E06 and E17 (Fig.2).

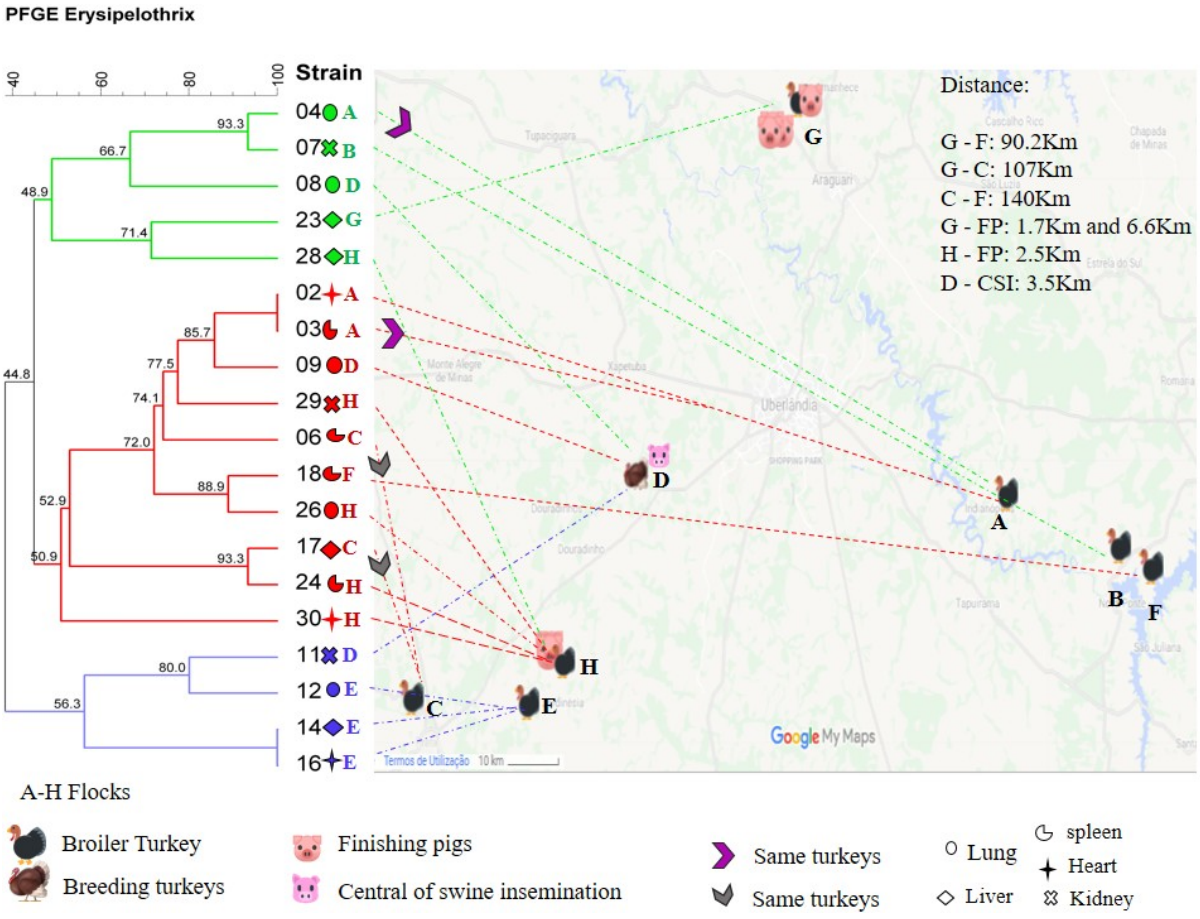
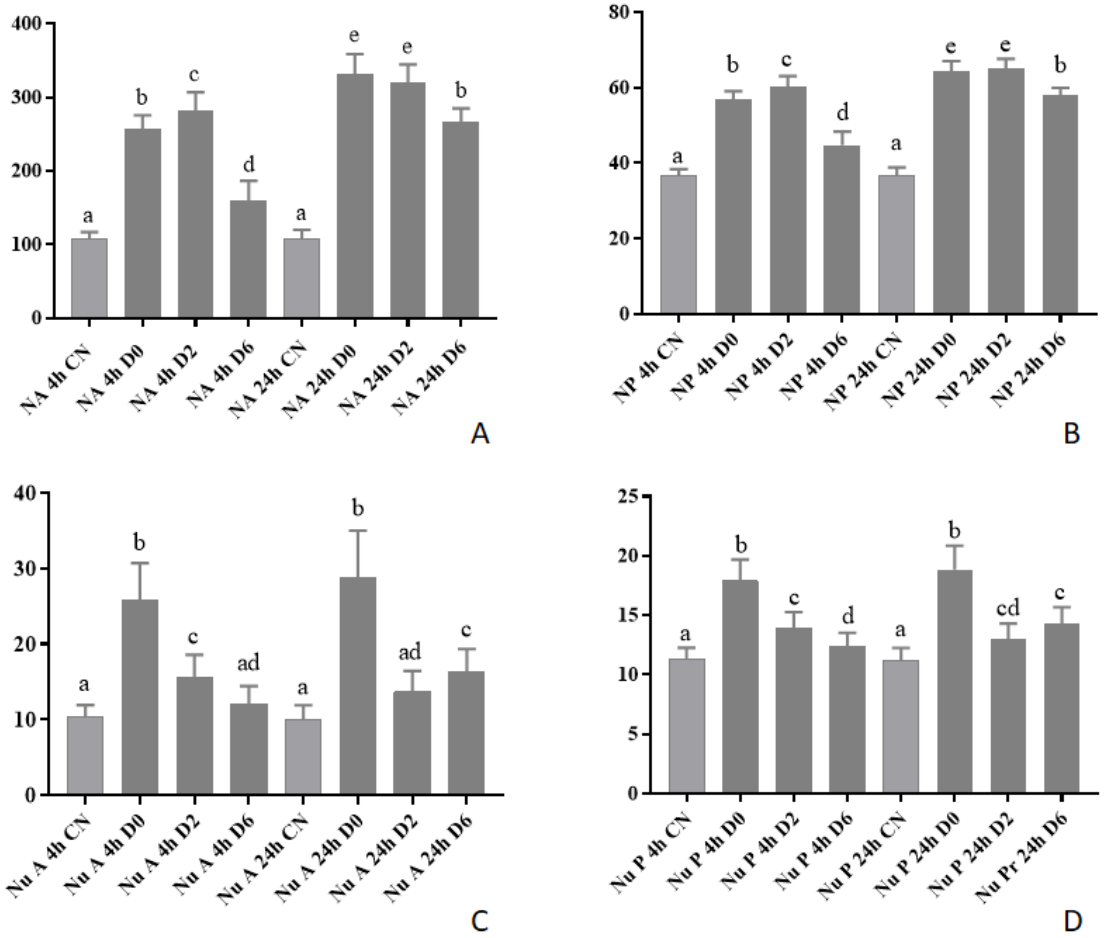


