UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CÂMPUS DE JABOTICABAL

UTILIZAÇÃO DE PCR EM TEMPO REAL QUANTITATIVO PARA DIAGNÓSTICO DIFERENCIAL ENTRE *Salmonella enterica* SUBESP. *enterica* SOROVARES ENTERITIDIS, TYPHIMURIUM E GALLINARUM (BIOVARES GALLINARUM E PULLORUM) EM AVES DOMÉSTICAS (*Gallus gallus*)

Marcela da Silva Rubio
Médica Veterinária

2017

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Orientador: Prof. Dr. Angelo Berchieri Junior
Coorientador: Dr. Rafael Antonio Casarin Penha Filho

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Medicina Veterinária, área: Patologia Animal.

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Câmpus de Jaboticabal

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TÍTULO DA TESE: UTILIZAÇÃO DE PCR EM TEMPO REAL QUANTITATIVO PARA DIAGNÓSTICO DIFERENCIAL ENTRE *Salmonella enterica* SUBESP. *enterica* SOROVARES ENTERITIDIS, TYPHIMURIUM E GALLINARUM (BIOVARES GALLINARUM E PULLORUM) EM AVES DOMÉSTICAS (*Gallus gallus*)

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Jaboticabal, 11 de dezembro de 2017

DADOS CURRICULARES DO AUTOR

MARCELA DA SILVA RUBIO – Filha de Osvaldo da Rocha Rubio e Marcia Regina da Silva Rubio, nascida em 11 de Dezembro de 1989, na cidade de Ilha Solteira, São Paulo. Foi graduada em Medicina Veterinária pela Faculdade de Ciências Agrárias de Andradina (F.C.A.A), em dezembro de 2011. Obteve o título de mestre em Ciência e Tecnologia Animal, Área de Concentração: Nutrição de Monogástricos, na Faculdade de Engenharia de Ilha Solteira, UNESP, câmpus de Ilha Solteira, SP, sob a orientação do Prof. Dr. Antonio Carlos de Laurentiz, com a dissertação intitulada: Efeitos da adição de *Piper cubeba* na dieta de frangos de corte em substituição ao antibiótico melhorador de desempenho, em fevereiro de 2014. Em março de 2014, ingressou no curso de doutorado em Medicina Veterinária, Área de Concentração: Patologia Animal, na Faculdade de Ciências Agrarias e Veterinárias, UNESP, câmpus de Jaboticabal, SP, é bolsista de doutorado da Fundação de Amparo à Pesquisa do Estado de São Paulo, sob orientação do Prof. Dr. Angelo Berchieri Junior e coorientação do Prof. Dr. Rafael Antonio Casarin Penha Filho. Em fevereiro de 2017, iniciou a atividade de docência ministrando a disciplina de Ornitopatologia e Pequenas Criações de Interesse Comercial, pelo Centro Universitário Moura Lacerda, câmpus de Ribeirão Preto, SP.

“Na ocorrência da vida, são muitos os contratempos. As bactérias, os vírus e os fungos são apenas uma lembrança de que os erros e os descuidos estão dentro de nós mesmos!”

Eneo

DEDICATÓRIA

Dedico este trabalho aos meus pais, Osvaldo da Rocha Rubio e Marcia Regina da Silva Rubio, que lutaram cada dia desses dez anos de vida acadêmica para me proporcionar a oportunidade de estudar e de me dedicar integralmente aos estudos, sempre acreditando que o esforço deles não seria em vão e que o maior retorno seria com a minha satisfação em ser Médica Veterinária com titulação de Doutora.

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Protocolo nº 01354/15 do trabalho de pesquisa intitulado "Utilização de PCR em tempo real quantitativo para diagnóstico diferencial entre *Salmonella enterica* subesp. *enterica sorovares* ENTERITIDIS, TYPHIMURIUM e GALLINARUM (biovares GALLINARUM e PULLORUM) em aves domésticas (*Gallus gallus)*", sob a responsabilidade do Prof. Dr. Angelo Berchieri Júnior está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 02 de fevereiro de 2015.

Jaboticabal, 02 de fevereiro de 2015.

Prof.ª Dr.ª Paola Castro Moraes
Coordenadora – CEUA

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UTILIZAÇÃO DE PCR EM TEMPO REAL QUANTITATIVO PARA DIAGNÓSTICO DIFERENCIAL ENTRE *Salmonella enterica* SUBESP. *enterica* SOROVARES ENTERITIDIS, TYPHIMURIUM E GALLINARUM (BIOVARES GALLINARUM E PULLORUM) EM AVES DOMÉSTICAS (*Gallus gallus*)

RESUMO – Produtos avícolas são frequentemente associados a surtos alimentares ocasionados por *Salmonella* spp. e atualmente, existem 2659 sorovares de *Salmonella*. Os programas existentes para prevenção e controle da infecção por essa bactéria, são complexos devido às várias fontes de contaminação presentes na indústria avícola. A obtenção de um teste diagnóstico rápido para a identificação de bactérias é crucial, para implementar rapidamente as medidas de controle, a fim de conter a propagação bacteriana, reduzir as perdas na produção animal e evitar riscos de infecções transmitidas por alimentos à saúde humana. O objetivo do presente estudo foi realizar a padronização e aplicação da técnica da PCR em tempo real para identificação e quantificação da carga bacteriana em órgãos e fezes de aves comerciais infectadas por *Salmonella enterica* subesp. *enterica* sorovares Enteritidis, Typhimurium e Gallinarum (biovares Gallinarum e Pullorum), em diferentes momentos pós-infecção. O presente estudo realizou a padronização de duas reações multiplex qPCR utilizando SYBr Green, uma reação para quantificação e diagnóstico diferencial entre *S*. Gallinarum e *S*. Pullorum, e outra para quantificar e diferenciar *S*. Enteritidis e *S*. Typhimurium . Após a padronização da qPCR, quatro grupos experimentais foram inoculados com cada estirpe estudada, por via oral, na 13ª semana de vida das aves. Aos quatro e sete dias após o desafio, três aves de cada grupo foram submetidas à eutanásia, para obtenção de amostras de fígado e conteúdo cecal. Após a colheita das amostras, foram realizadas identificação e quantificação bacteriana pela PCR em tempo real e pela microbiologia convencional. De acordo com a padronização das reações multiplex qPCR, as temperaturas de *Melting* (*Tm*) foram avaliadas para obtenção do diagnóstico diferencial. Para a primeira reação, o iniciador pSGP (97pb) obteve *Tm* de 78°C, para ambos biovars. O iniciador pSG (273pb) obteve 86,2°C de *Tm* para identificação de *S*. Gallinarum e o iniciador pSP (260pb) com *Tm* de 84,8°C para *S*. Pullorum. Para a segunda reação, observou-se uma *Tm* de 85°C (206pb) para *S*. Enteritidis e uma *Tm* de 79°C (62pb)

para *S*. Typhimurium. Com a obtenção da curva padrão, foi possível evidenciar a alta sensibilidade do teste proposto, uma vez que possibilitou a obtenção de oito pontos de quantificação, iniciando em 10^8 UFC/mL e finalizando em 10^1 UFC/mL. Quando as reações foram aplicadas em amostras provenientes de aves infectadas com as respectivas bactérias, foi possível comprovar a alta sensibilidade e especificidade de qPCR, especialmente quando as amostras foram provenientes de fezes, pois permitiu a obtenção de resultados positivos mesmo em amostras com grande quantidade de contaminantes e baixa carga bacteriana. Em decorrência do presente estudo, reações multiplex qPCR com alta sensibilidade, especificidade e com base na fluorescência de SYBr Green foram padronizadas. Além disso, esta metodologia alia baixo custo ao resultado diagnóstico rápido, tornando esse método atraente para aplicação em análises de rotina laboratorial.

Palavras-chave: Curva de dissociação, curva padrão, salmoneloses aviárias, SYBr green.

USE OF QUANTITATIVE REAL-TIME PCR FOR DIFFERENTIAL DIAGNOSIS BETWEEN *Salmonella enterica* SUBESP. *enterica* SEROVARS ENTERITIDIS, TYPHIMURIUM AND GALLINARUM (BIOVARS GALLINARUM AND PULLORUM) IN DOMESTIC BIRDS (*Gallus gallus*)

ABSTRACT – Poultry products are often associated with food outbreaks from *Salmonella* spp. and currently there are 2659 *Salmonella* serovars. Existing programs for disease prevention and control are complex due to the various sources of contamination within the poultry industry. Obtaining a rapid diagnostic test for identification of bacteria is crucial in order to rapidly implement control measures to contain bacterial spread, to reduce losses in animal production and to avoid risks from food-borne infections to human health. The objective of the present study was to perform a standardization and application of the real-time PCR for indirect diagnosis and quantification of bacterial load in organs and feces of commercial birds infected by *Salmonella enterica* subesp. *enterica* sorovares Enteritidis, Typhimurium and Gallinarum (biovares Gallinarum and Pullorum), at different post-infection moments. The present work describes the development of two multiplex qPCR using a low-cost DNA dye (SYBr Green), one for quantification and differential diagnosis between *S*. Gallinarum and *S*. Pullorum, and another to quantify and differentiate *S*. Enteritidis and *S*. Typhimurium. After qPCR standardization, four experimental groups were inoculated with each strains, orally at the 13th week of life of the birds. At four and seven days post challenge, three birds from each group were euthanasia for liver and cecal content samples collection. After the sampling, was carried out bacterial identification and quantification by real-time PCR and conventional microbiology. According to the multiplex qPCR standardization, the melting temperatures (*Tm*) was evaluated to obtain the differential diagnosis. For the first reaction, the pSGP amplicon (97 bp) showed *Tm* of 78°C for both biovars. The pSG amplicon (273 bp) showed a *Tm* of 86.2°C for *S*. Gallinarum and pSP amplicon (260 bp) dissociated at 84.8°C for *S*. Pullorum identification. For the second reaction, a *Tm* of 85°C (206bp) amplified product for *S*. Enteritidis and *Tm* of 79°C (62bp) amplified product for *S*. Typhimurium. The standard curve showed the high sensitivity of the proposed test, since it was possible to obtain eight quantification points,

starting at 10^8 CFU/mL and ending at 10^1 CFU/mL. When the reactions were applied in samples from birds infected with each respective bacteria, it was possible to evidence the high sensitivity and specificity of qPCR, especially when the samples came from feces, because obtained positive results even from samples with a large number of contaminants and low bacterial load. As a result of the present study, multiplex qPCR reactions with high sensitivity, specificity and based on the fluorescence of SYBr Green was standardized. In addition, this methodology aligns low cost to the faster diagnostic result, making it attractive for application in routine laboratory analyzes.

Keywords: Avian salmonelosis, melting curve, standard curve, SYBr green.

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1 Introdução

Salmonella spp. pertence à família Enterobacteriaceae e 2659 sorovares diferentes foram identificados (ISSENHUTH-JEANJEAN et al., 2014). Todos os sorovares são patogênicos, no entanto, a doença nas aves é classificada em três categorias diferentes, o tifo aviário (*S*. Gallinarum), a pulorose (*S.* Pullorum), os quais são hospedeiro-específicos de aves (LÖFSTRÖM et al., 2010; FENG et al., 2013) e, o paratifo aviário, que ocorre por qualquer outro sorovar que não seja *S*. Gallinarum e *S*. Pullorum, como por exemplo, os sorovares *S*. Enteritidis e *S*. Typhimurium, além de acometer diversos tipos de hospedeiros (BARLETTA et al., 2013).

Diferentes fatores, tais como o epitélio intestinal, a interação com o organismo do hospedeiro, a alta capacidade de invasão, a sobrevivência intracelular em macrófagos infectados e a evasão da resposta imune estão envolvidos com o desenvolvimento da patogenia da infecção por essas bactérias (SORIA et al., 2013). As infecções ou surtos provocados por bactérias desse gênero são de notificação obrigatória à Organização Mundial de Saúde Animal (OIE), devido à ocorrência de mortalidade nos animais, os prejuízos causados e o risco de disseminação para outras localidades (OIE, 2012).

O método classificado como padrão-ouro para o diagnóstico de *Salmonella* spp. são a cultura microbiológica, caracterização bioquímica e sorotipagem dos isolados (SANTANA et al., 2011; MA et al., 2014). Além da metodologia empregada para o isolamento bacteriano (método direto), os métodos indiretos são importantes para estudo e monitoramento das condições sanitárias e epidemiológicas dos lotes avícolas. Os testes sorológicos, como os testes de ELISA, imunocromatografia e soro-aglutinação rápida (SAR) baseiam-se na detecção de antígenos ou anticorpos contra *Salmonella* spp. presentes no soro sanguíneo. Já as provas moleculares, possuem a capacidade de detectar e identificar partículas especificas de DNA do patógeno (ANDRADE et al., 2010).

O exame microbiológico de bactérias do gênero *Salmonella* spp. torna-se mais laborioso e com mais etapas a serem cumpridas quando comparado às técnicas moleculares. Entre estes, a identificação direta das bactérias é difícil devido ao crescimento de outros microrganismos presentes na amostra, especialmente da microbiota natural do hospedeiro. A sua identificação e caracterização completa depende da análise bioquímica e sorológica de cada isolado, o que aumenta o tempo e o custo para obter resultados com essa metodologia (CHEN et al., 2010). O diagnóstico pela técnica de ELISA produz resultados dentro de dois dias. No entanto, esta técnica se baseia na maioria das vezes na detecção de anticorpos e exige a confirmação do diagnóstico pela microbiologia convencional, estendendo o custo e o tempo gasto para obtenção do resultado diagnóstico final (DICKEL et al., 2005).

A técnica de PCR foi estabelecida com sucesso para o diagnóstico rápido e confiável de diferentes patógenos, incluindo bactérias de difícil diagnóstico. Os resultados podem ser obtidos em no máximo dois dias, os quais não necessitam de confirmação pela microbiologia convencional, assim como os sorológicos (LIN et al., 2011). Por se tratar de um método caracterizado por alta especificidade e sensibilidade, a sua utilização em estudos epidemiológicos tem sido realizada com sucesso durante as últimas décadas (CHEN et al., 2010).

A PCR em tempo real foi desenvolvida mais recentemente para o diagnóstico de patógenos, com maior sensibilidade que a técnica de PCR convencional e com a utilização de menos etapas na reação, obtendo resultados mais rápidos (PARK et al., 2011; MA et al., 2014).

2 Revisão de literatura

2.1 Taxonomia do gênero *Salmonella* spp.

O gênero *Salmonella* pertence à família Enterobacteriaceae e é subdivido em duas espécies, *S. enterica* e *S. bongori* (LE MINOR; POPOFF, 1987), com 2659 sorovares identificados. A espécie *S. enterica* (2.637 sorovares) é subdividida em seis subespécies: *S. enterica* subesp. *enterica* (1.586 sorovares), *S. enterica*

subesp. *salamae* (522 sorovares), *S. enterica* subesp. *arizonae* (102 sorovares), *S. enterica* subesp. *diarizonae* (338 sorovares), *S. enterica* subesp. *houtenae* (76 sorovares), *S. enterica* subesp. *indica* (13 sorovares). Os demais 22 sorovares pertencem à espécie *S. bongori* (ISSENHUTH-JEANJEAN et al., 2014).

Salmonella enterica subespécie *enterica* abrange os sorovares mais relatados e importantes para a produção avícola, representados polos sorovares *S.* Gallinarum, *S.* Enteritidis e *S.* Typhimurium. Na classificação atual, o sorovar *S*. Gallinarum é subdivido em *S.* Gallinarum e *S*. Pullorum, que são considerados biovares de um mesmo sorovar (GRIMONT; WEILL, 2007). A nomenclatura adequada para relatar bactérias do gênero *Salmonella* spp. descreve que o gênero, espécie e subespécie devem ser escritos em itálico e apenas o gênero deve conter a primeira letra em maiúscula. O sorovar e o biovar devem conter a primeira letra em maiúsculo e sem itálico. Dessa forma, A nomenclatura completa dos biovares é *Salmonella enterica* subespécie *enterica* sorovar Gallinarum biovar Gallinarum e biovar Pullorum. No entanto, podem ser descritos de maneira resumida, ou seja, apenas o gênero e o sorovar (*S.* Gallinarum e *S.* Pullorum) (POPOFF et al., 1996).

2.2 Genética e tipificação de bactérias do gênero *Salmonella* spp.

As espécies e subespécies de *Salmonella* spp. evoluíram a partir de um mesmo ancestral. A razão disso é a habilidade genética que possuem, caracterizada pela aquisição e perdas de genes, o que ocasionou a formação de linhagens separadas, fato pelo qual se atribui a principal razão da sua diferenciação. Devido à associação entre o histórico evolutivo e a proximidade filogênica do gênero, há a representação da alta similaridade entre os sorovares do gênero, relatado em estudos de genética comparativa (MCQUISTON et al., 2008).

A caracterização dos sorovares é realizada pela combinação das estruturas antigênicas presentes na parede celular bacteriana, sendo 46 antígenos somáticos (O), comum a todos os sorovares, e 114 antígenos flagelares (H), encontrados apenas nos sorovares flagelados ou móveis (GRIMONT; WEILL, 2007; MCQUISTON et al., 2008).

2.3 Salmoneloses aviárias

Produtos de origem avícola são frequentemente associados com surtos de DTA causados por *Salmonella* spp. As aves infectadas por sorovares não hospedeiro-específicos podem permanecer portadoras por longos períodos e excretar a bactéria nas fezes. Os biovares *S*. Pullorum (SP) e *S*. Gallinarum (SG) são hospedeiro-específicos de aves, semelhantes geneticamente, imóveis e causam as doenças pulorose e tifo aviário, respectivamente (O'REGAN et al., 2008). Os sorovares flagelados, os quais não possuem especificidade aos hospedeiros, causam o paratifo em aves e mamíferos (LÖFSTRÖM et al., 2010).

Diferentes fatores, tais como a interação com o epitélio intestinal, a alta capacidade de invasão, a sobrevivência intracelular em macrófagos infectados e evasão da resposta imune estão envolvidos na patogênese dessas bactérias (SORIA et al., 2013). Por se tratar de um agente intracelular facultativo, multiplicar-se dentro de macrófagos é fundamental para a sua sobrevivência nas células hospedeiras infectadas e para a implementação da infecção sistêmica (BARROW; HUGGINS; LOVELL, 1994).

Possuem grande importância para toda a cadeia da indústria avícola, por provocarem grande perda econômica, pois além das altas taxas de mortalidade, os índices de produção e a qualidade dos produtos e subprodutos caem drasticamente nos animais infectados ou sobreviventes (BATISTA et al., 2013)

2.3.1 Pulorose

A pulorose é caracterizada como doença septicêmica e aguda em aves jovens, que resulta em um quadro clínico severo. Ocasiona alta mortalidade em lotes afetados e o pico de mortalidade ocorre em aves com duas a três semanas de vida. Eventualmente, as aves que conseguirem conter a progressão da doença, se tornarão aves adultas com infecção persistente, devido ao potencial de transmissão horizontal e vertical (GONG et al., 2013). Em geral, a infecção inicial é proveniente da transmissão vertical de aves adultas infectadas subclinicamente. Porém, a

transmissão horizontal contribui para a rápida disseminação dentro de um lote. (SHIVAPRASAD, 2000).

O quadro clínico da doença em animais adultos varia de acordo com a sua qualidade nutricional e imunológica, que se estiverem dentro dos padrões preconizados pelo tipo de produção, dificilmente sinais clínicos serão observados. Todavia, se houver o aparecimento de sintomatologia clinica, esta poderá ser confundida com o tifo aviário, o paratifo aviário ou outras enfermidades bacterianas (BARROW; FREITAS NETO, 2011; BERCHIERI JUNIOR; FREITAS NETO, 2009).

A doença também pode estar relacionada à diminuição das taxas de nascimento nos incubatórios ou a ocorrência de alta mortalidade em neonatos, devido à ocorrência de transmissão transovariana. Além disso, essa via de transmissão atua efetivamente na disseminação bacteriana para as granjas (PINHEIRO et al., 2001; BERCHIERI JUNIOR; FREITAS NETO, 2009).

2.3.2 Tifo aviário

O tifo aviário é caracterizado como doença septicêmica aguda ou crônica, o que gera altas taxas de mortalidade, de acordo com a patogenicidade da estirpe infectante. Acomete aves de todas as idades, todavia, é observada com maior frequência em aves adultas (BERCHIERI JUNIOR; FREITAS NETO, 2009; BATISTA et al., 2013).

A doença acarreta em elevados índices de morbidade e mortalidade, que podem corresponder até 80% das aves infectadas. Tais índices variam de acordo com a dose e a patogenicidade da estirpe infectante, a idade da ave, a linhagem, o manejo sanitário, o estado nutricional e a presença de outras doenças concomitantes nos animais afetados (SHIVAPRASAD, 2000). Em geral, o aparecimento da mortalidade em lotes infectados ocorre em torno de cinco a sete dias após a infecção e pode durar até 14 dias após o inicio da infecção. Aves de linhagens leves são consideradas resistentes ao tifo aviário, entretanto, atuam efetivamente como disseminadoras da doença, por se tornarem portadoras da bactéria (BERCHIERI JUNIOR et al., 2001; DE OLIVEIRA et al., 2005).