Figure 2. Location of the positive farms, swine and the relation with the results of the PFGE

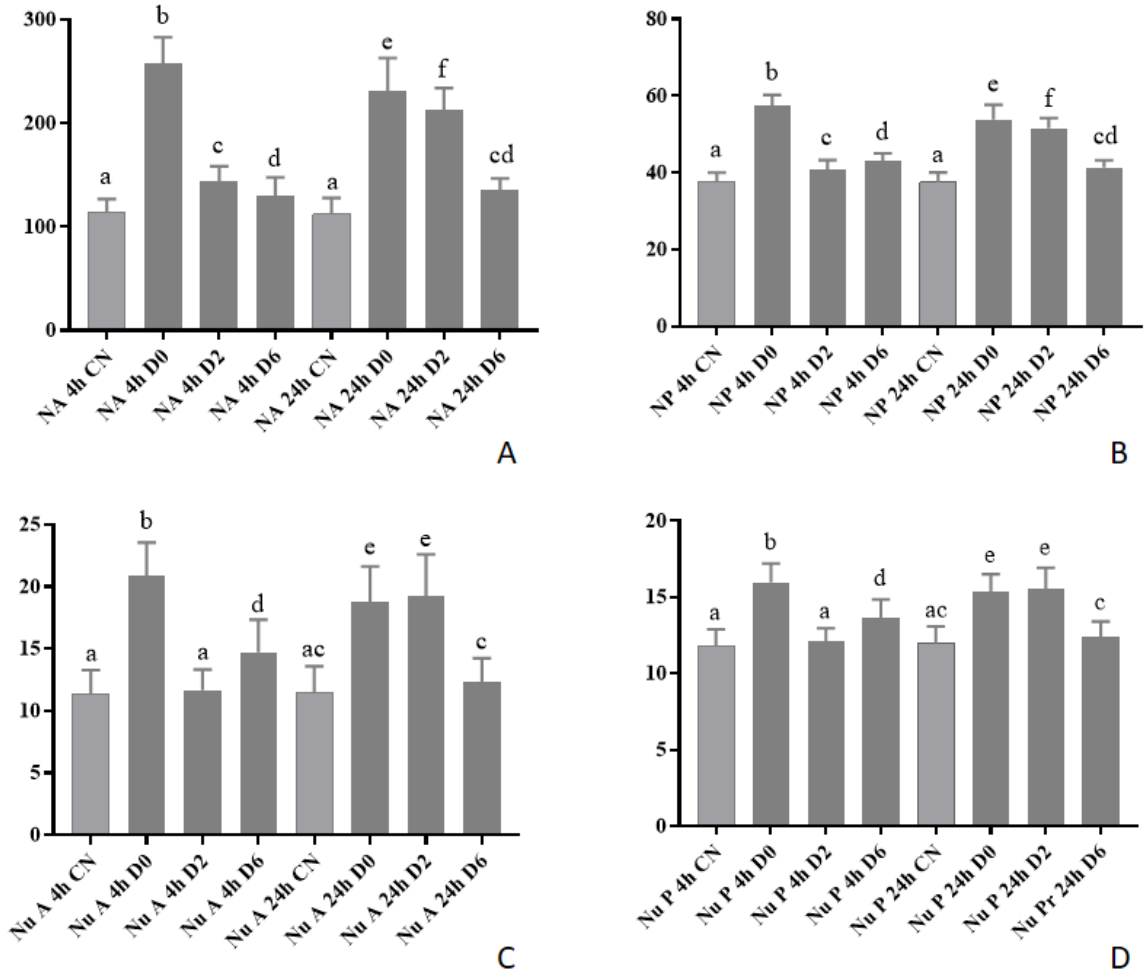
3.4 Morphometry in Vero cells

We infected Vero cell with Est06 and Est07 in order to verify changes in the cellular morphometry. After 4 and 24 hours (h) post infection (pi) in all doses of bacterium evaluated, the treatment with strains Est06 and Est07 resulted in a significant increase of all parameters measured such as nucleus area, nucleus perimeter, nucleolus area and nucleolus perimeter (Figure 3, 4 and 5). We observed that the smallest amount of bacteria (D0) was the most efficient in increasing the measurement parameter of nucleus at 4 and 24 h pi in Est07 (Figure 4A, 4B), nucleolus 4 and 24 h pi in Est06 (Figure 3B, 3C), nucleolus 4 h pi in Es07 (Figure 4C, 4D). The amout of bacteria D0 and D2 was more efficient at increasing the parameter of nucleus 4 and 24 pi in Est06 (Figure 3A, 3B).



425