2.3.3 Paratifo aviário

Nos últimos anos, muitas discussões sobre as salmoneloses aviárias foram geradas, devido à sua relevância como zoonose. A importância do seu controle em todas as escalas da cadeia avícola se dá pelo fato de serem capazes de infectar as aves comerciais. Consequentemente, a contaminação dos produtos de origem avícolas que posteriormente serão destinados ao consumo humano (LÖFSTRÖM et al., 2010).

Os sorovares flagelados, que são a grande maioria (por exemplo, *S*. Enteritidis e *S*. Typhimurium), causam infecção paratifóide em diferentes hospedeiros, incluindo os seres humanos, mamíferos, répteis e aves, pronunciada colonização gastrointestinal (BARLETTA et al., 2013). Provocam o paratifo aviário em aves de produção e por se tratar de animais suscetíveis a infecção, atuam como fonte de infecção para disseminação desse patógeno (OMICCIOLI et al., 2009).

Os sinais clínicos da enfermidade variam de acordo com a idade e a condição imunológica da ave, o sorovar e a intensidade da infecção. Em geral, são mais aparentes em aves com sistema imune imaturo, ou seja, em aves jovens (GAST; HOLT, 1998; GAMA; BERCHIERI JUNIOR; FERNANDES, 2003). Nessa fase, aparecem a partir do quarto dia após a infecção, com a observação de aves apáticas, com as penas arrepiadas, asas caídas, amontoadas e diarreia (BARROW, 2000). Em aves com mais de 14 dias de vida, normalmente não há o aparecimento de sinais clínicos, ou aparecem de maneira discreta no lote acometido. Ocorre retardo no crescimento e diminuição dos índices de desempenho dos animais acometidos, sejam eles destinados à produção de carne ou de ovos, devido ao acometimento do trato intestinal, com um quadro leve de enterite (BERCHIERI JUNIOR; FREITAS NETO, 2009).

A principal forma de transmissão do paratifo aviário ocorre pela via horizontal, pelo contato direto do ovo com as fezes contaminadas, seja durante a sua passagem pela cloaca durante a postura, ou pelo contato no ambiente contaminado (MESSENS, 2005). Também pode ocorrer a transmissão por contato direto da ave com materiais contaminados ou outros animais infectados, como ração, água, veículos, outras aves, seres humanos, moscas e roedores (BERCHIERI JUNIOR;

FREITAS NETO, 2009). A transmissão vertical também pode ocorrer, pois a bactéria pode colonizar os folículos ovarianos, o que levaria a infecção dos ovos durante o seu desenvolvimento no oviduto (THIAGARAJAN et al. 1994; POPPE, 2000).

2.3.3.1 Importância em saúde pública

A produção avícola é um dos mais importantes setores agropecuários do mundo, com grande relevância em mercados consumidores e exportadores, como Estados Unidos da América, União Européia e Brasil (CHAND et al., 2012). Ao aliar baixo custo de produção a técnicas de nutrição, melhoramento genético, manejo e controle sanitário (RIZZO et al., 2010), possibilita a obtenção de proteína de origem animal de alta qualidade e de maneira acessível a todos os públicos ou classes do mercado consumidor (SAMANTA et al., 2011).

A incidência de doenças transmitidas por alimentos (DTA), causadas pelo gênero *Salmonella* spp., em países industrializados é frequentemente alta em levantamentos epidemiológicos (MA et al. 2014). Ao menos 1,3 bilhão de casos de salmonelose humana são reportadas anualmente no mundo, e cerca de 3 milhões de pacientes morrem da doença (LIN et al., 2011). Os surtos de DTAs relacionados às salmoneloses provocam danos significativos à saúde de animais e seres humanos infectados, além do grande prejuízo econômico (CHEN et al. 2010).

Este patógeno pode infectar várias espécies de animais e também o homem, devido ao consumo de água ou alimentos crus de origem animal contaminados, como ovos, leite e carne proveniente de bovinos, aves, suínos, peixes e frutos do mar (MCGUINNESS et al., 2009). No humano, a doença provoca um quadro de enterite, com diferentes níveis de patogenicidade, dependendo do sorovar e da imunidade do hospedeiro (BARLETTA et al., 2013).

Nos Estados Unidos da América, as bactérias provenientes do gênero ocupam o segundo lugar no *ranking* de patógenos causadores de DTAs (MA et al. 2014). De acordo com relatório emitido pelo "*Centers for Desease Control and Prevention" (*CDC), 99% dos isolados notificados de *Salmonella* spp. são provenientes de *S. enterica* subsp. *enterica*. Conforme levantamento de dados epidemiológicos, no país estima-se que, os surtos provocam uma média anual de

1,2 milhão de enfermos, 15 mil hospitalizações e 480 mortes. Ao analisar a incidência dos sorovares envolvidos nesses surtos, os relatados com maior frequência foram *S.* Enteritidis (19%), *S.* Typhimurium (11%) e *S*. Newport (10%) (CDC, 2014).

Na China, as salmoneloses são a causa de aproximadamente 40% da incidência de DTAs provenientes de bactérias (CHEN et al. 2010). Na União Européia (UE), estima-se que 320 mil casos de DTAs são reportados anualmente e dentre eles, mais de 100 mil (31,2%) são provocados pelas salmoneloses, com uma taxa de incidência de 35 casos notificados a cada 100 mil habitantes. Os sorovares isolados com maior frequência nesses surtos são *S.* Enteritidis (66,7%) e *S.* Typhimurium (6,5%). De acordo com o *"European Food Safety Authority"* (EFSA), é estimado um prejuízo anual de 3 bilhões de euros por ano com os surtos provocados por este patógeno (EU, 2016).

No Brasil, em relatório publicado no ano de 2015, pela Secretaria de Vigilância em Saúde (SVS), foi relatado que, embora a incidência de DTAs no país tenha reduzido 35% em relação ao último relatório publicado em 2014, em 58,8% dos surtos não foi realizado o isolamento e identificação dos patógenos envolvidos nesses surtos. Entretanto, quando o agente foi identificado, 14,3% desses isolados foram provenientes de bactérias do gênero *Salmonella* spp., sendo o patógeno mais isolado dentre as amostras analisadas (SVS, 2015). Segundo o Instituto Adolfo Lutz, no ano de 2014, os principais isolados no Brasil foram semelhantes aos isolados nos EUA e na UE, com maior prevalência de *S.* Typhimurium (63,9%) e *S.* Enteritidis (15%) (BERCHIERI JUNIOR; PENHA FILHO, 2016).

2.4 Prevenção e controle

Os programas existentes para prevenção e controle da doença são complexos, devido às diversas fontes de contaminação dentro da indústria avícola. Entre estas fontes, destacam-se os alimentos derivados de aves, matérias primas para produção de rações, roedores, aves silvestres, insetos, veículos de transportes, gaiolas e galpões contaminados e o homem. Com isso, estratégias de intervenção

em todos os segmentos da cadeia alimentar são necessárias, para obtenção de um controle eficaz dessa bactéria (ANTUNES et al., 2003).

A adoção de rígidos programas de controle, por parte dos países desenvolvidos, proporcionaram estratégias bem sucedidas para controle de *Salmonella* spp. em seus plantéis. Esses programas são fundamentados, na adoção de medidas profiláticas, que envolvem higiene, limpeza e desinfecção de instalações, controle de pragas (roedores e insetos), retirada de aves mortas, identificação do patógeno atuante e abate sanitário dos animais doentes e portadores (BERCHIERI JUNIOR; FREITAS NETO, 2009). Os casos de infecções ou surtos são de notificação obrigatória à Organização Mundial de Saúde Animal (OIE), devido à mortalidade dos animais envolvidos, os prejuízos causados e o risco de disseminação para outros plantéis do país (OIE, 2012).

No Brasil, no ano de 1994, foi implementado o Programa Nacional de Sanidade Avícola (PNSA), a fim de normalizar as medidas de controle e prevenção para *Salmonella* spp. O plano estabelece que quando há a ocorrência nos plantéis avícolas de *S*. Gallinarum, *S*. Pullorum, *S*. Enteritidis e *S*. Typhimurium, a sua notificação é obrigatória. A partir disso, aves reprodutoras devem ser livres de *S*. Gallinarum e *S*. Pullorum e aves matrizes podem ser livres ou sob controle de *S*. Enteritidis e *S*. Typhimurium. Devido a isso, o PNSA descreve a obrigatoriedade na realização do teste de soroaglutinação rápida (SAR), com a finalidade de garantir a ausência de *S*. Gallinarum e *S*. Pullorum nos planteis, que deve ser realizado em 100% do lote de aves reprodutoras de linhagem pura, bisavós e avós em início de produção (BRASIL, 2009). Visando evitar a propagação dessas enfermidades, quando há a ocorrência de um surto de pulorose ou tifo aviário em uma granja, deve ser realizado o abate sanitário de todo o lote e adoção de medidas de desinfecção no aviário e nos equipamentos que possam atuar como possíveis fontes de contaminação para outros lotes (BERCHIERI JUNIOR; FREITAS NETO, 2009).

2.5 Diagnóstico

O método classificado como padrão ouro para diagnóstico de pulorose, tifo e paratifo aviário baseia-se no isolamento microbiológico, identificação e

caracterização bioquímica e sorológica de *Salmonella* spp. (BATISTA et al., 2013). Essa metodologia deve ser complementada pelo histórico, sinais clínicos, lesões e dados epidemiológicos dos animais ou lotes acometidos (SANTANA et al., 2011). Pode ter interpretação dificultada, devido ao crescimento de outros microrganismos presentes na amostra analisada. Além de possuir custo elevado, devido à quantidade de meios de cultura e antissoros necessários. O tempo necessário para obtenção dos resultados é dependente dos períodos de incubação e crescimento da bactéria, o que pode levar de 7 a 10 dias para ser concluído (CHEN et al. 2010).

Além dos métodos diretos utilizados para isolar o agente, os métodos indiretos são importantes para levantamentos epidemiológicos e na rotina de vigilância dos lotes de produção animal. Os testes sorológicos, como ELISA e soroaglutinação rápida são capazes de detectar anticorpos e os testes moleculares que identificam o material genético do agente etiológico pesquisado (ANDRADE et al., 2010).

Segundo Dickel et al. (2005), o diagnóstico obtido pela técnica de ELISA gera resultados em até dois dias. No entanto, esta técnica se baseia na detecção de anticorpos e, quando o resultado é positivo, há a necessidade da sua confirmação pela microbiologia convencional, o que prolonga o tempo e os custos para adquirir o resultado diagnóstico.

Um dos critérios mais importantes para obtenção de um método rápido de identificação de *Salmonella* spp., é a capacidade de detectar e diferenciar todos os sorovares, sem falso negativos e que permita que as amostras sejam rapidamente analisadas (MOORE; FEIST, 2007). Atualmente, existe a necessidade de identificação rápida de mais de um patógeno, utilizando as múltiplas plataformas de análise disponíveis (OMICCIOLI et al., 2009).

O gênero foi relatado em diversos estudos que caracterizavam as diferenças entre a utilização da microbiologia convencional e as técnicas moleculares para sua identificação e diferenciação em amostras laboratoriais, de alimentos e da produção animal (DICKEL et al., 2005; MALORNY et al., 2007; O'REGAN et al., 2008; MCGUINNESS et al., 2009; OMICCIOLI et al., 2009; ANDRADE et al., 2010; LÖFSTRÖM et al., 2010; PARK et al., 2011; BARLETTA et al., 2013; DOBHAL et al., 2014; LI et al., 2014; MA et al., 2014). A comparação entre as técnicas permite

aperfeiçoar cada vez mais os testes utilizados na rotina laboratorial. Além disso, a obtenção de um teste diagnóstico rápido para identificação de bactérias é crucial, a fim de implementar rapidamente as medidas de controle para conter a sua disseminação, a fim de reduzir as perdas decorrentes da produção animal e evitar os riscos causados por infecções de origem alimentar para a saúde humana (CHEN et al., 2010; CHERAGHCHI et al., 2014).

2.5.1 Testes Moleculares

Desde a década de 1980, as reações moleculares tornaram-se metodologias valiosas para a detecção de patógenos animais, alimentares e ambientais devido à sua velocidade de execução, sensibilidade e especificidade, aliado ao baixo custo, quando comparados aos métodos microbiológicos convencionais (DOBHAL et al., 2014).

2.5.1.1 Extração de DNA

As técnicas de extração de DNA têm papel limitante para a realização de testes moleculares, pois pode interferir no resultado final, devido à produção de inibidores para a PCR e a degradação de DNA presente na amostra. Os principais inibidores são obtidos a partir de irregularidades com o processo de lise celular, por captura e degradação do ácido nucleico, e por compostos que inibem a atividade de polimerase necessários para a amplificação do DNA alvo (ELIZAQUÍVEL; AZNAR, 2008).

Amostras biológicas têm sido relatadas como laboriosas, quando para a obtenção de amostras de DNA de qualidade para a utilização na PCR. Entre essas amostras, destacam-se os alimentos, tais como produtos cárneos e ovos, e amostras que avaliam a sanidade da produção, tais como órgãos e fezes (WANG et al., 2004).

Dobhal et al. (2014), Kawase et al. (2014) e Hanabara; Ueda (2016) descreveram que para obter amostras de DNA a partir de fezes, para utilização na PCR, são necessários utilizar métodos de purificação em coluna para remoção dos

inibidores, no entanto são metodologias mais onerosas, quando comparado as metodologias de extração de utilizadas para culturas puras ou tecidos. Similarmente, Wang et al. (2004) e Elizaquível; Aznar (2008) relataram que para se extrair DNA de amostras provenientes de alimentos, etapas devem ser incluídas durante o processo, afim de purificar adequadamente essas amostras e não degradar o DNA, como filtração e separação imunomagnética. No entanto, para as análises de rotina laboratorial, métodos de extração padronizados e rápidos são desejáveis, a fim de permitir que um elevado número de amostras sejam processadas diariamente, o que seria dificultado com a implementação dessas etapas.

Além disso, a viabilidade das bactérias a partir de amostras artificialmente contaminadas a partir de culturas podem se diferir das amostras naturalmente contaminadas, devido a uma variedade de condições desfavoráveis a que possam ter sido submetidas, como por exemplo, problemas durante o transporte e armazenamento (MYINT et al., 2006). Wang et al. (2015) relataram a importância e necessidade de se realizar enriquecimento em amostras contaminadas com *Salmonella* spp. para se obter o resultado diagnóstico, pois se trata de uma bactéria que mesmo em baixas concentrações podem resultar em doença nos humanos, além de pertencer a uma microbiota altamente competitiva e com microrganismos similares.

Portanto, a eficácia da técnica da PCR depende diretamente do processo de extração e purificação de DNA, a fim de remover todas às substancias inibidoras provenientes das amostras biológicas e proporcionar um bom rendimento do DNA purificado (DE MEDICI et al., 2003).

2.5.1.2 Reação em Cadeia da Polimerase - PCR

A técnica da reação em cadeia da polimerase (PCR) obtêm resultados altamente específicos, devido a utilização de iniciadores exclusivos a cada patógeno, com resultados gerados em um a dois dias e não necessita de confirmação pela microbiologia convencional (PLETTIG et al., 2015).

Hoorfar et al. (1999) compararam a técnica de cultura microbiológica e posterior análise bioquímica, com a técnica da PCR, para identificação de 36

amostras de *Salmonella* spp. Os autores obtiveram os mesmos resultados em ambas as técnicas analisadas. No entanto a obtenção dos resultados foi mais rápida com a utilização da técnica de PCR. Os autores relataram também, que a técnica da PCR detecta genes específicos e não depende de variações fenotípicas, evitando, assim, resultados falso-negativos, que são frequentemente obtidos pela analise microbiológica.

A especificidade de detecção dos testes moleculares depende tanto da singularidade da sequência de nucleotídeos alvos de anelamento dos iniciadores para o agente patogênico de interesse, quanto da ligação específica dos iniciadores (*primers*) ou de sondas para o gene referente ao patógeno de interesse. Esses são fatores cruciais para a eficácia de um método de detecção molecular (CHEN et al., 2010).

Ribeiro et al. (2009) e Batista et al. (2013) descreveram reações que diferenciam bactérias do gênero de *Salmonella* spp. classificadas pelos seus respectivos sorovares com a utilização da técnica da PCR convencional. No entanto, duas ou mais reações são necessárias para a obtenção do resultado diferencial.

2.5.1.3 PCR em tempo real - qPCR

Para uma abordagem qualitativa e quantitativa de patógenos, a PCR em tempo real, tem sido o foco principal do mercado com âmbito em diagnóstico. As vantagens da técnica incluem: reação executada de maneira rápida, a opção de detecção de mais de um patógeno ao mesmo tempo, detecção imediata do produto dentro de placas fechadas, eliminando a necessidade de eletroforese em gel após a PCR e evitando a contaminação do ambiente com produtos tóxicos e teratogênicos, como o brometo de etídeo, além da possibilidade de quantificar o patógeno atuante em determinada amostra (LEE et al., 2014).

Ma et al. (2014) demonstraram a alta sensibilidade, especificidade, e precisão em estudo desenvolvido com carcaças suínas contaminadas artificialmente por *Salmonella* spp., *Shigella* spp. e *S. aureus*, com obtenção de 99,2%, 100%, e 99,5% de amostras positivas, respectivamente. Esses autores evidenciaram também que o limite de detecção de 2,0 UFC/g de carcaça contaminada para o teste proposto.

A PCR em tempo real pode ser classificada em dois grupos principais, com base em reações que utilizam sondas como fluoróforo e reações que utilizam intercalantes de DNA, como o fluoróforo SYBr Green I. As sondas em geral são produtos caros e exigem mais tempo e conhecimento para serem sintetizadas (CHENG et al., 2013). Já as reações que utilizam a SYBr Green I como intercalante de DNA, que é um fluoróforo universalmente aplicável a qualquer sequência alvo representam uma opção mais barata para o desenvolvimento de protocolos de qPCR. Além disso, a utilização desse fluoróforo é especialmente atraente para aqueles que possuem metodologias padronizadas para a PCR convencional e desejam converter para o formato em tempo real (GOMES et al., 2014).

Os laboratórios de diagnóstico de rotina possuem ampla necessidade de técnicas que identifiquem patógenos designados a estudos de vigilância epidemiológica e que podem ser utilizadas como ferramentas rápidas de triagem em surtos de infecções alimentares. Com isso, os estudos voltados a padronização das reações múltiplas (multiplex qPCR) tem demonstrado grande potencial para suprir essa necessidade (O'REGAN et al., 2008). O recente foco está na utilização de reações padronizadas e validadas, que podem, simultaneamente, amplificar mais do que duas sequências de genes em uma única reação e tem sido usado para identificar e diferenciar grande variedade de agentes patogênicos (MA et al., 2014).

De maneira geral, a aplicabilidade da reação por diferentes laboratórios em todo o mundo, depende dos custos que envolvem a execução da reação. Ao considerar que a reação multiplex já reduz os custos com a redução das reações, é necessário considerar o custo com a utilização do fluoróforo na reação. Assim, considera-se que os intercalantes de DNA, são mais atrativos perante os custos com as reações, que quando se utiliza as sondas de hidrólise (PAFUNDO et al., 2010; GOMES et al., 2014; SINGH; MUSTAPHA, 2014). Apesar de as reações multiplex que utilizam intercalantes de DNA não proporcionarem índices de especificidade tão elevados quanto aos obtidos com a utilização de sondas, a sua confiabilidade pode ser verificada semi-qualitativamente por meio da análise da curva de dissociação dos iniciadores (*Tm*). Esse tipo de metodologia tem se demonstrado sensível, específico e barato para a detecção simultânea de múltiplos agentes patogênicos (CHENG et al., 2013).

O uso do sistema SYBR Green I para a reação multiplex qPCR tem sido relatada em diversos estudos para identificação de patógenos, ingredientes alergênicos, violações microbiológicas em alimentos contaminados e dos genes de resistência a antibióticos (FUKUSHIMA et al., 2003; PONCHEL et al., 2003; VARGA; JAMES, 2005; LIU et al., 2006; FAN et al., 2007; PAFUNDO et al., 2010; RAJTAK et al., 2011; KAGKLI et al., 2012; CHENG et al., 2013; GOMES et al., 2014; SINGH; MUSTAPHA, 2014).

Li et al. (2014) relataram a dificuldade ao se obter uma reação multiplex para o gênero *Salmonella* spp., que ao padronizarem uma reação hexaplex para diferenciação de cinco sorovares provenientes do gênero, demonstraram a necessidade de estudos comparativos com outros sorovares mais raros, para que a reação seja realmente validada e a fim de garantir a inexistência de reações cruzadas. Em estudo realizado por O'Regan et al. (2008) com amostras de frango artificialmente contaminadas com quatro sorovares de *Salmonella* spp., demonstraram eficiência de 100% das amostras testadas, tanto pelo método microbiológico tradicional, quanto para a multiplex qPCR com utilização de sondas.

3 Objetivos

3.1 Objetivo Geral

Padronização e aplicação da técnica da PCR em tempo real para identificação e quantificação da carga bacteriana em órgãos e fezes de aves comerciais infectadas por *Salmonella enterica* subesp. *enterica* sorovares ENTERITIDIS, TYPHIMURIUM e GALLINARUM (biovares GALLINARUM e PULLORUM), em diferentes momentos pós-infecção.

3.2 Objetivos Específicos

- Padronizar a reação multiplex qPCR em tempo real para diagnóstico, diferenciação e quantificação dos biovares SG e SP.

- Padronizar a reação multiplex qPCR em tempo real para diagnóstico, diferenciação e quantificação dos sorovares SE e STM.

- Avaliar a infecção sistêmica e excreção fecal por meio da contagem bacteriana pelas técnicas de microbiologia convencional e qPCR em tempo real, em amostras de fígado e conteúdo cecal de aves poedeiras experimentalmente infectadas por SG, SP, SE e STM.

4 Referências

ANDRADE, R.B.; GEMELLI, T.; ONDER, L.P.D.; CRISTINA, K.; BRITO, T.; BARBOZA, A.A.L.; BRITO, B.G. Métodos diagnósticos para os patógenos alimentares: *Campylobacter* sp., *Salmonella* spp. e *Listeria monocytogenes*. **Arquivos do Instituto Biológico**, São Paulo, v. 77, n. 4, p.741-750, 2010.

ANTUNES, P.; RÉU, C.; SOUSA, J.C.; PEIXE, L.; PESTANA, N. Incidence of Salmonella from poultry products and their susceptibility to antimicrobial agents. **International Journal of Food Microbiology**, v. 82, p.97-103, 2003.

BARLETTA, F.; MERCADO, E.H.; LLUQUE, A.; RUIZ, J.; CLEARY, T.G.; OCHOA, T.J. Multiplex real-time PCR for detection of *Campylobacter*, *Salmonella*, and *Shigella*. **Journal of Clinical Microbioly**, v. 51, n. 9, p.2822-2829, 2013.

BARROW, P. A., HUGGINS, M. B.; LOVELL, M. A. Host specificity of *Salmonella* infection in chickens and mice is expressed *in vivo* primarily at the level of the reticuloendothelial system. **Infection and Immunity**, Washington, v. 62, n, 10, p. 4602-4610, 1994.

BARROW, P.A. The paratyphoid *Salmonella*e. **Revue scientifique et technique**, Paris, v.19, n.2, p. 339-352, 2000.

BARROW, P.A.; FREITAS NETO, O.C. Pullorum disease and fowl typhoid – new thoughts on old diseases: a review. **Avian Pathology**, London, v. 40, n. 1, p. 1-13, 2011.

BATISTA, D.F.A.; FREITAS NETO, O.C.; LOPES, P.D.; ALMEIDA, A.M.; BARROW, P.A. BERCHIERI JR., A. Polymerase chain reaction assay based on *ratA* gene allows differentiation between *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovars Gallinarum and Pullorum. **Journal of Veterinary Diagnostic Investigation**, v. 25, n. 2, p.259-262, 2013.

BERCHIERI JUNIOR, A.; MURPHY, C. K.; MARSTON, K.; BARROW, P. A. Observations on the persistence and vertical transmission of *Salmonella enterica*

serovars Pullorum and Gallinarum in chickens: effect of bacterial and host genetic background. **Avian Pathology**, London, v. 30, n. 3, p. 221-231, 2001.

BERCHIERI JUNIOR, A.; FREITAS NETO, O. C. Salmoneloses.In: BERCHIERI JUNIOR, A.; SILVA, E. N.; FÁBIO, J. D.; SESTI, L.; ZUANAZE, M. A. F. (Eds.). **Doenças das Aves**. 2. Ed. Campinas: FACTA, 2009, p. 435-453

BERCHIERI JUNIOR, A.; PENHA FILHO, R.C. Encarte Especial: Os conhecimentos atuais sobre o Paratifo Aviário no Brasil. **AviSite**. n.04, p. 7-9, 2016.

BRASIL. Normas Técnicas para Controle e Certificação de Núcleos e Estabelecimentos Avícolas como livres de *Salmonella* Gallinarum e de *Salmonella* Pullorum e Livres ou Controlados para *Salmonella* Enteritidis e para *Salmonella* Typhimurium. Atos legais. Instrução Normativa n° 78. Diário Oficial da República Federativa do Brasil, Poder Executivo, Brasília – DF, 03 nov 2003. Seção 1, p 1-9. D

BRASIL, **Ministério da Agricultura, Pecuária e Abastecimento**. Manual de Legislação : Programa nacional de sanidade avícola / Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Departamento de Saúde Animal. Brasília : MAPA/SDA/DSA, p.171-241, 2009.

CDC. **Centers for Disease Control and Prevention**. National Enteric Disease Surveillance: Salmonella Annual Report, 2013. Georgia: US Departiment of Health and Human Services; 2014.

CHAND, N.; JAVED, Y.; KHAN, S.; MUSHTAQ, M.; SULTAN, A.; TANWEER, A.J. Comparative efficacy of different schedules of administration of medicinal plants mixed infusion on hematology of broiler chicks. **Sarhad Journal of Agriculture**, v. 28, n. 2, p.327-331, 2012.

CHEN, J.; ZHANG, L.; PAOLI, G.C.; SHI, C.; TU S.; SHI, X. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. **International Journal of Food Microbiology**, v. 137, p.168–174, 2010.

CHENG, J.; JIANG, Y.; RAO, P.; WU, H.; DONG, Q.; WU, Z.; DING, X.; GUO J. Development of a single-tube multiplex real-time PCR for detection and identification of five pathogenic targets by using melting-curve analysis with EvaGreen. **Archives of Virology**, v. 158, p.379-386, 2013.

CHERAGHCHI, N.; KHAKI, P.; BIDHENDI, S.M.; SABOKBAR, A. Identification of isolated *Salmonella enterica* serotype *gallinarum* biotype *Pullorum* and *Gallinarum* by PCR-RFLP. **Jundishapur Journal of Microbiology**, v.7, p. 1-4, 2014.

DE MEDICI, D.; CROCI, L.; DELIBATO, E.; DI PASQUALE, S.; FILETICI, E.; TOTI, L. evaluation of DNA extraction methods for use in combination with SYBR green I real-time PCR to detect *Salmonella enterica* serotype enteritidis in poultry. **Applied and Environmental Microbiology**, v. 69, n. 6, p.3456–3461, 2003.

DE OLIVEIRA, G. H.; BERCHIERI JÚNIOR, A. FERNANDES, A. C. Experimental infection of laying hens with *Salmonella enterica* serovar Gallinarum. **Brazilian Journal of Microbiology**, São Paulo, v. 36, n. 1, p. 51-56, 2005.

DICKEL, E.L.; RODRIGUES, L.B.; SANTOS, L.R.; VALLE, S.F.; PILOTTO, F.; RODEMBUSH, C.; WALD, V.B.; CANAL, C.W.; NASCIMENTO, V.P. Análise comparativa entre microbiologia convencional, ELISA e PCR para detecção de *Salmonella enteritidis*, *S. typhimurium*, *S. gallinarum* e *S. pullorum* em carne de frango contaminada artificialmente. **Revista Brasileira de Ciência Veterinária**, v. 12, n. 1/3, p.5-10, 2005.

DOBHAL, S.; ZHANG, G.; ROHLA, C.; SMITH, M.W.; MA, L.M. A simple, rapid, cost-effective and sensitive method for detection of Salmonella in environmental and pecan samples. **Journal of Applied Microbiology**, v. 117, p.1181-1190, 2014.

ELIZAQUÍVEL, P.; AZNAR, R. Comparison of four commercial DNA extraction kits for PCR detection of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, and *Staphylococcus aureus* in fresh, minimally processed vegetables. **Journal of Food Protection**, v.71, n.10, p. 2110–2114, 2008.

EU. RASFF - The Rapid Alert System for Food and Feed: 2015 annual report. European Commission. Health and Food Safety 2016.

FAN, W.; HAMILTON, T.; WEBSTER-SESAY, S.; NIKOLICH, M.P.; LINDLER, L.E. Multiplex real-time SYBr Green I PCR assay for detection of tetracycline efflux genes of gram-negative bacteria. **Molecular and Cellular Probes**, v.21, p. 245-256, 2007.

FENG, Y.; JOHNSTON, R. N; LIU, G.; LIU, S. Genomic comparison between *Salmonella* Gallinarum and Pullorum: differential pseudogene formation under common host restriction. **PLoS ONE**, v. 8, p. 1-6, 2013.

FUKUSHIMA, H.; TSUNOMORI, Y.; SEKI, R. Duplex real-time SYBr green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. **Journal of Clinical Microbiology**, v.41, p. 5134-5146, 2003.

GAMA, N. M. S. Q.; BERCHIERI Jr., A.; FERNANDES, S. A. Occurrence of *Salmonella* sp. in laying hens. **Revista Brasileira de Ciência Avícola**, Campinas, v. 5, n. 1, p. 15-21, 2003.

GAST, R. K.; HOLT, P. S. Persistence of *Salmonella* enteritidis from one day of age until maturity in experimentally infected layer chickens. **Poultry Science**, Champaign, v. 77, n. 12, p.1759-1762, 1998.

GOMES, T.S.; GARCIA, M.C.; CUNHA, F.S.; MACEDO, H.W.; PERALTA, J.M.; PERALTA, R.H.S. Differential diagnosis of *Entamoeba* spp. in clinical stool samples using SYBR Green real-time polymerase chain reaction. **The Scientific World Journal**, v. 2014, n. 645084, p.1-8, 2014.

GONG, J.; XU, M.; ZHU, C.; MIAO, J.; LIU, X.; XU, B.; ZHANG, J.; YU, Y.; JIA, X. Antimicrobial resistance, presence of integrons and biofilm formation of Salmonella Pullorum isolates from eastern China (1962–2010). **Avian Pathology**, v. 42, n. 3, p.290-294, 2013.

GRIMONT, P. A. D.; WEILL, F. X. Antigenic formulae of the *Salmonella* serovars." **WHO Collaborating Centre for Reference and Research on Salmonella**, Institut Pasteur, Paris, France, 2007.

HANABARA, Y.; UEDA, Y. A rapid and simple real-time PCR assay for detecting foodborne pathogenic bacteria in human feces. **Japanese Journal of Infectious Diseases**, v. 69, p.471–476, 2016.

HOORFAR, J.; BAGGESEN, D.L.; PORTING, P.H. A PCR-based strategy for simple and rapid identification of rough presumptive *Salmonella* isolates. **Journal of Microbiological Methods**, v.35, p.7-84, 1999.

ISSENHUTH-JEANJEAN, S.; ROGGENTIN, P.; MIKOLEIT, M.; GUIBOURDENCHE, M.; PINNA, E.; NAIR, S.; FIELDS, P.I.; WEILL, F. Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme. **Research in Microbiology**, v.165, p. 526-530, 2014.

KAGKLI, D.; FOLLONI, S.; BARBAU-PIEDNOIR, E.; EEDE, G.V.D.; BULCKE, M.V.D. Towards a pathogenic *Escherichia coli* detection platform using multiplex SYBr Green real-time PCR methods and high resolution melting analysis. **PLoS ONE**, v. 7, 1-12, 2012.

KAWASE, J.; KUROSAKI, M.; KAWAKAMI, Y.; KASHIMOTO, T.; TSUNOMORI, Y.; SATO, K.; IKEDA, T.; YAMAGUCHI, K.; WATAHIKI, M.; SHIMA, T.; KAMEYAMA, M.; ETOH, Y.; HORIKAWA, K.; FUKUSHIMA, H.; GOTO, R.; SHIRABE, K. Comparison of two methods of bacterial DNA Extraction from Human Fecal Samples Contaminated with *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Campylobacter jejuni*. **Japanese Journal of Infectious Diseases**, v. 67, p.441-446, 2014.

LE MINOR, L.; POPOFF, M.Y. Designation of *Salmonella enterica* sp. nov., nom. rev., as the Type and Only Species of the Genus *Salmonella*: Request for an Opinion. **International journal of systematic and evolutionary microbiology, Reading**, v. 37, n. 4, p. 465-446, 1987.

LEE, Y.; KIM, D.; LEE, K.; CHUN, J.; Single-channel multiplexing without melting curve analysis in real-time PCR. **Nature**, v. 4, p.1-6, 2014.

LI, R.; WANG, Y.; SHEN, J.; WU, C. Development of a novel hexa-plex PCR method for identification and serotyping of *Salmonella* species. **Foodborne Pathogens and Disease**, v. 11, n. 1, p.75-78, 2014.

LIN, L.H.; TSA, C. Y.; HUNG M.H.; FANG, Y.T.; LING, Q.D. Rectal swab sampling followed by an enrichment culture-based real-time PCR assay to detect *Salmonella enterocolitis* in children. **Clinical Microbiology and Infection**, v. 17, p.1421–1425, 2011.

LIU, J.I.; YAN, M.; WANG, Z.; WANG, L.; ZHOU, Y.; XIAO, B. Molecular diagnosis of α-thalassemia by combining real-time PCR with SYBr Green1 and dissociation curve analysis. **Translational Research**, v. 148, p.6-12, 2006.

LÖFSTRÖM, C.; HANSEN, F.; HOORFAR, J. Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. **Veterinary Microbiology**, v. 144, p.511–514, 2010.

MA, K.; DENG, Y.; BAI, Y.; XU, D.; CHEN, E.; WU, H.; LI, B.; GAO, L. Rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork using a multiplex real-time PCR assay based on immunomagnetic separation. **Food Control**, v. 42, p.87-93, 2014.

MALORNY, B.; BUNGE, C.; HELMUTH, R. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. **Journal of Microbiological Methods**, v. 70, p.245–251, 2007.

MCGUINNESS, S.; MCCABE, E.; O'REGAN, E.; DOLAN, A.; DUFFY. G.; BURGESS, C.; FANNING, S.; BARRY, T.; O'GRADY, J. Development and validation of a rapid real-time PCR based method for the specific detection of *Salmonella* on fresh meat. **Meat Science**, v. 83, p.555–562, 2009.

MCQUISTON, J. R.; HERRERA-LEON, S.; WERTHEIM, B. C.; DOYLE, J.; FIELDS, P. I.; TAUXE, R. V.; LOGSDON Jr, J. M. Molecular phylogeny of the *Salmonellae*: Relationship among *Salmonella* species and subspecies determined from four housekeepeing genes and evidence of lateral gene transfer events. **Journal of Bacteriology**, Washington, v. 190, n. 21, p. 7060-7067, 2008.

MESSENS, W. Eggshell penetration by *Salmonella*: A review. **World's PoultryScience Journal**, Cambridge, v. 61, p. 71–84, 2005. Disponível em: <http://dx.doi.org/10.1079/WPS200443>.

MOORE, M.M.; FEIST, M.D. Real-time PCR method for *Salmonella* spp. targeting the stn gene. **Journal of Applied Microbiology**, v. 102, p.516–530, 2007.

MYINT, M.S.; JOHNSON, Y.J.; TABLANTE, N.L.; HECKERT, R.A. The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated *Salmonella* in raw poultry compared to conventional culture. **Food Microbiology**, v. 23, p.599–604, 2006.

OMICCIOLI, E.; AMAGLIANI, G.; BRANDI, G.; MAGNANI, M. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. **Food Microbiology**, v. 26, p.615–622, 2009.

O'REGAN, E.; MCCABE, E.; BURGESS, C.; MCGUINNESS, S.; BARRY, T.; DUFFY, G.; WHYTE, P.; FANNING, S. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. **BMC Microbiology**, v. 8, p.1-11, 2008.

OIE, World Organisation for Animal Health. Fowl typhoid and Pullorum disease, capítulo 2.3.11. In: OIE (Ed.). **Manual of Diagnostic Tests and Vaccines for Terrestrial Animals**, 7th Ed. Paris: OIE, 2012. p. 538-548.

PAFUNDO, S.; GULLÌ, M.; MARMIROLI, N. Multiplex real-time PCR using SYBr GreenER™ for the detection of DNA allergens in food. **Analytical and Bioanalytical Chemistry**, v. 396, p.1831-1839, 2010.

PARK, S.H.; HANNING, I.; JARQUIN, R.; MOORE, P.; DONOGHUE, D.J.; DONOGHUE, A.M.; RICKE, S.C. Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. **FEMS Microbiology Letters**, v. 316, p.7-15, 2011.

PINHEIRO, L. A.; DE OLIVEIRA, G. H.; BERCHIERI JUNIOR, A. Experimental *Salmonella enterica* serovar Pullorum infection in two commercial varieties of laying hens. **Avian Pathology**, London, v. 30, n. 2, p. 129-133, 2001.

PLETTIG, R.; NOWAK, A.; BALAU, V.; HAHNENKAMP, K.; USICHENKO T. Prospective comparison of a PCR assay and a microbiological culture technique for identification of pathogens from blood and non-blood samples in septic patients. **Journal of Intensive Care**, v. 3, n. 51, p.1-8, 2015.

PONCHEL, F.; TOOMES, C.; BRANSFIELD, K.; LEONG, F.T.; DOUGLAS, S.H.; FIELD, S.L.; BELL, S.M.; COMBARET, V.; PUISIEUX, A.; MIGHELL, A.J.; ROBINSON, P.A.; INGLEHEARN, C.F.; ISAACS, J.D.; MARKHAM, A.F. Real-time PCR based on SYBr-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. **BMC Biotechnology**, v. 3, p.1-13, 2003.

POPPE, C. *Salmonella* Infections in the Domestic Fowl. In: WRAY, C.; WRAY, A. *Salmonella* in domestic animals. New York: CABI Publishing, 2000. cap. 7, p. 107-132.

POPOFF, M. Y.; BOCKEMÜHL, J.; HICKMAN-BRENNER, F. W. Kauffmann- White Scheme. **Research in Microbiology**, Paris, v. 147, n. 39, p. 765-769, 1996.

RAJTAK, U.; LEONARD, N.; BOLTON, D.; FANNING, S. A real-time multiplex SYBr Green I polymerase chain reaction assay for rapid screening of *Salmonella* serotypes prevalent in the European Union. **Foodborne Pathogens and Disease**, v. 8, p.769-779, 2011.

RIBEIRO, S.A.M.; PAIVA, J.B.; ZOTESSO, F. Molecular differentiation between *Salmonella enterica* subsp *enterica* serovar Pullorum and *Salmonella enterica* subsp

enterica serovar Gallinarum. **Brazilian Journal of Microbiology**, v. 40, p.184-188, 2009.

RIZZO, P.V.; MENTEN, J.F.M.; RACANICCI, A.M.C.; TRALDI, A.B.; SILVA, C.S.; PEREIRA, P.W.Z. Extratos vegetais em dietas para frangos de corte. **Revista Brasileira de Zootecnia**, v.39, n.4, p.801-807, 2010.

SAMANTA, B.; GHOSH, P.R.; BISWAS, A.; DAS, S.K. The effects of copper supplementation on the performance and hematological parameters of broiler chickens. **Journal of Animal Science**, Asian-Aust., v. 24, n. 7, p. 1001-1006, 2011.

SANTANA, E.S.; OLIVEIRA, F.H.; ROCHA, T.M.; SANTANA, R.R.; ANDRADE, M.A. Abordagem sobre *Salmonella* sp. com enfoque na caracterização, patogênese e métodos de diagnóstico em aves. **Enciclopédia Biosfera**, Centro Científico Conhecer - Goiânia, v. 7, n. 12, 23f, 2011.

SHIVAPRASAD, H.L. Fowl typhoid and pullorum disease. **Revue Scientifique et Technique**, v.19, p. 405-424, 2000.

SINGH, P.; MUSTAPHA, A. Development of a real-time PCR melt curve assay for simultaneous detection of virulent and antibiotic resistant *Salmonella*. **Food Microbiology**, v. 44, p.6-14, 2014.

SORIA, M.C.; SORIA, M.A.; BUENO, D.J.; TERZOLO, H.R. Comparison of 3 culture methods and PCR assays for *Salmonella gallinarum* and *Salmonella pullorum* detection in poultry feed. **Poultry Science**, v.92, p. 1505-1515, 2013.

SVS. **Secretaria de Vigilância em Saúde**. Doenças Transmitidas por Alimentos. Ministério da Saúde. Secretaria de Vigilância em Saúde 2015. Disponível em: http://u.saude.gov.br/images/pdf/2015/novembro/09/Apresenta----o-dados-gerais-DTA-2015.pdf.

THIAGARAJAN, D.; SAEED, A. M.; ASEM, E. K. Mechanism of transovarian transmission of *Salmonella* Enteritidis in laying hens. **Poultry Science**, Champaign, v. 73, n. 1, p. 89-98, 1994.

VARGA, A.; JAMES, D. Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBr Green and melting curve analysis: a rapid method for strain typing. **Journal of Virological Methods**, 123, 213-220, 2005.

WANG, X.; JOTHIKUMAR, N.; GRIFFITHS, M.W. Enrichment and DNA extraction protocols for the simultaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR. **Journal of Food Protection**, v. 67, n. 1, p.189–192, 2004.

WANG, H.; GILL, V.S.; CHENG, C.; GONZALEZ-ESCALONA, N.; IRVIN, K.A.; ZHENG, J.; BELL, R.L.; JACOBSON, A.P.; HAMMACK, T.S. Evaluation and comparison of rapid methods for the detection of *Salmonella* in naturally

contaminated pine nuts using different pre enrichment media. **Food Microbiology**, v. 46, p.58-65, 2015.