426 **Figure 3.** Morphometry of Vero cells 24 hours post infection with Est06 at different
427 concentrations. A. NA: Nucleus Area; B. NP: Nucleus Perimeter; C. Nu A: Nucleolus Area;
428 D. Nu P: Nucleolus Perimeter (uM). Different letters in the same column represent statistical
429 difference ($p<0.05$).



430

431 **Figure 4.** Morphometry of Vero cells 24 hours post infection with Est07 at different
432 concentrations. A. NA: Nucleus Area; B. NP: Nucleus Perimeter; C. Nu A: Nucleolus Area;
433 D. Nu P: Nucleolus Perimeter (uM). Different letters in the same column represent statistical
434 difference ($p<0.05$).

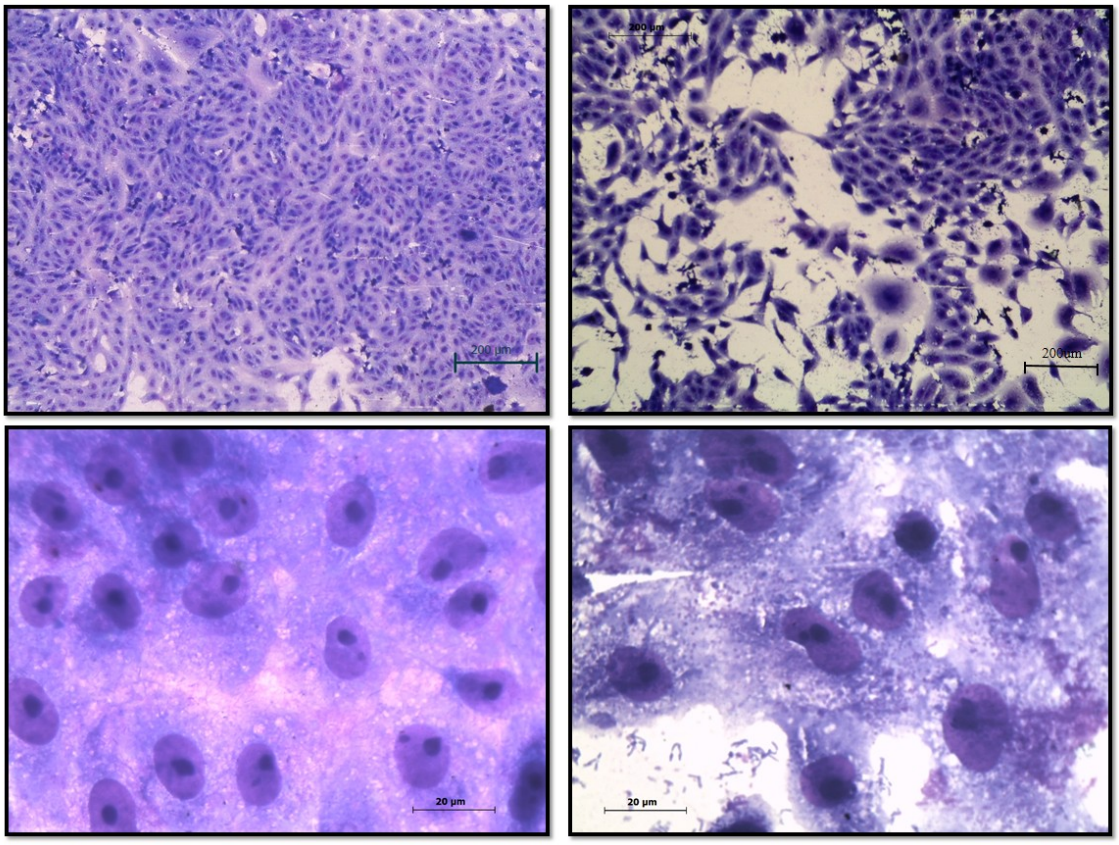


Figure 5. Vero cells stained with panoptic dye. A. Vero Cell control group 4 hours (10-fold increase). B. Vero Cell D0 Est06 group 4 hours pi. (10-fold increase). C. Vero Cell control group 4 hours (100-fold increase). B. Vero Cell D0 Est06 group 4 hours pi. (100-fold increase).

3.5. Apoptose

There was a higher percentage of cells labeled with YP in Est06 and Est07 treated groups than in those treated with LB or without any treatment. The Est07 group 4h pi had higher percentage of cell labelled with YP than other groups. Treated cells with Est06 and Est07 also had higher numbers of PI-labeled cells (Figure 5, 6).

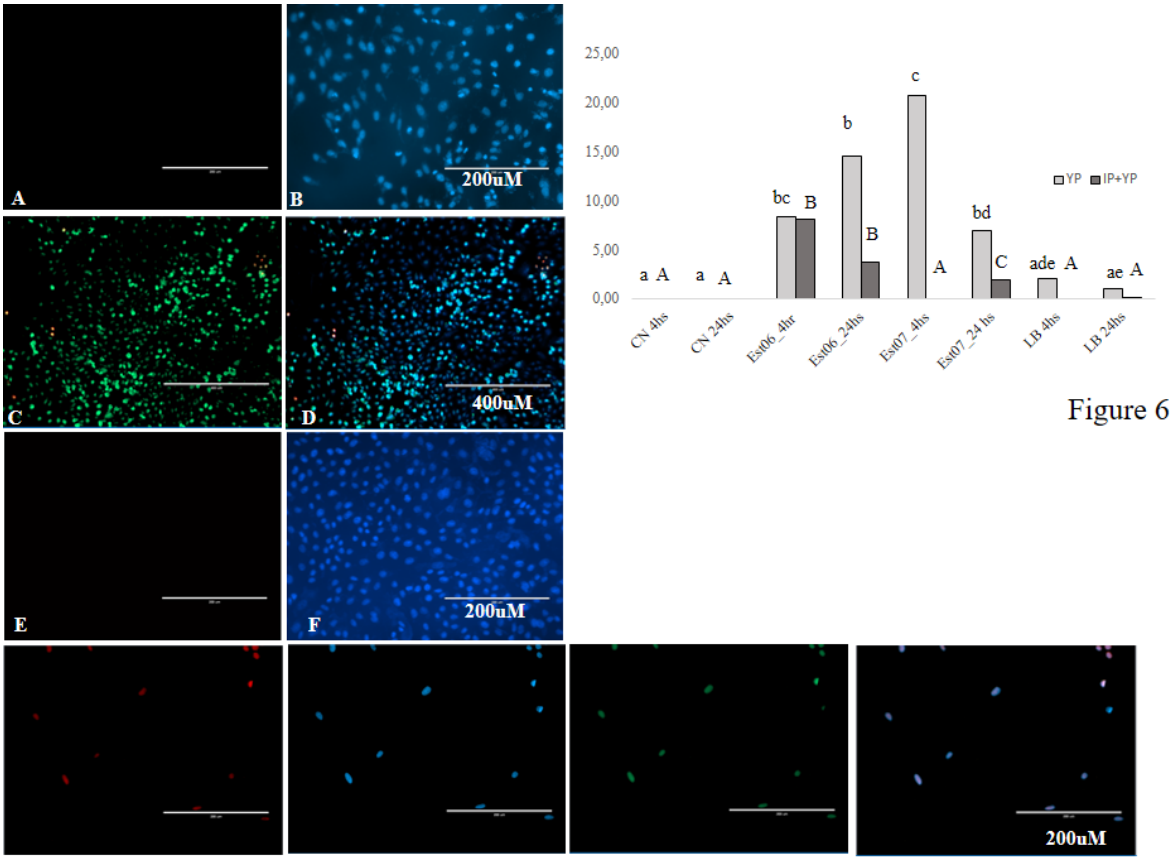


Figure 6

Figure 7

Figure 6. Percentage of cells labeled with YP and IP 4 and 24 hrs p.i. Different lowercase letters indicate statistical difference for YP markings. Different capital letters indicate statistical difference for marking with YP and IP.

Figure 7. Fluorescence imaging of YP, PI and hoechst labeled Vero cells. Scale bar is 200 µm or 400 µm. A and B: cells from the *Lactobacillus* spp. control group 4 hours showing only the Hoechst-labeled nuclei. B. cells were not marked with PI and YP (A).; C and D: cells from the Est06 group 4 hours showing IP and YP-labeled (C) and, YP, PI Hoechst-labeled nuclei (D). E and F: cells from the Negative control group 24 hours showing only the Hoechst-labeled nuclei (F) cells were not marked with PI and YP (E). G, H, I, J: cells from the Est07 24 hours showing PI-labeled nuclei (G), Hoeschst-labeled (H), YP-labeled (I) andall markers (J).