CAPITULO 2 – Development of a multiplex qPCR in real time for quantification and differential diagnosis of *Salmonella* Gallinarum and *Salmonella* Pullorum

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ABSTRACT – Currently there are 2659 Salmonella serovars. The host-specific biovars *Salmonella* Pullorum and *Salmonella* Gallinarum cause systemic infections in food-producing and wild birds. Fast diagnosis is crucial to control the dissemination in avian environments. The present work describes the development of a multiplex qPCR in real time using a low-cost DNA dye (SYBr Green) to identify and quantify these biovars. Primers were chosen based on genomic regions of difference (RoD) and optimized to control dimers. Primers pSGP detect both hostspecific biovars but not other serovars and pSG and pSP differentiate biovars. Three amplicons showed different melting temperatures (Tm), allowing differentiation. The pSGP amplicon (97 bp) showed Tm of 78°C for both biovars. The pSG amplicon (273 bp) showed a Tm of 86.2°C for *S*. Gallinarum and pSP amplicon (260 bp) dissociated at 84.8°C for *S*. Pullorum identification. The multiplex qPCR in real time showed high sensitivity and was capable of quantifying 10^8–10^1 CFU of these biovars.
Keywords: Fowl typhoid; pullorum disease; SYBr green; melting curve; ROD; identification; biovar

1 Introduction

Salmonella enterica belongs to the family Enterobacteriaceae and, currently, 2659 different serovars have been identified (ISSENHUTH-JEANJEAN et al., 2014). All serovars are pathogenic. However, diseases in birds are classified in three different categories. The flagellated serovars, which are the vast majority (e.g. *S*. Enteritidis and *S*. Typhimurium), cause paratyphoid infections in different hosts including humans, mammals, reptiles and birds, with pronounced gastrointestinal colonization (BARLETTA et al., 2013). Poultry are susceptible to paratyphoid infections and are frequently associated as source of infection in foodborne disease

in humans through the consumption of contaminated meat or eggs (OMICCIOLI et al., 2009).

The other two categories of diseases in birds are fowl typhoid caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (*S*. Gallinarum) and pullorum disease caused by *Salmonella enterica* serovar Gallinarum biovar Pullorum (*S*. Pullorum). Both biovars are closely related genetically, non-motile and are host-specific pathogens of birds (LÖFSTRÖM et al., 2010; FENG et al., 2013). Infections or outbreaks are of compulsory notification to the World Organisation for Animal Health (OIE), due to the mortality of animals, losses caused and risk of dissemination to other countries.

S. Pullorum affects newly hatched young chicks, causing septicaemic colonization, with high mortality in affected flocks. Infection initiates mainly from vertical transmission originating from subclinically infected adult breeders. However, horizontal transmission contributes to fast dissemination within the flock. *S*. Gallinarum is horizontally transmitted and evidence points to a vertical dissemination route (CELIS-ESTUPINAN et al., 2017). Moreover, the infection causes high morbidity and up to 80% of mortality in young or adult chickens (SHIVAPRASAD, 2000). Different factors, such as the interaction with the intestinal epithelium, high invasiveness, intracellular survival in infected macrophages and evasion of the immune response, are involved in the pathogenesis of these bacteria (SORIA et al., 2013).

The gold standard method used for the diagnosis of *S*. Gallinarum and *S*. Pullorum during outbreaks is microbiological culture, for isolation, biochemical identification and serotyping of the isolate (SANTANA et al., 2011; MA et al., 2014). In addition to direct methods used to isolate these bacteria, indirect methods are important for epidemiological studies and monitoring of the sanitary conditions in flocks. Serological tests such as ELISA and rapid sero-agglutination test can detect antibodies against *Salmonella* spp. and PCR is capable of detecting and identifying the DNA of the pathogen (ANDRADE et al., 2010).

The bacterial culture of *Salmonella* spp. has a few disadvantages compared to molecular biology techniques. Among these, the direct identification of the bacteria is difficult due to the growth of other microorganisms present in the sample,

especially from microbiota. The complete identification depends on biochemical and serological analysis of each isolate, increasing the time and cost to obtain results with this method (CHEN et al., 2010). The diagnosis by ELISA technique produces results within two days. However, this technique relies on the detection of antibodies and may require confirmation by conventional microbiology, which prolongs obtaining a diagnosis (DICKEL et al., 2005).

The PCR technique has been successfully established for fast and reliable diagnosis of different pathogens, including fastidious bacteria. The results may be obtained within two days or less. However, positive results do not require the confirmation by conventional microbiology. The result analysis follows an established pattern of interpretation (LIN et al., 2011). Many epidemiological surveys were successfully conducted during the last decades using PCR, because this method can be designed for high specificity and sensitivity (CHEN et al., 2010).

Real-time PCR has been more recently developed for diagnosis of pathogens, with the features of higher sensitivity than conventional PCR and faster protocols to obtain the results. Thus, in the present study, we describe the development of a multiplex real-time PCR to specifically detect and differentiate closely related biovars *S*. Gallinarum and *S*. Pullorum and simultaneously quantify the bacterial numbers in samples.

2 Material and methods

2.1 Bacterial strains for validation of the real-time PCR assay

Twenty-eight isolates from biovar *S*. Gallinarum and 18 isolates from biovar *S*. Pullorum were used for DNA extraction, development and validation of the multiplex quantitative PCR in real time, including the sequenced strains *S*. Gallinarum SG9 (accession number: CM001153.1), *S*. Gallinarum 287/91 (a.n.: AM933173.1), *S*. Pullorum FCAV198 (a.n.: AZRG00000000). Additionally, DNA from 59 different *Salmonella* isolates (including non-typhoidal and typhoidal serovars) and six other Enterobacteriaceae (Supplemental Table 1) were used as negative controls for validation of the primers' specificity. Isolates were obtained from two National Reference Laboratories or from the Laboratory of Avian Pathology at the School of

Agricultural and Veterinary Sciences (FCAV-Unesp/Jaboticabal/SP) and stored at −80°C.

2.2 Bacterial DNA extraction

Each isolate was cultured in Luria-Bertani broth (LB) at 37°C, shaking at 100 rpm for a period of 18 hours, including the bacteria used as negative controls. Before DNA extraction, all *S*. Gallinarum and *S*. Pullorum cultures were quantified by plating serial dilutions in Brilliant Green agar (Oxoid CM0263; Oxoid Ltd, Basingstoke, UK) of the culture with subsequent incubation at 37°C for 24 h. After incubation, the bacterial numbers of each culture were evaluated and the colony forming units per ml (CFU/ml) were transformed to Log_{10}. DNA extraction of all bacterial cultures was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purity and concentration of bacterial DNA was analysed by spectrophotometry (Nanodrop 1000 Thermo Fisher Scientific, Waltham, MA, USA).

2.3 Primer design for differential diagnosis between *S*. Gallinarum and *S*. Pullorum

Three pairs of primers were used and the sequences and details are in Table 1. The primer pSGP was designed to detect the *S. enterica* serovar Gallinarum, including both biovars, without detection of other typhoidal or non-typhoidal *Salmonella* sp. serovars. The primers pSG and pSP were designed to differentiate *S*. Gallinarum biovar Gallinarum (pSG) and *S*. Gallinarum biovar Pullorum (pSP) based on the analysis of regions of difference (RoDs) from each biovar (BATISTA et al., 2013). Primers were designed using the Primer 3 software (Free Software Foundation, Boston, MA, USA), based on the complete genome sequence of *S*. Gallinarum and *S*. Pullorum available at the NCBI database (http://www.ncbi.nlm.nih.gov/genome/) and validated *in silico* using the primer BLAST tool, to check for non-specific annealing with other sequenced microorganisms.

Supplemental Table 1. Bacterial strain used to multiplex real-time qPCR validation for differential diagnosis between *S.* Gallinarum and *S.* Pullorum

Strain	Code	qPCR	Strain	Code	qPCR	Strain	Code	qPCR
***Salmonella* spp. strain**			***Salmonella* spp. strain**			**Non *Salmonella* spp. strain**		
S. Gallinarum	SG 257/87-a	+	*S.* Typhimurium	STM 986	-	*Escherichia coli*	Field strains**	-
S. Gallinarum	SG 257/87-b	+	*S.* Typhimurium	STM 985/11	-	*Citrobacter freundii*	Field strains**	-
S. Gallinarum	SG 291/90	+	*S.* Abony	AV431.2/07	-	*Listeria Monocitogenes*	FC00266	-
S. Gallinarum	SG 292/90	+	*S.* Agona	CR104/10	-	*Pseudomonas* sp.	Field strains**	-
S. Gallinarum	SG 293/90	+	*S.* Alachua	CR310/06	-	*Shigella sonnei*	ATCC23931	-
S. Gallinarum	SG 297/91	+	*S.* Albany	CR189/07	-	*Yersinia enterocolitica*	ATCC9610	-
S. Gallinarum	HAKIM 2X LEBANM	+	*S.* Anatum	CR114/10	-			
S. Gallinarum	72-805 NANABI	+	*S.* Bareilly	CR306/08	-			
S. Gallinarum	372 GREEK	+	*S.* Belem	CR69/10	-			
S. Gallinarum	SG 5441-a	+	*S.* Brandenburg	CR140/10	-			
S. Gallinarum	SG 5441-b	+	*S.* Brendeney	CR46/05	-			
S. Gallinarum	SG 7285-b	+	*S.* Cerro	CR116/10	-			
S. Gallinarum	IOC3558/99 RS	+	*S.* Chester	CR332/08	-			
S. Gallinarum	IOC3554/99 RS	+	*S.* Choleraesuis	ATCC1078	-			
S. Gallinarum	IOC1780/96 SP	+	*S.* Corvallis	CR118/10	-			
S. Gallinarum	IOC4623/98 MG	+	*S.* Cubana	CR411/08	-			
S. Gallinarum	IOC4295/02 MG	+	*S.* Derby	CR436/07	-			
S. Gallinarum	IOC2003/01 MT	+	*S. enterica* 6,8:ch:-	CR21/08	-			
S. Gallinarum	SG 10	+	*S. enterica* O:16,14,24,25:y:-	CR230/08	-			
S. Gallinarum	SG 188-1c	+	*S. enterica* O:3,10	CR376/08	-			
S. Gallinarum	ATCC9184	+	*S. enterica* O:6,7:r:-	CR361/08	-			
S. Gallinarum	SG 1137/11-14	+	*S. enterica* subsp. *enterica* (O:13,23:i:-)	CR91/10	-			
S. Gallinarum	SG 2750/10-22	+	*S. enterica* subsp. *enterica* (O:9,12:-:1,5)	CR222/09	-			
S. Gallinarum	SG 345/11	+	*S. enterica* subsp. *enterica* (O:6,7:b:-)	CR016/10	-			
S. Gallinarum	SG 188-2	+	*S. enterica* subsp. *arizonae*	ATCC13314	-			
S. Gallinarum	IBB1811**	+	*S.* Gafsa	C427/07	-			
S. Gallinarum	IBB0212**	+	*S.* Grumpensis	CR325/07	-			
S. Gallinarum	IBB0412**	+	*S.* Hadar	CR111/10	-			
S. Pullorum	SP 21	+	*S.* Havana	CR517/06	-			
S. Pullorum	SP 449/87	+	*S.* Heidelberg	CR239/08	-			
S. Pullorum	IOC332/01 SC	+	*S.* Infantis	CR142/10	-			
S. Pullorum	IOC1776/96 SP	+	*S.* Kentucky	CR135/10	-			
S. Pullorum	IOC1467/97 SP	+	*S.* Kottbus	AV155/02, 4	-			
S. Pullorum	IOC1775/96 SP	+	*S.* Livingstone	CR288/08	-			
S. Pullorum	IOC1783/96 SP	+	*S.* London	AV214/99, 3	-			
S. Pullorum	IOC9771/04 RS	+	*S.* Minnesota	CR102/10	-			
S. Pullorum	IOC1771/96 SP	+	*S.* Montevideo	CR303/08	-			
S. Pullorum	SP 11	+	*S.* Muenchen	CR011/10	-			
S. Pullorum	ATCC9120	+	*S.* Newport	CR044/10	-			
S. Pullorum	CR31/06	+	*S.* Ohio	CR98/10	-			
S. Pullorum	CR32/06	+	*S.* Orion	CR124/10	-			
S. Pullorum	CR350/06	+	*S.* Panama	CR138/10	-			
S. Pullorum	CR135/07	+	*S.* Paratyphi B	CR423/06	-			
S. Pullorum	AV363/07	+	*S.* Rissen	CR412/08	-			
S. Pullorum	CR31/06-1	+	*S.* Saintpaul	CR325/08	-			
S. Pullorum	CR366/08	+	*S.* Schwarzengrund	CR121/10	-			
S. Enteritidis	P125088	-	*S.* Senftenberg	CR119/10	-			
S. Enteritidis	SE 147/139-2	-	*S.* sp (8,20:z4:z23:-)	CR128/05	-			
S. Enteritidis	SE 16742	-	*S.* Tennessee	CR404/08	-			
S. Enteritidis	SE 174	-	*S.* Thompson	AV424/07, 29	-			
S. Typhimurium	STM 1116	-	*S.* Typhi	ATCC10749	-			
S. Typhimurium	STM 723	-	*S.* Worthington	CR345/08	-			
S. Typhimurium	STM F98	-						

*Bacterial isolates were obtained from: The American Type Culture Collection (ATCC), Oswaldo Cruz Foundation - FIOCRUZ (IOC/FC), National Agricultural and Livestock Laboratories – LANAGRO (CR/AV). ** Field strains isolated and maintained at School of Agricultural and Veterinary Sciences, São Paulo State University, FCAV/UNESP, Jaboticabal Campus, SP, Brazil. ***(+) positive test result, (−) negative test result.

2.4 Cloning of the target DNA amplicon

The target genome regions used for the specific amplification and diagnosis of each biovar were cloned into plasmids for use as positive controls and to obtain the quantitative standard curve in the real-time PCR. Briefly, the amplicons obtained after conventional PCR using the primers pSG for *S*. Gallinarum and pSP for *S*. Pullorum were digested with BamHI and subsequently cloned into plasmids. After cloning, the accurate copy number of the target amplicons was calculated and the DNA of each clone was diluted, corresponding to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU of *S*. Gallinarum or *S*. Pullorum, to obtain the quantitative standard curve.

Table 1. Specifications of the primers used in the study

Primer	Sequence	Product Size (pb)	Concentration (µM)	Amplicon *Tm**
pSGP F	TGGCCTTGACGTTATTCGTT	97	0.025	78.0°C
pSGP R	AGCATTTTGGTCACCTGCAA			
pSG F	ATGGCGAGTCCGCCCAGAGT	273	0.05	86.2°C
pSG R	GGCGGTATGCTGGTTGCCGT			
pSP F	CCGCCTGCGCGATGGCTTTA	260	0.05	84.8°C
pSP F	TCTGGTTGACGGCGTGGGGA			

**Tm*: Melting temperature.

2.5 Conditions of the qPCR in real time

The multiplex real-time PCR was performed using the following optimized reaction mix of 13 µl Luminoct ® SYBr® Green qPCR Readymix (Sigma-Aldrich, St. Louis, MI, USA), containing 1.25 U of Taq DNA polymerase and adding 0.1 µM of the primer pSGP, 0.05 µM of primers pSG and pSP, 2.5 µl of serially diluted DNA or eluted DNA after extraction from tested samples, and ultrapure water to make the final volume 25 µl. The first dilution (10^{-1}) corresponded to 100 ng of DNA and the following dilutions were made in $\log_{10}$ up to 10^{-8} from the initial DNA sample. The qPCR in real time was performed in the C1000 TouchTM Thermal Cycler (Bio-Rad, Hercules, CA, USA), with cycling protocol starting with one denaturation cycle at 94°C for 2 min, followed by 40 cycles at 94°C for 15 s and 63°C for 30 s. After amplification, the melting curve was performed to obtain the specific melting temperature (*Tm*) of each amplicon for the differential diagnosis between *S*. Gallinarum and *S*. Pullorum.

2.6 Standard curve for bacterial quantification

Serially diluted plasmid DNA containing the cloned DNA from biovars *S*. Gallinarum and *S*. Pullorum was used to prepare the quantitative standard curve. The reactions followed the optimized PCR conditions for the multiplex reactions. The Software CFX ManagerTM 3.1 (Bio-Rad) was used to calculate the regression coefficients, standard deviation and standard curves efficiency ratio. The linear standard curve was used for quantification of number of genes copies that corresponded to the number of CFU of each biovar. The reactions were acceptable for analysis when R^2 value was higher than 0.99.

3 Results and discussion

The genus *Salmonella* spp. has often been reported in studies using different microbiological and molecular techniques for the identification and differentiation in samples from company and food-producing animals (DICKEL et al., 2005; MALORNY et al., 2007; O'REGAN et al., 2008; MCGUINNESS et al., 2009; OMICCIOLI et al., 2009; ANDRADE et al., 2010; LÖFSTRÖM et al., 2010; PARK et al., 2011; BARLETTA et al., 2013; DOBHAL et al., 2014; LI et al., 2014; MA et al., 2014). The fast diagnosis and identification of these bacteria is crucial for the control of bacterial dissemination, reduction of losses in animal production and risks to human health caused by foodborne infections (CHEN et al., 2010; CHERAGHCHI et al., 2014).

Currently there are 2659 pathogenic serovars identified and most cause gastrointestinal infections in a large number of hosts (ISSENHUTH-JEANJEAN et al., 2014). However, a restricted number of serovars are host-specific. Considering the high pathogenicity of the host-specific biovars *S*. Gallinarum and *S*. Pullorum to birds, the differentiation from others is important to take the correct measures in commercial poultry farms. In Brazil, the presence of either of these two biovars in breeder flocks of chickens condemns the entire flock to elimination, sanitation and replacement of the birds. Thus, the diagnosis has to be fast and accurate, to avoid false positives, as the presence of other nontyphoidal serovars allow treatments and milder biosafety measures in these flocks.

Different PCR methods have been described for the differential diagnosis of a few *Salmonella* serovars, including *S*. Gallinarum and *S*. Pullorum and research on this topic is continuously developing (KISIELA et al., 2005; JEON et al., 2007; BATISTA et al., 2016; REN et al., 2017; XIONG et al., 2017). Currently, modern diagnostic tools, such as real-time PCR, have become accessible to researchers and diagnostic laboratories. However, the design of specific probes and primers for this technique is a limitation as it depends on availability of genome sequences and bioinformatics analysis. The present work shows an extensive analysis and validation of a real-time PCR, using all its data, including the melting curve for differentiation of each PCR product by the respective Tm, and consequently for specific diagnosis of each biovar. As shown in Table 1, the sequences of the three primers and the length of each amplicon obtained have different Tm when the melting curve is evaluated, allowing differentiation of the serovar *Salmonella enterica* serovar Gallinarum from 2659 other serovars (ISSENHUTH-JEANJEAN et al., 2014), and also the differentiation between the biovars *S*. Gallinarum and *S*. Pullorum, which share large homologous genome regions (BATISTA et al., 2015).

The applicability of this reaction by different laboratories worldwide depends on the costs of the reaction. Thus, we considered all the available DNA fluorescent dyes available and opted to use SYBr Green instead of probes. The use of the SYBr Green system in multiplex PCR in real time has been reported in different studies to identify pathogens, allergens, microbiological violations in contaminated food and antibiotic-resistant genes (FUKUSHIMA et al., 2003; PONCHEL et al., 2003; VARGA; JAMES, 2005; LIU et al., 2006; FAN et al., 2007; PAFUNDO et al., 2010; RAJTAK et al., 2011; KAGKLI et al., 2012; CHENGET al., 2013; GOMES et al., 2014; SINGH; MUSTAPHA, 2014). However, this fluorescent dye, differently from probe systems, binds indistinctly to double-stranded DNA, emitting interfering levels of fluorescence in case of nonspecific amplification. Despite the *in silico* validation of the primers showing a specific site of annealing, the multiplex qPCR in real time described here in was developed after extensive titration of the primer concentrations and validation of annealing temperatures, to avoid nonspecific amplifications and primer dimers.

All three primer pairs used in the study were tested and validated with control strains, to determine the optimal concentration for each primer and the appropriate

annealing temperature, in order to reach amplification and prevent the occurrence of non-specific reactions. Based on the results obtained with individually tested primers the multiplex qPCR in real time was performed. The optimized annealing temperature for this reaction was set at 63°C, which offered good conditions for the annealing of primers and amplification of the DNA, without the occurrence of any nonspecific fluorescence. The results obtained from the standardization of individual primer pairs or multiplex qPCR in real time were checked by agarose gel electrophoresis to confirm correct amplification based on the length of the products and the absence of nonspecific reactions as shown in Figures 1 and 2, respectively. The results of the multiplex qPCR in real time with positive control DNA samples are shown in Figure 3.