3.6 Bacterial multiplication

After 24 hours, there was *Erysipelothrix* sp. strain 2 multiplication in cell culture medium since we infected 3 log CFU/well and after 24 hours we found 7,72 log CFU/well in strain Est06 and 7,11 log CFU/well in strain Est07. We did not find *Lactobacillus* spp (table

3) in the Vero cell culture medium. Cells were four times washed with PBS and in the fourth wash, there was another bacterial counting. There was no difference between the counts of strain Est06 and Est07 (table 3). The cells were treated with trypsin for the removal the plate's cells, then centrifuged (1500rpm/10min), treated with triazole for cell membrane disruption and release of intracellular bacteria and then four times washed (1500rpm/10min), and the bacteria was counted again. There was a statistical difference between the number of bacteria after the integrate cells wash and the number of bacteria after the triton membrane dissolution (table 3). This indicates that the bacteria remained in the intracellular environment after 24 hours of inoculation.

Table 3. Mean of number of *Erysipelotrhix sp* strain 2 and LB (log CFU/well) in cutlture medium after 24 hours and after several washes and treatment with triton

	Strain 06	Strain 07	LB
Initial inoculums	3.00 a	3.00 a	3.00
In the culture cell medium	7.22 b A	7.11 b A	0
After four washes	2.89 a B	2.81 a B	0
After treatment with triton	3.13 a C	3.23 a C	0

Lowercase letters should be interpreted in relation to the initial inoculum. Upper case letters represent the evaluation against bacterial count in the cell culture medium after 24 hours of inoculation. Different letters (lower or upper case) indicate statistical difference.

4 Discussion

All sick turkeys were infected with *Erysipelothrix* sp. strain 2, which has not been described causing infections in turkeys to date. The main agent, which causes the erysipelas in turkeys, is the *E. rhusiopathiae* (Bricker and Saif, 2013). The clinical signs and macroscopic lesions in *Erysipelothrix* sp. strain 2-positive turkeys in this study were like those caused by *E. rhusiopathiae*.

The ultrastructural characteristics of the *Erysipelothrix sp*. strain 2 in TEM images reveals a typical rod-shaped (longitudinal section) and circular-shaped bacterium (cross section). For the first time, in this work we measured the *Erysipelothrix sp.* strain 2 size, which was 2.16X0.55um. As medidas obtidas pela Microscopia Eletrônica de transmissão apresentam uma bactéria de pequena dimensão, o que também ocorre em bactérias de outros gêneros como é o caso da Campylobacter que possui 0,2–0,8 µm × 0,5–5 µm (Silva et al., 2011). Outros microrganismos apresentam diametros maiores como Staphylococcus com aproximadamente 0,5 a 1,5 µm de diâmetro (KONEMAN, 2001). The bacterial wall and cellular membrane could be visualized by the photomicrograph. These structures were also visualized by (Boerner, et al. 2004) in an ultrastructure examination of *Erysipelothrix* spp. by MET; they checked bacteria rounded up in cross section and in format of short and fine bacilli in longitudinal sections, with thick cellular wall and they were not presenting wrinkled extern membranes. In this paper, we have a photomicrograph of *Erysipelothrix sp*. strain 2 by the first time. These images are important for future work in which in an assessment of the

association between pathogen and host cell, the integrity of bacterial structures can be better identified. However, it is important to consider in future analyzes other types of contrasts for better visualization of other bacteria structures by MEV.

There was a positive correlation between the mortality rate and the presence of the bacteria with r = 0.8027 (table 2) indicating strong correlation (Mukaka, 2012). The final mortality rate for the *Erysipelothrix* sp. strain 2-positive turkeys was an average 13.26% ranging from 7.93% to 19.82%. This variation is related to the time interval when the farmer perceived the disease, the veterinarian was informed, and the medication was started. However, all flocks with clinical symptomatology were treated with antimicrobial agents. It is important to emphasize that we had access only to the samples and to the informed field data, and we have not interfered with farm management, thus any low mortality rates caused by minor management errors or ambience in negative flocks were not removed from the analysis. Thus, since we have field data, there are several variables that interfere with our analysis and so, this value (r=0.8027) is representative for us.

The mean age of turkeys in outbreak farms was 120.5 days old for commercial turkeys (only turkeys aged from 73 to 145 days old became ill on commercial turkeys' flocks). The outbreak in breeding turkeys' flocks occurred at 210 days old, when the birds are at the beginning of egg production. Flocks where the outbreak did not occur, the average age was 94 days old. The age of birds showed a positive correlation with *Erysipelothrix* sp. strain 2 presence (r=0.3967) (table 2), which is considered a weak correlation (Mukaka, 2012). However, in our study we had only turkeys older than 35 days old (4 samples) and all others with 62 days old in negative birds. As poult flocks in the first weeks of age show many management variables related to older animals and we did not find birds with clinical symptoms similar to those of older turkeys, they were not considered for analysis. Although, Hollifield, et al. (2000) identified *E. rhusiopathiae* on newly hatched turkeys, typically, erysipelas mostly occur in older turkeys (Bricker and Saif, 2013). However, there are no studies about the age of *Erysipelothrix* sp. strain 2-infecting turkeys.

The PFGE of *Erysipelothrix sp*. strain 2 showed high genetic variability (Figure 2 and supplementary figure 1). There are many forms of dissemination of bacterium to turkeys' flocks, such as the flow of people, materials, feed, rodents and wild animals. Our primary aim on this study was the characterization of the outbreak pathogen, *Erysipelothrix sp*. strain 2, and therefore, this work was not initially designed to track the environmental sources of infection nor how this bacterium reached the infected flocks. However, the genetic diversity of isolates shown in this work suggests that there is not a single source of infection for all turkey flocks. *Erysipelothrix* spp. have been isolated from environmental sources, such as dust, water nipples and manure (Bender et al., 2010; Eriksson et al., 2014), suggesting that the bacteria may be widespread in the environment, which may serve as a source of infection for nearby flocks and may contribute to the genetic variability of isolates.

Turkey breeding, even with high biosafety status, were also infected at 210 days old at the same time of commercial turkeys. Breeding turkeys at 210 days old are at the beginning of egg production and that's why *Erysipelotrix sp*. strain 2 positive breeding turkeys did not originate commercial turkeys for this study. So, this agent transmission via egg is unlikely. Associated with this, only turkeys older than 83 days old became ill, which suggests that vertical transmission was not a route of infection of the turkeys in this work. There is no description of the transmission routes for *Erysipelotrix sp*. strain 2 but for E. *rhusiopathiae*,

542 the vertical transmission is not reported. However, Hollifield et al. (2000) isolated *E.*
543 *rhusiopathiae* in yolk sac. Future studies should consider the age of infection of
544 *Erysipelothrix sp*. strain 2.

545 We have found association among the turkey flocks and proximity to swine farms (up
546 7 Km). There was a close distance between the swine flocks (including the commercial swine
547 production and the central of swine insemination) and the turkeys' flocks (1,7 km-7 km).
548 Swine are the domestic animals most affected by *Erysipelotrix* spp. and they are considered
549 the most important reservoir but several other animals as turkeys, chickens and ducks are also
550 susceptible (Wang, et al. 2010). It is important to note that turkey and swine flocks in Farm H
551 were from the same owners. Beside this, the feed for all turkeys and swine flocks were from
552 the same animal's food factory. Pigs can be carriers of *E. rhusiopathiae* (Opriessnig and
553 Wood, 2012) and this bacterium is isolated from several samples of swine farms like drinking
554 fountains, walls, ration and water (Bender et al., 2010). Similar serotypes of *E. rhusiopathiae*
555 can be found in swine and turkey flocks (Croos and Claxton, 1979)

556 In one study, *E. rhusiopathiae* was isolated of contaminated dust samples from
557 exhaust fans, which led the authors to speculate about the possibility of this microorganism to
558 leave the environment of commercial turkeys and disseminate to wild animals (Eriksson et al.,
559 2014). As previously mentioned, it is difficult for us to establish the initial focus of infection
560 and the forms of dissemination due to innumerable number of variables. However, it is
561 important to highlight that in the region where there was an outbreak there was an intense
562 commercial production of chickens, turkeys and swine and there were also many properties
563 where swine and poultry were present backyard.

564 To verify the alteration of the cellular morphometry, we infected Vero cells with
565 several bacteria amount. Images were produced by this study based on techniques of
566 microscopy and morphometric cellular. In accordance with study carried out by Falk et al.
567 (2018), these analyses favour greater statistical rigor. There was significant increase of the
568 area and perimeter of the nucleus and nucleolus during 4- and 24-hours pi (Figure 3,4,5). This
569 results show that *Erysipelothrix* sp. strain 2 leads to cellular cytotoxicity. Although we have
570 observed an increase of nucleus and nucleolus, we did not identify the virulence protein (s)
571 that can generate this cellular alteration. At cellular alterations can be basis for other studies
572 about this bacterium. In gram-negative bacterium (such as Shigella spp. and *Campylobacter*
573 *jejuni*) (Jinadasa, et al. 2011) there are Cytolethal Distending Toxin (CDT) that lead to the
574 enlarged or distended cells and then promotes cell apoptosis but there are not similar toxin
575 related in gram positive bacteria. However, proteins like alpha-hemolisin (Menzies and
576 Kourteva, 2000) and pyrogenic exotoxin B (Tsai et al., 1999) are examples of proteins known
577 to induce apoptosis in gram-positive bacteria.

578 In this study in general, the smallest amount of the inoculated bacterium was able to
579 increase the cellular parameters related to the highest amount of *Erysipelotrix sp.* strain 2. We
580 hypothesized that this event occurred because the smallest amount of bacteria found more
581 culture medium and free cells to infect. However, this work draws attention to the probable
582 mechanism of pathogenesis of *Erysipelotrhix sp*. strain. 2. The increase of nucleus and
583 nucleolus dimensions led us to evaluate the apoptotic index of these cells (Figure 5 and 6).

The increase of nucleus and nucleolus dimensions led us to evaluate the apoptotic index of these cells (Figure 6 and 7). For this, we used YP that can labels cells that are still metabolically active but that have compromised plasma membranes that is, it can detects cells in earlier stages of apoptosis (cells in which DNA fragmentation has not yet occurred) (Fujisawa et al., 2014). While PI detects cells that are already in the death stage because delay in PI penetration, compared to YP.

If we consider the clinical form of the erisipela in the turkeys in this study, the possible apoptosis caused by this bacterium may be the key to a better understanding of the pathogenesis of *Erysipelotrhix sp.* strain 2. Apoptosis may be an important mechanism of microorganisms to circumvent the immune response in particular the innate reducing the inflammatory lesion. Erysipelas leads sepsis in turkeys with some animals dying with acute mortality. Apoptosis may be harmful to the host associated with enhanced virulence potential of the bacterium. This mechanism is considering a non-inducing inflammatory response (Kerr, 1972; Gallucci et al., 1999).