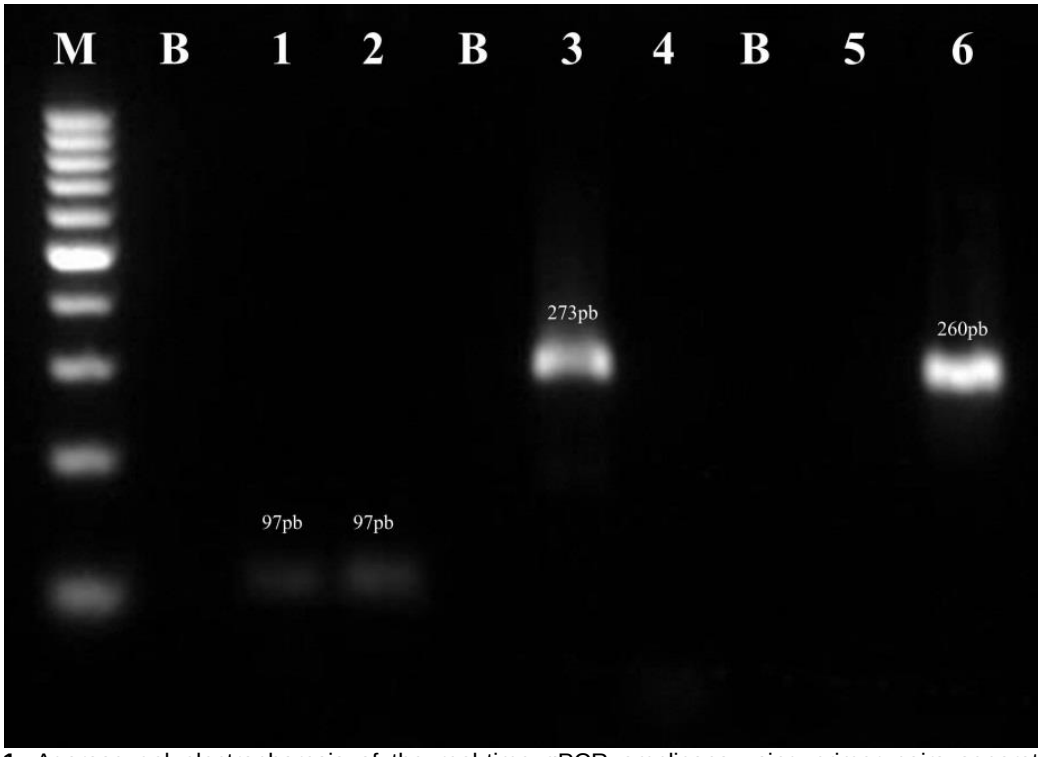

Figure 1. Agarose gel electrophoresis of the real-time qPCR amplicons using primer pairs separately. (M) Molecular Marker (100 bp); (B) Negative control (blank); (1) Primer pSGP with DNA of *S*. Gallinarum; (2) Primer pSGP with DNA of *S*. Pullorum; (3) Primer pSG with DNA of *S*. Gallinarum; (4) Primer pSG with DNA of *S*. Pullorum; (5) Primer pSP with DNA of *S*. Gallinarum; (6) Primer pSP with DNA of *S*. Pullorum.

For diagnosis of each biovar, one amplification curve is obtained by the multiplex qPCR in real time. However, the differentiation is further performed by the analysis of the melting curve, obtained after amplification. This post-analysis shows the dissociation *Tm* of each amplicon, which is represented by a peak of fluorescence loss, when approximately 50% of doublestranded DNA (dsDNA) from

amplicons is dissociated at the corresponding temperature and the SYBr Green reaction is lost. The evaluation of the *Tm* has shown consistency and sensitivity for the purpose of differential diagnosis of bacteria and virus with low mutation rates. Two factors influence the *Tm* of each amplicon: the length of the dsDNA fragment and the CG content. The higher values of these components require higher Tm for dissociation. The differential diagnosis between biovars in the qPCR in real time was performed using the *Tm* values. As shown in Figure 3(A) and 3(B) it is possible to notice two different dissociation peaks, corresponding to two different *Tm* in the melting curve after the multiplex PCR in real time. The first dissociation peak, with *Tm* of 78°C, refers to the amplicon with 97 bp obtained with primer pSGP, present exclusively in biovars *S*. Gallinarum and *S*. Pullorum (serovar *S*. Gallinarum), but not detected in any other *Salmonella* serovar. In the same multiplex qPCR also occurred the amplification for differentiation between biovars. As demonstrated in Figure 3(A), biovar *S*. Gallinarum is specifically detected by the primer pSG and the corresponding dissociation peak occurs at the Tm of 86.2°C, while the amplicon obtained with primers pSP designed for biovar *S*. Pullorum has a dissociation *Tm* of 84.8°C (Figure 3(B)). As carried out with the individual reactions, the efficacy, specificity and quality of the multiplex qPCR in real time were evaluated and confirmed by agarose gel electrophoresis, and the results are shown in Figure 2. The amplicons with 273 bp corresponded to biovar *S*. Gallinarum and with 260 bp corresponded to *S*. Pullorum.

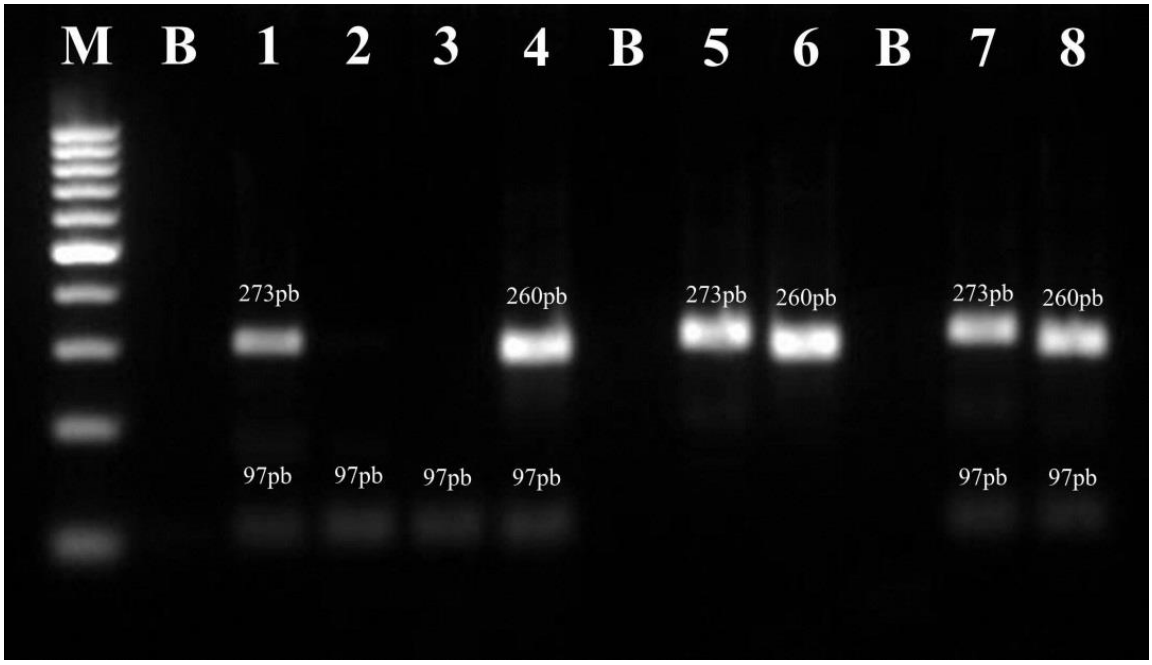

Figure 2. Agarose gel electrophoresis with amplicons from the multiplex qPCR in real time. (M) Molecular Marker (100 bp); (B) Negative control (blank); (1) Primers pSGP and pSG with *S*. Gallinarum DNA sample; (2) Primers pSGP and pSG with *S*. Pullorum DNA sample; (3) Primers pSGP and pSP with *S*. Gallinarum DNA sample; (4) Primers pSGP and pSP with *S*. Pullorum DNA sample; (5) Primers pSG and pSP with *S*. Gallinarum DNA sample; (6) Primers pSG and pSP with *S*. Pullorum DNA sample; (7) Primers pSGP, pSG and pSP with *S*. Gallinarum DNA sample; (8) Primers pSGP, pSG and pSP with *S*. Pullorum DNA sample.

As shown in Figure 2, the duplex qPCR in real time can identify the pathogen at the level of the serovar and the biovar. However, differential diagnosis was achieved by the addition of the third primer pair composing the multiplex reaction, as per results shown in lanes 7 and 8. The differential diagnosis of these two typhoidal biovars has been previously reported by conventional PCR and methods (RIBEIRO et al., 2009; BATISTA et al., 2013). However, the described techniques require two separate reactions and agarose gel electrophoresis for biovar differentiation, which affects time and costs for this reaction. In a single reaction the multiplex reaction was able to perform the identification and differentiation between the biovars, based on the specific *Tm* of the amplicons.

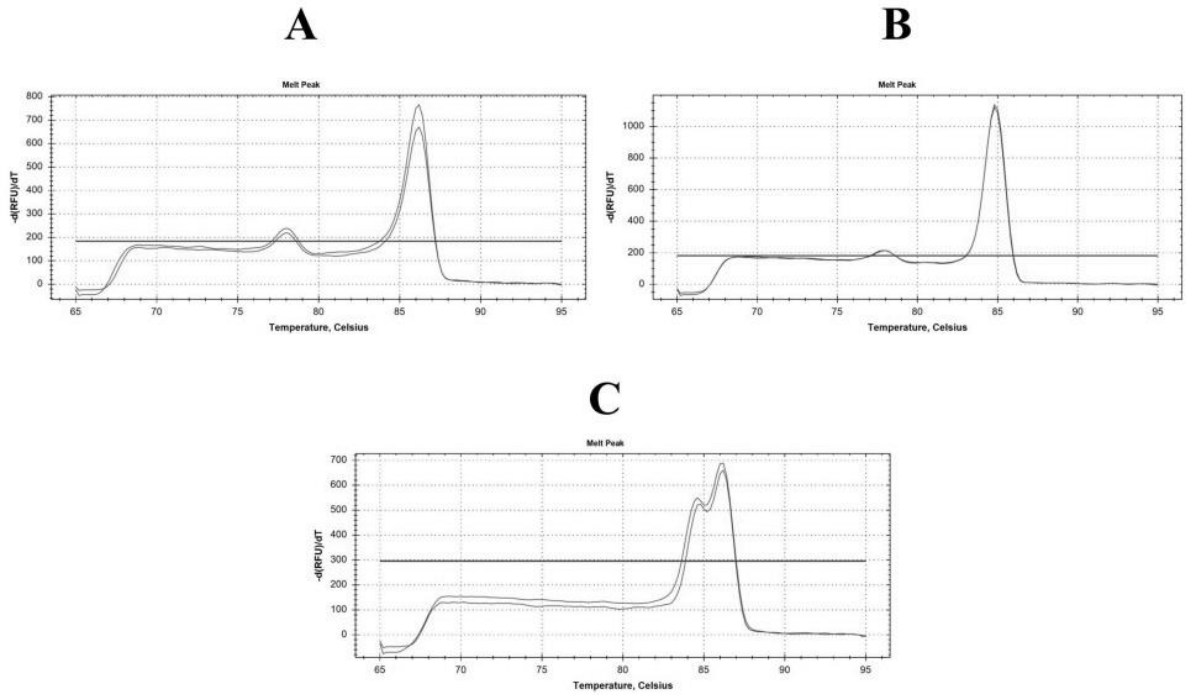

Figure 3. Melting curves and *Tm* peaks of the multiplex qPCR in real time for differential diagnosis between *S*. Gallinarum and *S*. Pullorum. (A) *Tm* peaks refer to the amplification of *S*. Gallinarum DNA samples with primers pSGP and pSG; (B) *Tm* peaks refer to the amplification of *S*. Pullorum DNA samples with primers pSGP and pSP; (C) *Tm* peaks refer to negative results using *S*. Enteritidis DNA samples in which no amplification with pSGP is noted (*Tm* 78°C), and amplifications with pSG, pSP occur together.

The specificity of the multiplex qPCR in real time for differential diagnosis between biovars was tested with DNA samples from non-typhoidal *Salmonella*. In such cases it is possible to notice the amplicons obtained with primers pSG (273 bp) or pSP (260 bp). However, the amplicon with 97 bp and *Tm* of 78°C is not present in non-typhoidal *Salmonella*. Thus, for differential diagnosis between these two typhoidal Salmonella biovars, it is necessary to include the primer pSGP for the differentiation from other non-typhoidal serovars. Moreover, as shown in Figure 3(C), amplification only with primers pSG and pSP may be used for diagnosis of other serovar from genus *Salmonella*, such as *S*. Enteritidis in cases when the pSGP amplicon with 97 bp (*Tm* peak of 78°C) is absent.

In addition to the amplification with positive diagnosis obtained with conventional PCR, another advantage of diagnosing with PCR in real time is the capacity of quantification of the subject of study. Based on the amplification data plotted, the cycle threshold (Cq) values are informative of the genetic material quantity, corresponding to the bacterial numbers in the sample. In the present study the multiplex qPCR in real time was developed to determine the sensitivity limit for

quantification of *Salmonella*. The standard curve with genomic and plasmid DNA containing the genes of interest for each biovar was prepared. The conventional quantification of *Salmonella* by microbiological culture has a detection limit of 10^2 CFU/ml and lower bacterial numbers may not be detected without sample enrichment (MALORNY et al., 2008). As shown in Figure 4, the standard curve was prepared with serially diluted DNA, and each dilution showed an interval of approximately four cycles for *S*. Gallinarum and three cycles for *S*. Pullorum (Table 2), corresponding to the dilution sequence of each biovar. Moreover, it was possible to detect and quantify all DNA samples, including the lowest dilution, corresponding to 10^1 CFU/ml from both biovars, demonstrating the high sensitivity obtained with the described reaction. Considering the high sensitivity, this diagnostic tool is capable of detecting low numbers of this pathogen, even without enrichment of the sample, consequently reducing time to obtain results.

Table 2. Mean of amplification cycles (Cq) for each serial dilution of multiplex real time qPCR reaction to differential diagnosis and absolute quantification of *S*. Gallinarum and *S*. Pullorum

		Mean of Amplification Cycles (Cq)	
Quantification Point	**CFU/mL***	***S*. Gallinarum**	***S*. Pullorum**
1	10^1	39.9	37.5
2	10^2	35.3	33.2
3	10^3	31.3	29.8
4	10^4	27.2	26.2
5	10^5	23.0	22.8
6	10^6	19.1	19.1
7	10^7	15.2	14.8
8	10^8	11.4	11.6

*CFU: Colony forming units

Poultry production is often affected by acute and subclinical infections caused by enterobacteriaceae of genus *Salmonella* spp. and these bacteria are responsible for health impacts and large economic losses worldwide (Dobhal et al., 2014). The outcome of the disease depends on different factors, such as age of birds, genetic resistance and immunosuppressive conditions. The option for treatment or elimination of the flock depends on the serovar. Thus, correct and fast diagnosis is

very important for the right decision. Currently, the available techniques used for diagnosis have limitations, considering the time for results and low sensitivity to reduced bacterial loads in organ or environmental samples. Therefore, the proposed multiplex qPCR in real time has shown the capacity to diagnose the two avian host-specific *Salmonella* biovars in a single reaction. Additionally, a large number of non-typhoidal serovars can be detected in the reaction, differentiating from *S*. Gallinarum and *S*. Pullorum. Furthermore, the described technique is also capable of quantifying unknown bacterial loads, in addition to the positive or negative results obtained with conventional methods.

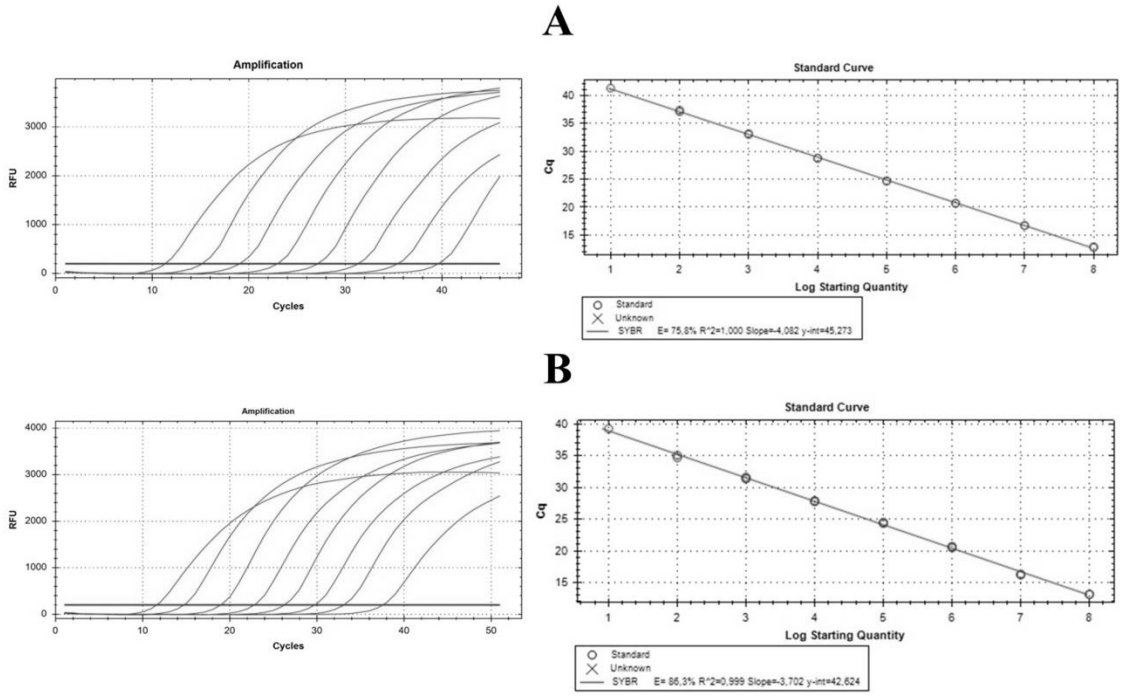

Figure 4. Amplification curves of serially diluted DNA using SYBr Green I (left) and quantitative standard curve (right) obtained by multiplex qPCR in real time for differential diagnosis and absolute quantification of *S*. Gallinarum and *S*. Pullorum. (A) *S*. Gallinarum DNA sample; (B) *S*. Pullorum DNA sample.

Overall, the present study demonstrated the possibility of using a low-cost DNA dye such as SYBr Green in a convenient multiplex qPCR in real time for identification and quantification of two major typhoidal *Salmonella* biovars, capable of infecting birds and causing systemic infection and mortality. Thus, the described technique may facilitate the diagnosis and accelerate protocols to control the spread of these pathogens, which are of worldwide occurrence and which are able to disseminate both vertically and horizontally among avian hosts.

References

ANDRADE, R.B.; GEMELLI, T.; ONDER, L.P.D.; CRISTINA, K.; BRITO, T.; BARBOZA, A.A.L.; BRITO, B.G. Métodos diagnósticos para os patógenos alimentares: *Campylobacter* sp., *Salmonella* spp. e *Listeria monocytogenes*. **Arquivos do Instituto Biológico**, v. 77, p.741-750, 2010.

BARLETTA, F.; MERCADO, E.H.; LUQUE, A.; RUIZ, J.; CLEARY, T.G.; OCHOA, T.J. Multiplex real-time PCR for detection of *Campylobacter*, *Salmonella*, and *Shigella*. **Journal of Clinical Microbiology**, v. 51, p.2822-2829, 2013.

BATISTA, D.F.; DE FREITAS NETO, O.C.; DE ALMEIDA, A.M.; BARROW, P.A.; DE OLIVEIRA BARBOSA, F.; BERCHIERI JR, A. Molecular identification of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovars Gallinarum and Pullorum by a duplex PCR assay. **Journal of Veterinary Diagnostic Investigation**, v. 28, p.419-422, 2016.

BATISTA, D.F.A. (2013). Análise comparativa dos genomas de Salmonella enterica subsp. enterica sorovar Gallinarum biovares Gallinarum 287/91 e Pullorum 449/87 para identificação de regiões de diferenças (RODs). 2013. xii, 77 f. Dissertação (mestrado) – Universidade Estadual Paulista Júlio de Mesquita Filho, Faculdade de Ciências Agrárias e Veterinárias, 2013. <http://hdl.handle.net/11449/99844>

BATISTA, D.F.A.; DE FREITAS NETO, O.C.; LOPES, P.D.; DE ALMEIDA, A.M.; BARROW, P.A.; BERCHIERI JR, A. Polymerase chain reaction assay based on *ratA* gene allows differentiation between *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovars Gallinarum and Pullorum. **Journal of Veterinary Diagnostic Investigation**, v. 25, p.259-262, 2013.

BATISTA, D.F.A.; FREITAS NETO, O.C.; BARROW, P.A.; DE OLIVEIRA, M.T.; ALMEIDA, A.M.; FERRAUDO, A.S.; BERCHIERI JR, A. Identification and characterization of regions of difference between the *Salmonella* Gallinarum biovar Gallinarum and the *Salmonella* Gallinarum biovar Pullorum genomes. **Infection, Genetics and Evolution**, v. 30, p.74-81, 2015.

CELIS-ESTUPINAN, A.L.D.P.; BATISTA, D.F.A.; CARDOZO, M.V.; SECUNDO DE SOUZA, A.I.; RODRIGUES ALVES, L.B.; MARIA DE ALMEIDA, A.; BARROW, P.A.; BERCHIERI, A., JR.; CAETANO DE FREITAS NETO, O. Further investigations on the epidemiology of fowl typhoid in Brazil. **Avian Pathology**, v. 3, p.1-10, 2017.

CHEN, J.; ZHANG, L.; PAOLI, G.C.; SHI, C.; TU, S.; SHI, X. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. **International Journal of Food Microbiology**, v. 137, p.168-174, 2010.

CHENG, J.; JIANG, Y.; RAO, P.; WU, H.; DONG, Q.; WU, Z.; DING, X.; GUO, J. Development of a single-tube multiplex real-time PCR for detection and identification of five pathogenic targets by using melting-curve analysis with EvaGreen. **Archives of Virology**, v. 158, p.379-386, 2013.

CHERAGHCHI, N.; KHAKI, P.; MORADI BIDHENDI, S.; SABOKBAR, A. Identification of isolated *Salmonella enterica* serotype gallinarum biotype Pullorum and Gallinarum by PCR-RFLP. **Jundishapur Journal of Microbiology**, v. 7, p.1-4, 2014.

DICKEL, E.L.; RODRIGUES, L.B.; SANTOS, L.R.; VALLE, S.F.; PILOTTO, F.; RODEMBUSH, C.; WALD, V.B.; CANAL, C.W.; NASCIMENTO, V.P. Análise comparativa entre microbiologia convencional, ELISA e PCR para detecção de *Salmonella enteritidis*, *S. typhimurium*, *S. gallinarum* e *S. pullorum* em carne de frango contaminada artificialmente. **Revista Brasileira de Ciência Veterinária**, v. 12, p.5-10, 2005.

DOBHAL, S.; ZHANG, G.; ROHLA, C.; SMITH, M.W.; MA, L.M. A simple, rapid, cost-effective and sensitive method for detection of *Salmonella* in environmental and pecan samples. **Journal of Applied Microbiology**, v. 117, p.1181-1190, 2014.

FAN, W.; HAMILTON, T.; WEBSTER-SESAY, S.; NIKOLICH, M.P.; LINDLER, L.E. Multiplex real-time SYBr Green I PCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. **Molecular and Cellular Probes**, v. 21, p.245-256, 2007.

FENG, Y.; JOHNSTON, R.N.; LIU, G.; LIU, S. Genomic comparison between *Salmonella* Gallinarum and Pullorum: differential pseudogene formation under common host restriction. **PLoS ONE**, v. 8, p.1-6, 2013.

FUKUSHIMA, H.; TSUNOMORI, Y.; SEKI, R. Duplex real-time SYBr green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. **Journal of Clinical Microbiology**, v. 41, p.5134-5146, 2003.

GOMES, T.S.; GARCIA, M.C.; CUNHA, F.S.; MACEDO, H.W.; PERALTA, J.M.; PERALTA, R.H.S. Differential diagnosis of *Entamoeba* spp. in clinical stool samples using SYBr Green real-time polymerase chain reaction. **The Scientific World Journal**, v. 2014, p.1-8, 2014.

ISSENHUTH-JEANJEAN, S., ROGGENTIN, P., MIKOLEIT, M., GUIBOURDENCHE, M., DE PINNA, E., NAIR, S., FIELDS, P.I. & WEILL, F.X. Supplement 2008–2010 (no. 48) to the White-Kauffmann-Le Minor scheme. **Research in Microbiology**, v. 165, p.526-530, 2014.

JEON, S.; EO, S.K.; KIM, Y.; YOO, D.J.; KANG, S.H. Capillary and microchip gel electrophoresis for simultaneous detection of *Salmonella* Pullorum and *Salmonella* Gallinarum by rfbS allele-specific PCR. **Talanta**, v. 73, p.415-419, 2007.

KAGKLI, D.M.; FOLLONI, S.; BARBAU-PIEDNOIR, E.; VAN DEN EEDE, G.; VAN DEN BULCKE, M.; AHMED N. Towards a pathogenic Escherichia coli detection platform using multiplex SYBR® Green real-time PCR methods and high resolution melting analysis. **PLoS ONE**, v. 7, p.1-12, 2012.

KISIELA, D.; KUCZKOWSKI, M.; KICZAK, L.; WIELICZKO, A.; UGORSKI, M. Differentiation of *Salmonella* Gallinarum biovar Gallinarum from *Salmonella* Gallinarum biovar Pullorum by PCR-RFLP of the fimH gene. **Journal of Veterinary Medicine Series**, v. 52, p.214-218, 2005.

LI, R.; WANG, Y.; SHEN, J.; WU, C. Development of a novel hexa-plex PCR method for identification and serotyping of *Salmonella* species. **Foodborne Pathogens and Disease**, v. 11, p.845-852, 2014.

LIN, L.H.; TSA, C.Y.; HUNG, M.H.; FANG, Y.T.; LING, Q.D. Rectal swab sampling followed by an enrichment culture-based real-time PCR assay to detect *Salmonella enterocolitis* in children. **Clinical Microbiology and Infection**, v. 17, p.1421-1425, 2011.

LIU, J.I.; YAN, M.; WANG, Z.; WANG, L.; ZHOU, Y.; XIAO, B. Molecular diagnosis of α-thalassemia by combining real-time PCR with SYBr Green1 and dissociation curve analysis. **Translational Research**, v. 148, p.6-12, 2006.

LÖFSTRÖM, C.; HANSEN, F.; HOORFAR, J. Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. **Veterinary Microbiology**, v. 144, p.511-514, 2010.

MA, K.; DENG, Y.; BAI, Y.; XU, D.; CHEN, E.; WU, H.; LI, B.; GAO, L. Rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork using a multiplex real-time PCR assay based on immunomagnetic separation. **Food Control**, v. 42, p.87-93, 2014.

MALORNY, B.; BUNGE, C.; HELMUTH, R. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. **Journal of Microbiological Methods**, v. 70, p.245-251, 2007.

MALORNY, B.; LÖFSTRÖM, C.; WAGNER, M.; KRÄMER, N.; HOORFAR, J. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. **Applied and Environmental Microbiology**, v. 74, p.1299-1304, 2008.

MCGUINNESS, S.; MCCABE, E.; O'REGAN, E.; DOLAN, A.; DUFFY, G.; BURGESS, C.; FANNING, S.; BARRY, T.; O'GRADY, J. Development and validation of a rapid real-time PCR based method for the specific detection of *Salmonella* on fresh meat. **Meat Science**, v. 83, p.555-562, 2009.

OMICCIOLI, E., AMAGLIANI, G.; BRANDI, G.; MAGNANI, M. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. **Food Microbiology**, v. 26, p.615-622, 2009.

O'REGAN, E.; MCCABE, E.; BURGESS, C.; MCGUINNESS, S.; BARRY, T.; DUFFY, G.; WHYTE, P.; FANNING, S. Development of a real-time multiplex PCR

assay for the detection of multiple *Salmonella* serotypes in chicken samples. **BMC Microbiology**, v. 8, p.156, 2008.

PAFUNDO, S.; GULLÌ, M.; MARMIROLI, N. Multiplex real-time PCR using SYBr® GreenER™ for the detection of DNA allergens in food. **Analytical and Bioanalytical Chemistry**, v. 396, p.1831-1839, 2010.

PARK, S.H.; HANNING, I.; JARQUIN, R.; MOORE, P.; DONOGHUE, D.J.; DONOGHUE, A.M.; RICKE, S.C. Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. **FEMS Microbiology Letters**, v. 316, p.7-15, 2011.

PONCHEL, F.; TOOMES, C.; BRANSFIELD, K.; LEONG, F.T.; DOUGLAS, S.H.; FIELD, S.L.; BELL, S.M.; COMBARET, V.; PUISIEUX, A.; MIGHELL, A.J.; ROBINSON, P.A.; INGLEHEARN, C.F.; ISAACS, J.D.; MARKHAM, A.F. Real-time PCR based on SYBr-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. **BMC Biotechnology**, v. 3, p.1-13, 2003.

RAJTAK, U.; LEONARD, N.; BOLTON, D.; FANNING, S. A real-time multiplex SYBr Green I polymerase chain reaction assay for rapid screening of *Salmonella* serotypes prevalent in the European Union. **Foodborne Pathogens and Disease**, v. 8, p.769-780, 2011.

REN, X.; FU, Y.; XU, C.; FENG, Z.; LI, M.; ZHANG, L.; ZHANG, J.; LIAO, M. High resolution melting (HRM) analysis as a new tool for rapid identification of *Salmonella enterica* serovar Gallinarum biovars Pullorum and Gallinarum. **Poultry Science**, v. 96, p.1088-1093, 2017.

RIBEIRO, S.A.M.; DE PAIVA, J.B.; ZOTESSO, F.; LEMOS M.V.F.; BERCHIERI JÚNIOR Â. Molecular differentiation between *Salmonella enterica* subsp *enterica* serovar Pullorum and *Salmonella enterica* subsp *enterica* serovar Gallinarum. **Brazilian Journal of Microbiology**, v. 40, p.184-188, 2009.

SANTANA, E.S.; OLIVEIRA, F.H.; ROCHA, T.M.; SANTANA, R.R.; ANDRADE, M.A. Abordagem sobre *Salmonella* sp. com enfoque na caracterização, patogênese e métodos de diagnóstico em aves. **Enciclopédia Biosfera**, v. 7, p.1-23, 2011.

SHIVAPRASAD, H.L. Fowl typhoid and pullorum disease. **Revue Scientifique et Technique de l'OIE**, v. 19, p.405-424, 2000.

SINGH, P.; MUSTAPHA, A. Development of a real-time PCR melt curve assay for simultaneous detection of virulent and antibiotic resistant *Salmonella*. **Food Microbiology**, v. 44, p.6-14, 2014.

SORIA, M.C.; SORIA, M.A.; BUENO, D.J.; TERZOLO, H.R. Comparison of 3 culture methods and PCR assays for *Salmonella* Gallinarum and *Salmonella* Pullorum detection in poultry feed. **Poultry Science**, v. 92, p.1505-1515, 2013.

VARGA, A.; JAMES, D. Detection and differentiation of Plum pox virus using real-time multiplex PCR with SYBr Green and melting curve analysis: a rapid method for strain typing. **Journal of Virological Methods**, v.123, p.213-220, 2005.

XIONG, D.; SONG, L.; TAO, J.; ZHENG, H.; ZHOU, Z.; GENG, S.; PAN, Z.; JIAO, X. An efficient multiplex PCR-based assay as a novel tool for accurate inter-serovar discrimination of *Salmonella* Enteritidis, *S*. Pullorum/Gallinarum and *S*. Dublin. **Frontiers in Microbiology**, v. 8, p.1-9, 2017.

CAPITULO 3 – Duplex real-time PCR using SYBr green I for quantification and differential diagnosis between *Salmonella* Enteritidis and *Salmonella* Typhimurium

ABSTRACT – The incidence of foodborne diseases caused by the genus *Salmonella* spp. in industrialized countries is often high in epidemiological surveys. Obtaining a rapid diagnostic test for identification of bacteria is crucial in order to rapidly implement control measures to contain bacterial spread, to reduce losses in animal production and to avoid risks from food-borne infections to human health. The aim of this study was to standardize duplex real-time PCR using SYBr Green I for differential and quantitative diagnosis of *S*. Typhimurium and *S*. Enteritidis. According to the experiment, the melting temperature of 85°C was observed for a 206bp amplified product when *S*. Enteritidis DNA was added to the reaction. *S*. Typhimurium DNA showed that the melting temperature of 79°C when observed for a 62bp amplified product. The standard curve showed the high sensitivity of the proposed test, since it was possible to obtain eight quantification points, starting at 10^8 CFU/mL and ending at 10^1 CFU/mL. As a result of the present study, a real-time PCR duplex reaction with high sensitivity, specificity and based on the fluorescence of SYBr Green I was standardized. In addition, this methodology aligns low cost to the faster diagnostic result, in relation to other molecular tests, making it attractive for application in routine laboratory analyzes.

Keywords: Identification, melting curve, non-typhoidal salmonelosis, standard curve.

1 Introduction

The incidence of foodborne diseases caused by the genus *Salmonella* spp. in industrialized countries is often high in epidemiological surveys (Ma et al. 2014). At least 1.3 billion cases of human salmonellosis are reported annually worldwide, with 93.8 million cases of acute gastroenteritis, 155000 deaths and approximately 85% of which are estimated to be foodborne (FAO/WHO, 2016). These outbreaks cause significant damage to the health of infected animals and humans and economic losses (Chen et al. 2010).

In the United States of America this bacterium occupies the second place in the ranking of pathogens causing foodborne diseases (Ma et al. 2014), with annual average of 1.2 million patients, 15.000 hospitalizations and 480 deaths. Among the serovars involved in these outbreaks, the most frequently reported were *S*. Enteritidis (19%), *S*. Typhimurium (11%) and *S*. Newport (10%) (CDC, 2014). In China, salmonellosis is responsible for approximately 40% of all foodborne diseases caused by bacteria (Chen et al. 2010). According to the China CDC, the incidence of salmonellosis cases in the year 2015 was 1051 cases with absence of obits (CDC, 2015). European Union estimated that 320000 cases of foodborne diseases are reported annually and more than 100000 (31.2%) are caused by salmonellosis, with an incidence rate of 35 reported cases per 100000 inhabitants and estimated a loss of 3 billion euros annually. The most frequently isolated serovars are *S*. Enteritidis (66.7%) and *S*. Typhimurium (6.5%) (EU, 2016).

A report published in Brazil, by the Secretary of Health Surveillance (SVS), showed that the incidence of foodborne diseases in this country was reduced by 35% in comparison to the last report published in 2014, but in 58.8% of these outbreaks, the isolation and identification of the pathogens involved was not performed. However, when the agent was identified, 14.3% of these isolates came from bacteria of the genus *Salmonella* spp., being the most frequently isolated pathogen among the analyzed samples (SVS, 2015).

Products of poultry origin are often associated with food born pathogen outbreaks from *Salmonella* spp., because they are affected by both host-specific and non-specific serovars (O'Regan et al., 2008). The importance of their control at all scales of the poultry production is due to the fact that they are capable of infecting commercial birds. Consequently, there will be the contamination of poultry products which are subsequently destined for human consumption (Löfström et al., 2010).

A rapid diagnostic test for identification of bacteria is crucial in order to rapidly implement control measures to contain bacterial spread, to reduce losses in animal production and to avoid risks from food-borne infections to human health (Chen et al., 2010; Cheraghchi et al., 2014). With this purpose, the real-time PCR (qPCR) has been outstanding for the diagnosis of pathogens, since they obtain results faster than the microbiological diagnosis and with reduction of the stages used in the reaction,

associated with higher sensitivity, when compared to conventional PCR (Park et al., 2011; Ma et al., 2014). The aim of this study was to standardize duplex qPCR using SYBr Green for differential and quantitative diagnosis between *S*. Enteritidis and *S*. Typhimurium.

2 Material and methods

2.1 Bacterial strains for validation of the qPCR assay

Bacterial strains are used for standardization of the multiplex quantitative PCR in real time, included the sequenced strain *S*. Enteritidis P125/109 (accession number: ASM950v1) and *S*. Typhimurium F98 (Barrow et al., 1990). Although the primers had already undergone validation in published research (Malorny et al., 2007; Park et al., 2013), 21 *Salmonella* spp. and six non - *Salmonella* spp. strains were used as negative controls for validation of the primers and the multiplex reaction specificity (Table 1). All bacteria were stored at -80°C in the Laboratory of Avian Pathology at School of Agricultural and Veterinary Sciences (FCAV-Unesp/Jaboticabal/SP).

2.2 Bacterial DNA extraction

Each isolate was cultured in lysogeny broth (LB) at 37 °C, shaking at 100 rpm for a period of 18 hours, including the bacteria used as negative controls. Before DNA extraction, all *S.* Enteritidis and *S*. Typhimurium cultures were quantified by plating serial dilutions in Brilliant Green agar (Oxoid CM0263) of the culture with subsequent incubation at 37°C for 24h. After incubation, the bacterial numbers of each culture were evaluated and the colony forming units per mL (CFU/mL) were transformed to Log_{10}. DNA extraction of all bacterial cultures was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The purity and concentration of bacterial DNA was analyzed by spectrophotometry (Nanodrop 1000 ThermoFisher Scientific, USA).

2.3 Primers used for differential diagnosis between *S*. Enteritidis and *S*. Typhimurium

Two pairs of primers were used and the sequences and details are in Table 2. The primer pSE were based on Malorny et al. (2007) to detect the *S.* Enteritidis and the primer pSTM were based on Park et al. (2013) to detect *S.* Typhimurium. Primers were validated *in silico* using primer BLAST tool and with 21 *Salmonella* spp. and six non - *Salmonella* spp. strains. For the standardization of this reaction, the primers were submitted to a temperature and concentration gradient evaluation, to obtain the best annealing temperature for both primers and the best reaction inclusion concentration for each primer (not shown).

Table 1. Bacterial strains used to duplex real-time qPCR validation for differential diagnosis between *S.* Enteritidis and *S.* Typhimurium

Strain	**Code**	**Strain**	**Code**
***Salmonella* spp.**		**Non - *Salmonella* spp.**	
S. Agona	CR104/10	*Escherichia coli*	Field strains
S. Albany	CR189/07	*Citrobacter freundii*	Field strains
S. Enteritidis	P125088	*Listeria Monocitogenes*	FC00266
S. Enteritidis	SE 147/139-2	*Pseudomonas* sp.	Field strains
S. Enteritidis	SE 16742	*Shigella sonnei*	ATCC23931
S. Enteritidis	SE 174	*Yersinia enterocolitica*	ATCC9610
S. Gallinarum	SG 287/91		
S. Hadar	CR111/10		
S. Heidelberg	CR239/08		
S. Infantis	CR142/10		
S. Newport	CR044/10		
S. Paratyphi B	CR423/06		
S. Pullorum	SP FCAV198		
S. Saintpaul	CR325/08		
S. Schwarzengrund	CR121/10		
S. Senftenberg	CR119/10		
S. Typhimurium	STM 1116		
S. Typhimurium	STM 723		
S. Typhimurium	STM F98		
S. Typhimurium	STM 986		
S. Typhimurium	STM 985/11		

Bacterial isolates were obtained from: National Agricultural and Livestock Laboratories – LANAGRO (CR/AV); Field strains isolated and maintained at School of Agricultural and Veterinary Sciences, São Paulo State University, FCAV/UNESP, Jaboticabal Campus, SP, Brazil.

2.4 Cloning of the target DNA amplicon

The target genome regions used for the specific amplification and diagnostic of each biovar, were cloned into plasmids for use as positive controls and to obtain

the quantitative standard curve in qPCR, according to the adapted protocol described by Sambrook and Russel (2001). Briefly, the amplicons were obtained with conventional PCR which had as reaction mix 10X of Buffer, 2 µM of dNTP, 25 µM of $MgCl_2$, 4 µM of each primer, 2 µL de DNA, 5 U/mL de Taq DNA polimerase and ultrapure water to make the final volume 20 µL (Sigma-Aldrich, St. Louis, Missouri, USA), with cycling protocol starting with one denaturation cycle at 94 °C for 3 minutes, followed by 30 cycles at 94°C for 40 seconds, 63°C for 60 seconds and 72°C for 60 seconds and one final extension cycle at 72°C for 7 minutes.

The primer used for *S*. Enteritidis, were designed with SalI and PstI (Thermo Scientific, Waltham, Massachusetts, EUA); and for *S*. Typhimurium were designed with BamHI and HindIII (Thermo Scientific, Waltham, Massachusetts, EUA) subsequently both amplicons are cloned in plasmid (pMAL™-p4X; NEB #E8000). Were used the competent cell *E. coli* DH5α™, submitted to a thermal transformation reaction. After cloning, the accurate copy number of the target amplicons was calculated (NEBioCalculator™ v 1.6.0) and the DNA of each clone was diluted, corresponding to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 CFU of *S*. Enteritidis or *S*. Typhimurium, to obtain the quantitative standard curve.

Table 2. Specifications of the primers used in the study

Primer	Sequence (5' - 3')	Product Size (pb)	Concentration (µM)
pSE F	ATATCGTCGTTGCTGCTTCC	206	0.05
pSE R	CATTGTTCCACCGTCACTTTG		
pSTM F	GCGCACCTCAACATCTTTC	62	0.025
pSTM R	CGGTCAAATAACCCACGTTCA		

**Tm*: Melting temperature.

2.5 Conditions of the duplex qPCR

The duplex qPCR was performed using the following optimized reaction mix of 12.5 µL Luminoct® SYBr® Green qPCR Readymix (Sigma-Aldrich, St. Louis, Missouri, USA), 0.05 µM of the primer pSE, 0.025 µM of primers pSTM, 2.5 µL of serially diluted DNA or eluted DNA after extraction from tested samples, and ultrapure water (Sigma-Aldrich, St. Louis, Missouri, USA) to make the final volume 25 µL. The qPCR in real time was performed in the C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, California, USA) and were used Low-Profile 0.2 ml 8-Tube Strips

(Bio-Rad, Hercules, California, USA), with cycling protocol starting with one denaturation cycle at 94 °C for 2 minutes, followed by 40 cycles at 94°C for 15 seconds and 63°C for 21 seconds. After amplification, the melting curve was performed to obtain the specific melting temperature (*Tm*) of each amplicon for the differential diagnosis between *S*. Enteritidis and *S*. Typhimurium, in which were used 5°C per 5 seconds for the temperature and time range.

2.6 Standard curve for absolute bacterial quantification

Serially diluted plasmid DNA containing the cloned DNA from *S*. Enteritidis and *S*. Typhimurium, in triplicate, were used to prepare the quantitative standard curve. The reactions followed the optimized qPCR conditions for the multiplex reactions. The CFX Manager™ 3.1 Software (Bio-Rad, Hercules, California, USA) was used to calculate the regression coefficients, standard deviation and standard curves efficiency ratio. The linear standard curve was used for quantification of number of genes copies that corresponded to the number of CFU of each serovar.