The cellular survive and the indication of apoptosis in this research may be an important strategy of virulence of this bacterium. In order to persist in the host cell, pathogens have developed mechanisms of intracellular survive and/or apoptosis (Shimoji, 2000, Galan and Cossart, 2005; Mostowy and Cossart, 2012).

In this work, the turkeys died from septicemia (with organ damage) or an acute mortality. Thus, the characteristic of acute disease infers that somehow the bacterium may evade the animal's immune system. On the other hand, tissue injury caused by bacterium infection is important to inflammation (Matzinger, 1994). Necrosis activate cells of the immune system and inflammation response (Matzinger, 1994, 2002; Kono and Rock, 2008) and some situations, apoptotic cells can be pro-inflammatory (Cullen et al., 2013 and Kearney et al., 2013). These events may explain the inflammatory lesions in some animals.

There is no report about *Erysipelothrix sp*. strain 2 lifestile in cells. However, Días-Delgado et al., (2011) found 30−50% and 75% of *E. rhusiopathiae* in the intracellular and extracellular of liver and kidney of free-ranging Atlantic spotted dolphin with acute septicaemia. *E. rhusiopathiae* is a facultative intracellular pathogen and can survive inside polymorphonuclear leukocytes and macrophages (Shimoji et al., 1996; Wood, 1999). The highest number of *Erysipelothrix sp.* strain 2 in the extracellular medium shows that this bacterium can survive well in the extracellular environment, but bacteria were also found after treatment with triton in greater quantity than the previous washes of whole cells. This leads us to believe that this bacterium can also survive inside the Vero cells. The ability of the bacteria to live in the intracellular environment is an excellent strategy of dribbling the animal's immune system (Shimoji, 2000).

This work has brought several findings about *Erysipelothrix sp*. strain 2 but also left several doubts to be studied in the future. One of our questions is whether the isolates of *Erisipelotrix* spp. are identified in other field outbreaks. Most researchers mention that only *E. rhusiopathiae* causes erysipelas in avian but we isolated just *Erysipelothrix sp*. strain 2. It is possible that *Erysipelothrix* sp. strain 2 may be disseminated in other places, but not identified. Beside this, more studies about the pathogenicity of this bacterium are important, in order to identify the main virulence genes and the host pathogen relationship.

In this research, we have found turkeys older than 83 days old infected with *Erysipelothrix sp*. strain 2 with high mortality rate. For the first time we show that isolated of *Erysipelothrix sp*. strain 2 had high genetic variability even those coming from the same turkeys. *Erysipelothrix sp*. strain 2 leads to increased cell size, nucleus and nucleolus when it infects Vero cells, it increases the number of dead cells in apoptosis and necrosis, multiplies intensely in the extracellular environment and can survive inside the cells.

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6 Author contributions

BF have developed and planned the project, analysed and interpreted the data and wrote the manuscript. TR wrote the manuscript and she was the main executor of laboratory work. TR, EM, PP, DR performed PFGE analysis and interpreted the results. TR, EM, DR kept the bacterium. MB, TR and BF performed the cell culture analysis and TEM. PH associated the outbreak with the bacterium, isolated and identified the bacterium and conducted the revision of the English language. LG interpreted the data and carried out the revision of the scientific and English language essay. PB identified diseased turkeys, assessed macroscopic lesions, collected samples, evaluated epidemiological data, and conducted the revision of the English language. All authors read and approved the final manuscript.

7 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Contribution to the Field Statement

This study evaluated a specie of Erysipelothrix genus, little known and studied, during an outbreak in turkeys in Brazil. We have isolated during an outbreak the Erysipelothrix sp strain 2 that is different specie from the commonly isolated E. rhusiopathiae. Erysipelotrix sp strain 2 was associated with sepsis and high mortality in breeding and broiler turkeys. The disease have occurred in turkeys from 83 days old on and there was a relationship between the presence of the disease in turkey flocks and the proximity to swinw farms. During a genetic study, we have found high genetic variability among the Erysipelotrix sp. strain 2 isolated which shows the possibility of multiple outbreaks of infection. When we infected cells

663 with Erysipelotrix sp. strain 2, we checked for a cell increase. This shows that there is the
664 participation of some antigen from the bacterium with malefic effect to the cells.