3 Results and discussion

The effects in public health caused by bacteria of the genus *Salmonella* spp. are known to be harmful to animal productions, due to their high occurrence in reports of food outbreaks and in industrialized or non-industrialized countries, with higher incidence of *S*. Enteritidis and *S*. Typhimurium isolates (Aktas et al., 2007; Arshad et al., 2008; Freitas et al., 2010; Matheson et al., 2010; Favier et al., 2013; CDC, 2014; SVS, 2015; EU, 2016). Due to this, qualitative and quantitative studies with these bacteria are of great value, by properly identifying the pathogen that is occurring, the place from which they are derived and the bacterial amount that is necessary to cause damage to the animals and humans health (Ellingson et al., 2004).

With the standardization of the reaction, the results obtained in the present study (Figure 1) demonstrate the possibility to differentiate and quantify two serovars, *S*. Enteritidis and *S*. Typhimurium, in a single reaction and using the fluorophore SYBr Green I. Because it does not require the use of probes and more than one reaction to obtain the differential diagnosis, this protocol becomes less expensive

and faster, in order to enable the implementation of adequate control measures to eradicate these important pathogens from the public health view.

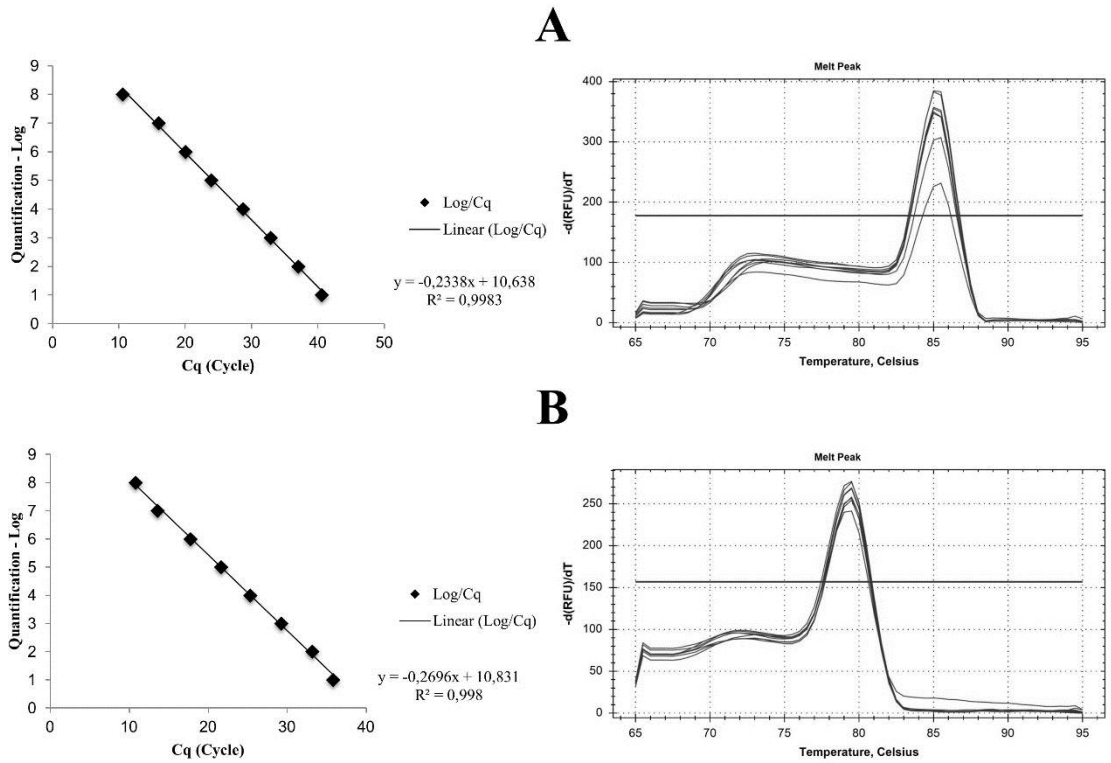

Figure 1. Standard curve (left), melting curves and *Tm* peaks (right) of the quantitative duplex real-time qPCR using SYBr Green I for differential diagnosis between *S*. Enteritidis and *S*. Typhimurium. A) *S*. Enteritidis DNA sample with *Tm* peak in 85°C; B) *S*. Typhimurium DNA sample with *Tm* peak in 79°C.

This methodology for the simultaneous detection of pathogens is frequently reported in the literature with the use of hydrolysis probes. However, the manufacture of this type of fluorophore has a higher cost than the fluorophore SYBr Green I. In the absence of probes, the specificity of the reaction and the differentiation of pathogens with the use of DNA intercalators is determined by the melting temperature of the amplified product (Nam et al., 2005). The differentiation of *S*. Enteritidis and *S*. Typhimurium serovars by the duplex reaction qPCR in the present work was possible due to the difference between the size generated for each amplified product and melting temperature (*Tm*) of the specific primers. According to the experiment, a *Tm* of 85°C was observed for a 206bp amplified product when *S*. Enteritidis DNA was added to the reaction. When added *S*. Typhimurium DNA, a *Tm* of 79°C was observed for a 62bp amplified product. In contrast, when carrying out the validation of

the reaction with the negative control samples, no *Tm* was observed, or it was different from the positive controls obtained for the duplex reaction.

Akiba et al. (2011) performed a multiplex conventional PCR reaction for differentiation of the *S*. Enteritidis and *S*. Typhimurium serovars, however, it required the step after PCR (electrophoresis), demanded 7 pairs of primers for its differentiation. In addition, due to the difficulty in interpreting the results, through the large number of primers used in the reaction, reported the occurrence of false negative reactions. Similarly, Freitas et al. (2010) standardized a multiplex reaction to differentiate these serovars by conventional PCR and although they reported the need for only 2 primers for their differentiation by viewing bands, the post-PCR step was also required, which requires more time and increases the risk of contamination or loss of the samples analyzed.

With a standard curve for quantification of samples for the duplex reaction qPCR showed the high sensitivity of the proposed test, since it was possible to obtain eight quantification points, starting at 10^8 CFU/mL and ending at 10^1 CFU/mL. This fact evidences the potential of detection that qPCR possesses, obtaining the diagnostic result from the DNA of 10^1 CFU/mL, which could not be done only by conventional microbiology, which according to Malorny et al. (2008), the limit of bacterial detection by the microbiological technique is 10^2 CFU/mL, using the most probable number (MPN) with reference.

In an epidemiological study in Michigan (USA), Arshad et al. (2008) reported that cases of *Salmonella* spp. are underreported, probably due to low bacterial load in faecal samples and presence of mild symptoms in the patients analyzed. However, they did not quantify the samples from the reported fact, reinforcing the importance of having a standard curve for quantification of this type of clinical sample and for epidemiological surveys, in order to adequately delineate the spread, course and predispositions of the disease.

Similar to the present study, Park and Ricke (2010) performed a reaction for *S*. Enteritidis and *S*. Typhimurium serovars differentiation and quantification of the samples. However, they performed the differentiation in conventional PCR and with specific primers for each serovar. The quantification of the samples was done by qPCR and with a primer specific only for the genus *Salmonella* spp. This fact

contrasts with the present study, which also differentiated and quantified the serovars and the diagnostic result was obtained in only one type of reaction, without the need for subsequent steps and allowing its quantification and differentiation in only one reaction.

Hein et al. (2006) to standardize a reaction based on the *inv*A gene for the detection of *Salmonella* spp., performed a quantification curve with *S*. Typhimurium DNA in order to evaluate the limit of detection of the reaction. At the end of the standardization, they reported a limit of detection of 10^2 CFU/mL for their reaction, a fact contrary to the present experiment, which allows the identification of samples with bacterial load referring to 10^1 CFU/ml. This fact evidences the high sensitivity obtained with the reaction of the present study.

As a result of the present study, a duplex qPCR with high sensitivity, specificity and based on the fluorescence of SYBr Green I was standardized for differential and quantitative diagnosis between *S*. Enteritidis and *S*. Typhimurium. In addition, this methodology aligns low cost to the speed in obtaining the diagnostic result, making it attractive for application in routine laboratory analyzes.

4 References

Akiba, M.; Kusumoto, M.; Iwata, T. Rapid identification of *Salmonella* enterica serovars, Typhimurium, Choleraesuis, Infantis, Hadar, Enteritidis, Dublin and Gallinarum, by multiplex PCR. **Journal of Microbiological Methods**, v. 85, p.9-15, 2011.

Aktas, Z.; Dayb, M.; Kayacan, C.B.; Diren, S.; Threlfall, E.J. Molecular characterization of *Salmonella* Typhimurium and *Salmonella* Enteritidis by plasmid analysis and pulsed-field gel electrophoresis. **International Journal of Antimicrobial Agents**, v. 30, p.541-545, 2007.

Arshad, M.M.; Wilkins, M.J.; Downes, F.P.; Rahbar, M.H.; Erskine, R.J.; Boulton, M.L. Younus, M.; Saeed, A.M. Epidemiologic attributes of invasive non-typhoidal *Salmonella* infections in Michigan, 1995-2001. **International Journal of Infectious Diseases**, v. 12, p.176-182, 2008.

Barrow, P.A.; Hassan, J.O.; Berchieri Junior A. Reduction in faecal excretion of *Salmonella typhimurium* strain F98 in chickens vaccinated with live and killed *S. typhimurium* organisms. **Epidemiology and Infection**, v.104, p. 413-426, 1990.

CDC. **Center for Diseases Control and Prevention**. National Enteric Disease Surveillance: *Salmonella* Annual Report, 2013. Georgia: US Departiment of Health and Human Services; 2014.

CDC. **Chinese Center for Disease Control and Prevention**. National notifiable disease situation in October 2015. Beijing: National Health and Family Planning Commission of the PRC; 2015.

Chen, J.; Zhang, L.; Paoli, G.C.; Shi, C.; Tu S.; Shi, X. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. **International Journal of Food Microbiology**, v. 137, p.168-174, 2010.

Cheraghchi, N.; Khaki, P.; Bidhendi, S.M.; Sabokbar, A. Identification of isolated *Salmonella enterica* serotype *gallinarum* biotype *Pullorum* and *Gallinarum* by PCR-RFLP. **Jundishapur Journal of Microbiology**, v.7, p. 1-4, 2014.

Ellingson, J.L.E.; Anderson, J.L.; Carlson, S.A.; Sharma, V.K. Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products. **Molecular and Cellular Probes**, v. 18, p.51-57, 2004.

EU. **European Union**. RASFF - The Rapid Alert System for Food and Feed: 2015 annual report. European Commission. Health and Food Safety 2016.

FAO/WHO. **Food and Agriculture Organization of the United Nations/World Health Organization**. Interventions for the control of non-typhoidal *Salmonella* spp. in beef and pork: Meeting report and systematic review. 2016. Microbiological Risk Assessment Series No. 30. Rome. 276pp.

Favier, G.I.; Estrada, C.S.M.L.; Otero, V.L.; Escudero, M.E. Prevalence, antimicrobial susceptibility, and molecular characterization by PCR and pulsed field gel electrophoresis (PFGE) of *Salmonella* spp. isolated from foods of animal origin in San Luis, Argentina. **Food Control**, v. 29, p.49-54, 2013.

Freitas, C.G.; Santana, A.P.; Silva, P.H.C.; Gonçalves, V.S.P.; Barros, M.A.F.; Torres, F.A.G.; Murata, L.S.; Perecmanis, S. PCR multiplex for detection of *Salmonella* Enteritidis, Typhi and Typhimurium and occurrence in poultry meat. **International Journal of Food Microbiology**, v. 139, p.15-22, 2010.

Hein, I.; Flekna, G.; Krassnig, M.; Wagner, M. Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. **Journal of Microbiological Methods**, v. 66, p.538-547, 2006.

Lin, L.H.; Tsa, C.Y.; Hung M.H.; Fang, Y.T.; Ling, Q.D. Rectal swab sampling followed by an enrichment culture-based real-time PCR assay to detect *Salmonella enterocolitis* in children. **Clinical Microbiology and Infection**, v. 17, p.1421-1425, 2011.

Löfström, C.; Hansen, F.; Hoorfar, J. Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. **Veterinary Microbiology**, v. 144, p.511-514, 2010.

Ma, K.; Deng, Y.; Bai, Y.; Xu, D.; Chen, E.; Wu, H.; Li, B.; Gao, L. Rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork using a multiplex real-time PCR assay based on immunomagnetic separation. **Food Control**, v. 42, p.87-93, 2014.

Malorny, B.; Bunge, C.; Helmuth, R. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. **Journal of Microbiological Methods**, v. 70, p.245-251, 2007.

Malorny, B.; Löfström, C.; Wagner, M.; Krämer, N.; Hoorfar, J. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. **Applied and Environmental Microbiology**, v. 74, p.1299-1304, 2008.

Matheson, N.; Kingsley, R.A.; Sturgess, K.; Aliyu, S.H.; Wain, J.; Dougan, G.; Cooke, F.J. Ten years experience of *Salmonella* infections in Cambridge, UK. **Journal of Infection**, v. 60, p.21-25, 2010.

Nam, H.; Srinivasan, V.; Gillespie, B.E.; Murinda, S.E.; Oliver, S.P. Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. **International Journal of Food Microbiology**, v. 102, p.161-171, 2005.

Ngan, G.J.Y.; Ng, L.M.; Lin, R.T.P.; Teo, J.W.P. Development of a novel multiplex PCR for the detection and differentiation of *Salmonella* enterica serovars Typhi and Paratyphi A. **Research in Microbiology**, v. 161, p.243-248, 2010.

O'Regan, E.; Mccabe, E.; Burgess, C.; Mcguinness, S.; Barry, T.; Duffy, G.; Whyte, P.; Fanning, S. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. **BMC Microbiology**, v. 8, p.1-11, 2008.

Park, S.H.; Hanning, I.; Jarquin, R.; Moore, P.; Donoghue, D.J.; Donoghue, A.M.; Ricke, S.C. Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. **FEMS Microbiology Letters**, v. 316, p.7-15, 2011.

Park, M.; Weerakoon, K.A.; Oh, J.; Chin, B.A. The analytical comparison of phage-based magnetoelastic biosensor with TaqMan-based quantitative PCR method to detect *Salmonella* Typhimurium on cantaloupes. **Food Control**, v. 33, p.330-336, 2013.

Park, S.H.; Ricke, S.C. Development of multiplex PCR assay for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *Salm*. Enteritidis, *Salm*.

Heidelberg and *Salm*. Typhimurium. **Journal of Applied Microbiology**, v. 118, n. 1, p.152-160, 2015.

Sambrook, J. and Russel, D. W. **Molecular Cloning**. New York: Cold Spring Harbor Laboratory Press, 2001. Cap. 6, p. 6.1-6.62.

SVS. **Secretaria de Vigilância em Saúde**. Doenças Transmitidas por Alimentos. Ministério da Saúde. Secretaria de Vigilância em Saúde 2015. Available at http://u.saude.gov.br/images/pdf/2015/novembro/09/Apresenta----o-dados-gerais-DTA-2015.pdf.

CAPITULO 4 – Real-time PCR and conventional microbiology for identification and quantification of *Salmonella* Gallinarum, *S.* Pullorum, *S.* Enteritidis and *S.* Typhimurium in liver and cecal content samples of experimentally infected laying hens

ABSTRACT – Poultry products are often associated with food outbreaks from *Salmonella* spp. and programs for disease prevention and control depends on the correct diagnosis. Thus, the objective of the present study was to identify and quantify *Salmonella* Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium in liver and cecal content of infected laying hens. Four experimental groups were used, inoculated with each strains, orally at the 13th week of life of the birds. At four and seven days post challenge, three birds from each group were euthanasia for liver and cecal content sample collection. After the sampling, was carried out bacterial identification and quantification by real-time PCR and conventional microbiology. It was possible to evidence the high sensitivity and specificity of qPCR, especially when the samples came from feces, because obtained positive results even from samples with a large number of contaminants and low bacterial load.
Keywords: Avian salmonelosis, melting curve, standard curve, SYBr Green I.

1 Introduction

Poultry products are often associated with food outbreaks caused by *Salmonella* spp., by the fact that they are affected by host-specific serovars as well by non-specific serovars. The biovars *S*. Pullorum e *S*. Gallinarum are host-specific for birds, genetically similar, immobile and cause the pullorum disease and fowl typhoid, respectively (O'REGAN et al., 2008). Flagellated serovars, which have no host specificity, cause non-typhoidal salmonelosis in birds and mammals (LÖFSTRÖM et al., 2010).

Because it is of great importance for the entire poultry industry chain, due to the great economic losses, on account of the high mortality rates, the reduction of production rates and the quality of products and by-products of infected or resilient animals (BATISTA et al., 2013). Existing programs for disease prevention and control are complex due to the various sources of contamination within the poultry industry

(ANTUNES et al., 2003). In recent years, many discussions on avian salmonellosis have been generated, owing to their relevance as zoonosis. The importance of their control in all scales of the poultry production is due to the fact of being able to infect the commercial birds, which consequently, will generate contamination in the products destined for the human consumption (LÖFSTRÖM et al., 2010).

The microbiological examination, which is recommended by government inspection organization, has been more laborious and with more steps to be followed, when compared to molecular techniques. Among these, direct identification of bacteria is difficult due to the growth of other microorganisms present in the sample, especially the host's natural microbiota. Their identification and complete characterization depends on the biochemical and serological analysis of each isolate, which increases the time and cost to obtain results with this methodology (CHEN et al., 2010).

The PCR technique was successfully established for the rapid and reliable diagnosis of different pathogens, including bacteria of difficult diagnose. The results can be obtained in one or two days and the positive results do not need confirmation by conventional microbiology (LIN et al., 2011). It has become a tool widely used in epidemiological studies, because it is a method characterized by high specificity and sensitivity (CHEN et al., 2010). Real-time qPCR was performed in a more recent way for the diagnosis of pathogens, with greater sensitivity than the conventional PCR technique and the use of fewer steps in the reaction, obtaining faster results (PARK et al., 2011; MA et al., 2014). Thus, the objective of the present study was to identify and quantify *Salmonella* Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium in liver and cecal content of infected laying hens by qPCR and microbiological analysis.

2 Material and Methods

2.1 Bacterial strains and preparation of inoculum

Were used four bacterial strains to inoculate the animals. The strains included *S*. Gallinarum 287/91 (a.n.: AM933173.1), *S.* Pullorum FCAV198 (a.n.: AZRG00000000), *S*. Enteritidis P125/109 (accession number: ASM950v1) and *S*. Typhimurium F98 (BARROW et al., 1990). All bacteria were obtained and stored at -

80°C in the Laboratory of Avian Pathology at School of Agricultural and Veterinary Sciences (FCAV-Unesp/Jaboticabal/SP). *Salmonella* strains used for inoculation were cultured in lysogeny broth (LB, Sparks, Maryland, USA) at 37°C, shaking at 100 rpm for a period of 18 hours as previously described by Rubio et al., 2017.

2.2 Experiment with the animals and sampling

A total of sixty laying hens of semi-heavy lineage from brown variety were housed with one day of life in climatized environment. At the time of arrival of the animals, meconium samples were collected from the inside of the boxes to search for *Salmonella* spp., following the methodology described by Zancan et al. (2000). The birds were divided into five groups (n=12), with an excess of six birds per group, considering the mortality risk. The supply of water, feed and light program was performed as recommended by the lineage manual. One group was used as negative control, where the birds were not infected. The other four groups were divided according to the inoculum administered, where the birds were infected with *S*. Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium. The experiment is in accordance with the ethical principles for animal experimentation, approved by the Ethics Committee on Animal Use (CEUA), present at the University (Process 01354/15; approved on 02 February 2015).

The inoculation of the animals was carried out with the administration of 2 mL of LB broth containing 10^8 CFU of the respective bacterium, orally at the 13th week of life of the birds. At four and seven days post infection (dpi), tree birds from each group were euthanased for liver and cecal content sample collection.

2.3 Quantification by conventional microbiology

The samples were submitted to the first serial dilution inside the tube, using buffered saline solution pH 7.4 (PBS), in the proportion of 1:10, where the liver samples were macerated and the cecal content homogenized, for further decimal serial dilution in PBS. From each dilution, 0.1 mL was taken, spreading on plates containing bright green agar (VB Nal), containing nalidixic acid (25 µg/mL) and incubated at 37°C for 24 h. At the end of the plating, 0.5 mL of selenite broth, containing novobiocin (0.04%) and prepared in double concentration, was added to

the tubes containing the homogenized samples in PBS (1:10), and incubated at 37°C for a further 24 h. After the first incubation, the bacterial numbers of each culture were evaluated and the colony forming units per mL (CFU/mL) were transformed to Log_{10}. In the absence of colonies for quantification at the end of the first incubation period, samples enriched in selenite broth were seeded in VB Nal, with subsequent incubation at 37ºC for 24 h.

2.4 Quantification by qPCR

2.4.1 DNA extraction of animal samples

Liver and cecal content samples were macerated and submitted to the enrichment process in 1 mL of selenite broth, in double concentration, for 4 hours at a temperature of 37ºC. After the incubation, DNA extraction of all bacterial cultures were performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer instructions. The purity and concentration of bacterial DNA was analyzed by spectrophotometry (Nanodrop 1000 ThermoFisher Scientific, USA).