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793 **SUPPLEMENTARY MATERIAL**
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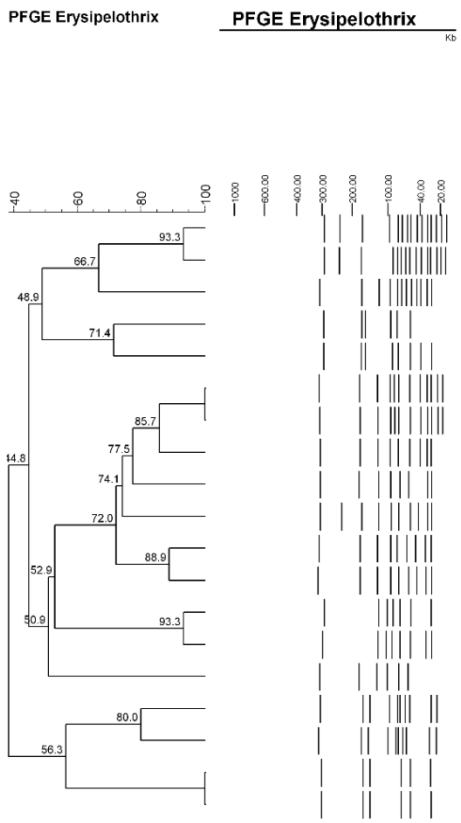

FIGURE S1. PFGE Dendrogram showing the relationship between *Erysipelotrix* sp. strain 2 isolates. The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.

Table S1. Distribution of the presence and absence of *Erysieplothrix* sp. strain 2 by age and organs in the flocks selected for the study.

Flock	Kind of creation	Number os strain	Erysipelothrix sp. strain 2	Swine (to 7km)	Final Mortality (%)	Age (Days)	Organ
A	broiler turkeys	Est01	Pres	0	19,82	123	Liver
A	broiler turkeys	Est02	Pres	0	19,82	123	Heart
A	broiler turkeys	Est03	Pres	0	19,82	123	Spleen
A	broiler turkeys	Est04	Pres	0	19,82	123	Lung
A	broiler turkeys	there are no*	Pres	0	19,82	102	Spleen
B	broiler turkeys	there are no*	Pres	0	11,09	88	Liver
B	broiler turkeys	there are no*	Pres	0	11,09	100	Heart
B	broiler turkeys	there are no*	Pres	0	11,09	100	Lung
B	broiler turkeys	there are no*	Pres	0	11,09	100	Liver
B	broiler turkeys	there are no*	Pres	0	11,09	100	Kidney
B	broiler turkeys	Est05	Pres	0	11,09	126	Liver
B	broiler turkeys	Est7	Pres	0	11,09	126	Kidney
C	broiler turkeys	Est6	Pres	0	12,54	116	Spleen
D	Breeding turkeys	Est8	Pres	1	13,33	210	Lung

D	Breeding turkeys	Est9	Pres	1	13,33	210	Lung
D	Breeding turkeys	Est10	Pres	1	13,33	210	Liver
D	Breeding turkeys	there are no*	Pres	1	13,33	210	Spleen
D	Breeding turkeys	Est11	Pres	1	13,33	210	Kidney
E	broiler turkeys	Est12	Pres	1	12,29	139	Lung
E	broiler turkeys	Est13, Est14	Pres	1	12,29	139	Liver
E	broiler turkeys	Est15, Est16	Pres	1	12,29	139	Heart
C	broiler turkeys	Est17	Pres	0	12,54	116	other
F	broiler turkeys	Est18	Pres	0	17,25	145	Spleen
F	broiler turkeys	Est19	Pres	0	17,25	145	Liver
G	broiler turkeys	Est20	Pres	1	7,93	141	Spleen
G	broiler turkeys	Est21	Pres	1	7,93	141	Lung
G	broiler turkeys	Est22	Pres	1	7,93	141	Heart
G	broiler turkeys	Est23	Pres	1	7,93	141	Liver
H	broiler turkeys	Est24	Pres	1	10,62	84	Spleen
H	broiler turkeys	Est25, Est30	Pres	1	10,62	84	Heart
H	broiler turkeys	Est26	Pres	1	10,62	84	Lung
H	broiler turkeys	Est27, Est28	Pres	1	10,62	84	Liver
H	broiler turkeys	Est29	Pres	1	10,62	84	Kidney
I	broiler turkeys	no	Absent	0	7,83	62	Absent in all organs
J	broiler turkeys	no	Absent	0	6,87	82	Absent in all organs
K	broiler turkeys	no	Absent	0	6,03	100	Absent in all organs
L	broiler turkeys	no	Absent	1	5,39	107	Absent in all organs
M	broiler turkeys	no	Absent	0	7,38	115	Absent in all organs
N	broiler turkeys	no	Absent	0	4,96	88	Absent in all organs
O	broiler turkeys	no	Absent	0	6,28	88	Absent in all organs
P	broiler turkeys	no	Absent	0	7,27	109	Absent in all organs
Q	broiler turkeys	no	Absent	0	6,8	35	Absent in all organs
R	broiler turkeys	no	Absent	0	5,99	100	Absent in all organs

811 **Table S2.** Summary of the number of samples in the flocks selected for the

	Age of material collection	Number of collection	Number of organs	Number of samples	Number of positive sample
A	102 e 123	2	7	14	5
B	88,100,126	3	7	21	7
C	116	1	7	7	2
D	210	1	7	7	5
E	73 e 139	2	7	14	3
F	145	1	7	7	2
G	141	1	7	7	4
H	84	1	7	7	5
I	62 e 144	2	7	14	0
J	82	1	7	7	0
K	85, 94, 103, 112	4	7	28	0
L	107	1	7	7	0
M	115	1	7	7	0
N	88	1	7	7	0
O	88	1	7	7	0
P	109	1	7	7	0
Q	88	1	7	7	0
R	100	1	7	7	0

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