2.4.2 Primer used for qPCR

Five pairs of primers were used and the sequences and details are in Table 1. The primer pSGP was designed to detect the *S. enterica* serovar Gallinarum, including both biovars, without detection of other typhoidal or non-typhoidal *Salmonella* sp. serovars. The primers pSG and pSP were designed to differentiate *S*. Gallinarum biovar Gallinarum (pSG) and *S*. Gallinarum biovar Pullorum (pSP) based on the analysis of regions of difference (RoDs) from each biovar (BATISTA et al., 2013). These three pairs of primers make up a reaction for differential diagnosis between *S*. Gallinarum and *S*. Pullorum, described by Rubio et al. (2017).

The pair of primer pSE was based on Malorny et al. (2007) and used for amplification of specific product of *S.* Enteritidis. The pair of primer pSTM was based on Park et al. (2013) to detect *S.* Typhimurium. These two pairs of primers make up a reaction for differential diagnosis between *S.* Enteritidis and *S.* Typhimurium.

2.4.3 Conditions of the qPCR

Two multiplex reactions were used to identify and quantify the isolates. One of the reactions for identification of SG and SP biovars, as described by Rubio et al. (2017) and the other for the identification of SE and STM serovars. Both reactions was performed using the following optimized reaction mix of 12.5 µL Luminoct® SYBr® Green qPCR Readymix (Sigma-Aldrich, St. Louis, Missouri, USA), 2.5 µL of serially diluted DNA or eluted DNA after extraction from tested samples, and ultrapure water to make the final volume 25 µL, with variation only in the primers inclusion, which for the first reaction corresponded to 0.1 µM of the primer pSGP, 0.05 µM of primers pSG and pSP and the second reaction corresponded to 0.05 µM of the primer pSE, 0.025 µM of primers pSTM.

The real-time qPCR was performed in the C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, California, USA), with cycling protocol stating with one denaturation cycle at 94 °C for 2 minutes, followed by 40 cycles at 94°C for 15 seconds and 63°C for 30 seconds. After amplification, the melting curve was performed to obtain the specific melting temperature (*Tm*) of each amplicon for the differential diagnosis between *Salmonella* isolates.

Table 1. Specifications of the primers used in the study

Primer	Sequence	Product Size (pb)	Concentration (µM)	Amplicon *Tm**
pSGP F	TGGCCTTGACGTTATTCGTT	97	0.025	78.0 °C
pSGP R	AGCATTTTGGTCACCTGCAA			
pSG F	ATGGCGAGTCCGCCCAGAGT	273	0.05	86.2 °C
pSG R	GGCGGTATGCTGGTTGCCGT			
pSP F	CCGCCTGCGCGATGGCTTTA	260	0.05	84.8 °C
pSP F	TCTGGTTGACGGCGTGGGGA			
pSE F	ATATCGTCGTTGCTGCTTCC	206	0.05	85°C
pSE R	CATTGTTCCACCGTCACTTTG			
pSTM F	GCGCACCTCAACATCTTTC	62	0.025	79°C
pSTM R	CGGTCAAATAACCCACGTTCA			

**Tm*: Melting temperature.

2.4.4 Standard curve for bacterial quantification

Serially diluted plasmid DNA containing the specific product of each bacterial strain was used to prepare the quantitative standard curve. The reactions followed the optimized PCR conditions for the multiplex reactions and was performed, for

each sample, in three replicates with one negative controls (ultra-pure water), four positive controls (*Salmonella* DNA strains) and eight diluted plasmid DNA. The Software CFX Manager™ 3.1 (Bio-Rad, Hercules, California, USA) was used to calculate the regression coefficients, standard deviation and standard curves efficiency ratio. The linear standard curve was used for quantification of number of genes copies that corresponded to the number of CFU of each serovar.

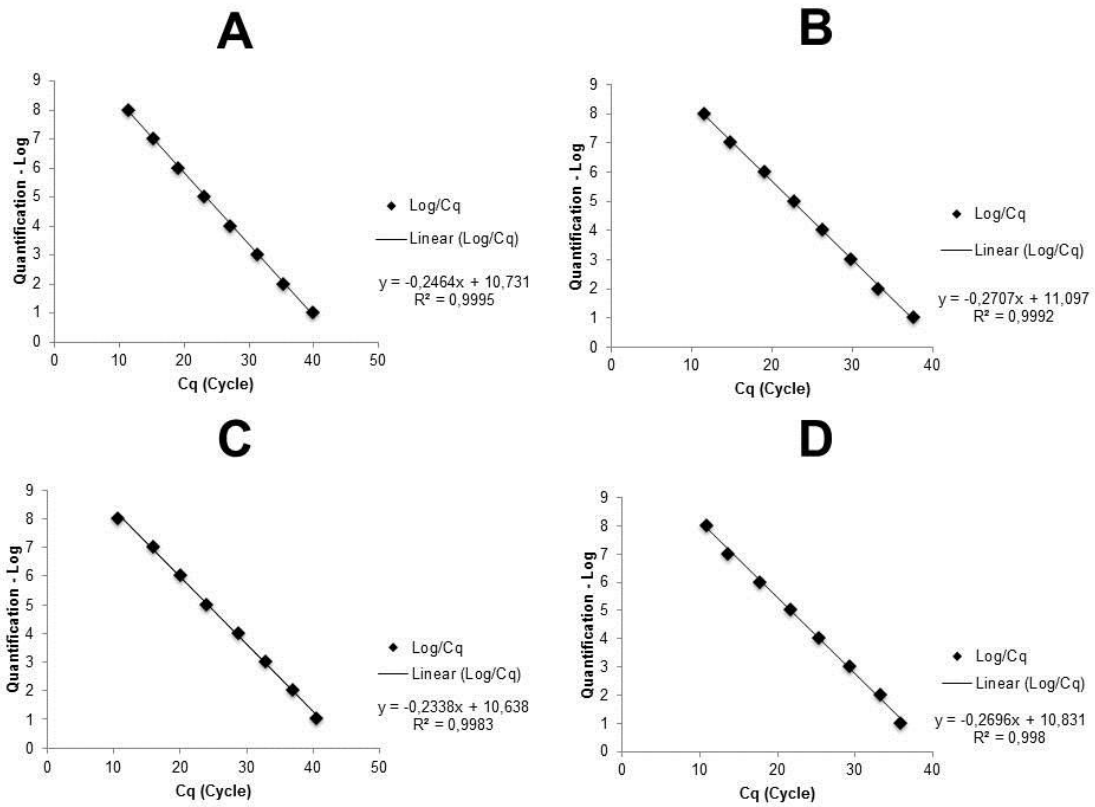

Figure 1. Standard curve for quantitative real-time qPCR. A) *S*. Gallinarum standard curve; B) *S*. Pullorum standard curve; C) *S*. Enteritidis standard curve; D) *S*. Typhimurium standard curve.

2.4.5 Quantification of samples

After qPCR, with the diagnostic result obtained, the amplification cycles (Cq) obtained for each sample were converted to Log_{10} (CFU/mL), considering the inclusion of the Cqs in the formula obtained with the standardized regression curve for each bacterium, to obtain the quantification of the samples.

2.5 Statistical analysis

Statistical differences amongst viable bacteria numbers recovered from liver and cecal contents were determined using Tukey's test (p<0.05), performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

3 Results and discussion

Salmonella spp. identification and quantification in animal organs and feces samples has been increasingly approached in scientific studies, since it evaluate the course of infection in the animal, in addition, it effectively acts in epidemiological surveys (FUKUSHIMA et al., 2003; MALORNY et al. 2008; WRIGHT et al., 2009; CHEN et al., 2010; POSTOLLEC et al. 2011; AGRIMONTI et al., 2013; CHERAGHCHI et al., 2014; HAMIDI et al., 2014; BONILAURI et al., 2016; HANABARA; UEDA, 2016). The present study analyzed liver and cecal contents samples of birds infected with *S*. Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium, in order to compare two laboratory techniques, qPCR and conventional microbiological analyses (Table 2).

Table 2. Comparison of real-time qPCR and conventional microbiology techniques of liver and cecal content samples of laying hens infected with *S.* Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium

Strain	Sample	4 days post infection		7 days post infection	
		qPCR	Microbiologic	qPCR	Microbiologic
***S.* Gallinarum**	**Liver**	2,70[b]	4,66[a]	2,50[b]	5,37[a]
	Cecal Content	1,66[a]	0,00[b]*	2,78[a]	0,00[b]*
***S.* Pullorum**	**Liver**	1,88	2,30*	0,26	1,33*
	Cecal Content	3,44[b]	5,15[a]	0,72	2,1*
***S.* Enteritidis**	**Liver**	1,60	2,00*	0,00	1,33*
	Cecal Content	4,58[b]	6,84[a]	4,20	4,65
***S.* Typhimurium**	**Liver**	0,95	1,99*	0,00	0,00*
	Cecal Content	6,24	5,33	4,59	4,65

Mean of Log_{10} values of the viable bacterial cells number (CFU/mL).
Different letters on the lines there was statistical significance by Tukey's test (p<0.05) between distinct reactions by day.
*Samples submitted to enrichment in Selenite broth for 24 hours.

In liver samples from *S*. Gallinarum infected group and in both periods, it was possible to detect 100% of positive samples were detect in both techniques. However, microbiology was more effective than qPCR for quantification of samples ($p<0.05$). As for cecal content samples in the same group, it was not possible to isolate the bacteria by conventional microbiology. An opposite result was observed in the qPCR performance, where it was possible to obtain positive samples, which shows a greater capacity and high sensitivity to isolate this biovar from this type of sample ($p<0.05$). This result reflects the findings of Malorny et al. (2008), who reported the difficulty of isolating a specific pathogen by conventional microbiology in fecal samples, due to the high microbial flora present in cecal sample. This result raises a worrying fact, because the birds, besides carriers, continue to eliminate the bacteria to the environment and consequently, spreading the disease to the rest of the lot, which would not be observed by the microbiological monitoring, what would inhibit an implementation of measures of prevention and control of the disease.

In the group inoculated with *S*. Pullorum, no significant differences ($p<0.05$) were observed between the liver samples, in both periods evaluated. However, to obtain positive results by microbiological examination, it was necessary to perform enrichment of the samples, which added 24 hours to release the final result. In addition, quantification of enriched samples is not performed and the value of 10^2 CFU/mL is considered, which may not adequately represent the amount of bacteria present in these samples. When analyzing the results of cecal samples, positivity was observed in all samples and periods evaluated, with a statistical significance for only 4 dpi, collected samples with a higher number of CFU/mL quantified by conventional microbiology examination, as observed in the *S*. Gallinarum group. However, at 7 dpi, the enrichment step was necessary for the microbiological test, as well as for liver samples. This fact reveals the weakness in the technique to isolate low amounts of bacteria.

These data disagree with the results reported by Löfström et al. (2010), who compared the qPCR and microbiological techniques, obtained 100% positive results for *S*. Enteritidis in both tests. However, they reported that this index was only reached when the inoculum quantification was higher than 10^2 CFU/mL. This justifies the negative results in cecal samples of animals inoculated with *S*. Gallinarum and

the need for enrichment step in the group inoculated with *S*. Pullorum by microbiological examination, that in addition to the large amount of feces contaminants, the fecal excretion of biovars after 4 dpi is low or null (BERCHIERI JUNIOR et al., 2001), highlighting the need for a diagnostic test with high sensitivity to monitor this important pathogens in poultry lots.

The results obtained with the quantification of liver and cecal contents samples of birds inoculated with *S*. Enteritidis and *S*. Typhimurium exhibited in a similar pattern of detection, with statistical difference only for cecal content samples of the *S*. Enteritidis group. Similar to the previous groups, the microbiological quantification of cecal contents stood out, when compared to qPCR.

The fact that conventional microbiology quantifies a greater quantity of bacteria than qPCR in most of the evaluated groups, can be attributed to the process of DNA extraction, according to a study conducted by Elizaquível and Aznar (2008), who evaluated four types of DNA extraction methods, reported that the extraction with commercial kit generates loss of genetic material as the samples are processed, which does not occur in the microbiological analysis. Similar result was observed in the present study, which an adaptation of the protocol proposed by the commercial kit for DNA extraction was necessary to possibilite the quantification of the evaluated samples.

In a study carried out by Wolffs et al. (2006), described the achievement of a quantitative threshold of 220 CFU/100 mL of artificially contaminated *S*. Typhimurium chicken skin samples. This limit of detection was attributed to the fact that they did not use the enrichment phase in the samples. This step was included in the present study, in order to improve the extraction methodology and decrease bacterial DNA losses, allowing a minimum mean of quantification, for the experiment as a whole, of 1,8 CFU/mL in liver samples at 7dpi, from the *S*. Pullorum group, evidencing the high sensitivity used by the qPCR technique. Similarly, Myint et al. (2006), when comparing conventional PCR with microbiological technique in naturally infected samples with *Salmonella* spp., reported 128 CFU/mL of the sample, although they included the enrichment step and the sensitivity was high, it did not reach the indices observed in this study.

The evaluation of two diagnostic techniques for bacterial genus identification is due to the need in which poultry production has to implement quickly and adequately the measures of control and eradication of this important pathogen, due the rapid growth and production cycles (HOORFAR et al., 1999; DICKEL et al., 2005; MYINT et al., 2006; MALORNY et al., 2007; O'REGAN et al., 2008; MCGUINNESS et al., 2009; LÖFSTRÖM et al., 2010; SORIA et al., 2013; TA et al., 2014; PLETTIG et al., 2015). In addition, *Salmonella* spp. are obligatory reporting bacteria in Brazil, where misdiagnosis can lead to the disposal of the whole lot when infected with these strains (BRASIL, 2009). This fact makes indispensable a reliable diagnostic test for the identification and differentiation of this pathogen, for having 2659 serovars isolated (ISSENHUTH-JEANJEAN et al., 2014).

In a retrospective study performed by Bonilauri et al. (2016) with the use of food samples for comparison of conventional microbiology methods and conventional PCR, the authors reported 2.18% (130/ 5962) positive samples for *Salmonella* spp. by PCR, against 0.43% (26/5962) by microbiological analyses, which highlights the greater sensitivity of the molecular tests in relation to the microbiological techniques. A similar result was observed in the present study, that when the amount of bacteria present in the sample was low in the qPCR, it was difficult to obtain the positive result by the microbiological tests.

Besides the high sensitivity shown by qPCR, the use of SYBr Green for the differential diagnosis of commercial and public health pathogens makes the use of this methodology even more attractive. Although not as specific as the hydrolysis probes (TaqMan), it does not require additional technical knowledge to perform its specific design, also to having a more attractive cost. Its specificity is due to the adequacy and thorough standardization of the melting curve (*Tm*) obtained from the reaction (DE MEDICI et al., 2003; PONCHEL et al., 2003; MACKAY, 2004; ESPY et al., 2006; KAGKLI et al., 2012). The multiplex qPCR reactions used in this study performed satisfactorily with their application in biological samples, allowing the differential diagnosis to be obtained even from samples with high levels of contaminants (cecal content).

The qPCR already has a recognized potential to obtain results that are more agile than microbiological methods, however, DNA extraction has been limiting the

effectiveness of the test (WOLFFS et al., 2006; RAJTAK et al., 2011; AGRIMONT et al., 2013). In the present study, it was inferred the occurrence of this problem, that even with the inclusion of the pre-enrichment step, it was not possible to avoid the DNA losses from the extraction process, which reduced the quantification potential of the samples.

The comparison between two diagnostic techniques showed that the microbiological analysis was more efficient in the quantification of samples from birds infected with *S*. Gallinarum, *S*. Pullorum, *S*. Enteritidis and *S*. Typhimurium. However, the qPCR was qualitatively highlighted, especially when the samples are feces, because positive results were obtained even from samples with a large number of contaminants and low bacterial load. This comparison for *Salmonella* spp. diagnosis should not delimit the use of one or the other, but rather favor reliability and applicability of obtaining the diagnostic result, with the adequacy of the needs observed in laboratories and animal production.

4 References

AGRIMONTI, C.; BORTOLAZZI, L.; MAESTRI, E.; SANANGELANTONI, A.M.; MARMIROLI, N. A Real-Time PCR/SYBR Green I Method for the Rapid Quantification of *Salmonella enterica* in Poultry Meat. **Food Analytical Methods**, n. 6, p.1004-1015, 2013.

ANTUNES, P.; RÉU, C.; SOUSA, J.C.; PEIXE, L.; PESTANA, N. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. **International Journal of Food Microbiology**, v. 82, p.97-103, 2003.

BARROW, P.A.; HASSAN, J.O.; BERCHIERI JUNIOR A. Reduction in faecal excretion of *Salmonella typhimurium* strain F98 in chickens vaccinated with live and killed *S. typhimurium* organisms. **Epidemiology and Infection**, v.104, p. 413-426, 1990.

BATISTA, D.F.A.; FREITAS NETO, O.C.; LOPES, P.D.; ALMEIDA, A.M.; BARROW, P.A. BERCHIERI JR., A. Polymerase chain reaction assay based on *ratA* gene allows differentiation between *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovars Gallinarum and Pullorum. **Journal of Veterinary Diagnostic Investigation**, v. 25, n. 2, p.259-262, 2013.

BERCHIERI JR, A.; MURPHY, C.K.; MARSTON, K.; BARROW P.A. Observations on the persistence and vertical transmission of *Salmonella enterica* serovars Pullorum and Gallinarum in chickens: Effect of bacterial and host genetic background. **Avian**

Pathology, v. 30, n. 3, p.221-231, 2001. Access in: <http://dx.doi.org/10.1080/03079450120054631>

BONILAURI, P.; BARDASI, L.; LEONELLI, R.; RAMINI, M.; LUPPI, A.; GIACOMETTI, F.; MERIALDI, G. Detection of food hazards in foods: comparison of real time polymerase chain reaction and cultural methods. **Italian Journal of Food Safety**, v. 5:5641, p.37-40, 2016.

BRASIL, **Ministério da Agricultura, Pecuária e Abastecimento**. Manual de Legislação : Programa nacional de sanidade avícola / Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Departamento de Saúde Animal. Brasília : MAPA/SDA/DSA, p.171-241, 2009.

CHEN, J.; ZHANG, L.; PAOLI, G.C.; SHI, C.; TU S.; SHI, X. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. **International Journal of Food Microbiology**, v. 137, p.168–174, 2010.

CHERAGHCHI, N.; KHAKI, P.; BIDHENDI, S.M.; SABOKBAR, A. Identification of isolated *Salmonella enterica* serotype *gallinarum* biotype *Pullorum* and *Gallinarum* by PCR-RFLP. **Jundishapur Journal of Microbiology**, v.7, p. 1-4, 2014.

DE MEDICI, D.; CROCI, L.; DELIBATO, E.; DI PASQUALE, S.; FILETICI, E.; TOTI, L. Evaluation of DNA extraction methods for use in combination with
SYBR Green I real-time PCR to detect *Salmonella enterica* serotype Enteritidis in poultry. **Applied and Environmental Microbiology**, v. 69, n. 6, p.3456-3461, 2003.

DICKEL, E.L.; RODRIGUES, L.B.; SANTOS, L.R.; VALLE, S.F.; PILOTTO, F.; RODEMBUSH, C.; WALD, V.B.; CANAL, C.W.; NASCIMENTO, V.P. Análise comparativa entre microbiologia convencional, ELISA e PCR para detecção de *Salmonella enteritidis*, *S. typhimurium*, *S. gallinarum* e *S. pullorum* em carne de frango contaminada artificialmente. **Revista Brasileira de Ciência Veterinária**, v. 12, n. 1/3, p.5-10, 2005.

ELIZAQUÍVEL, P.; AZNAR, R. Comparison of four commercial DNA extraction kits for PCR detection of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, and *Staphylococcus aureus* in fresh, minimally processed vegetables. **Journal of Food Protection**, v.71, n.10, p. 2110-2114, 2008.

ESPY, M.J.; UHL, J.R.; SLOAN, L.M.; BUCKWALTER, S.P.; JONES, M.F.; VETTER, E.A.; YAO, J.D.C.; WENGENACK, N.L.; ROSENBLATT, J.E.; COCKERILL, F.R.; SMITH, T.F. Real-time PCR in clinical microbiology: applications for routine laboratory testing. **Clinical Microbiology Reviews**, v. 19, n. 1, p.165-256, 2006. Acess in: <doi:10.1128/CMR.19.1.165–256.2006>

FUKUSHIMA, H.; TSUNOMORI, Y.; SEKI, R. Duplex real-time SYBr green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. **Journal of Clinical Microbiology**, v. 41, p.5134-5146, 2003.

HAMIDI, A.; IRSIGLER, H.; JAEGER, D.; MUSCHALLER, A.; FRIES, R. Quantification of water as a potential risk factor for cross-contamination with *Salmonella*, *Campylobacter* and *Listeria* in a poultry abattoir. **British Poultry Science**, v. 55, n. 5, p.585-591, 2014. Access in: <http://dx.doi.org/10.1080/00071668.2014.949622>

HANABARA, Y.; UEDA, Y. A rapid and simple real-time PCR assay for detecting foodborne pathogenic bacteria in human feces. **Japanese Journal of Infectious Diseases**, v. 69, p.471–476, 2016.

HOORFAR, J.; BAGGESEN, D.L.; PORTING, P.H. A PCR-based strategy for simple and rapid identification of rough presumptive *Salmonella* isolates. **Journal of Microbiological Methods**, v. 35, p.7-84, 1999.

ISSENHUTH-JEANJEAN, S.; ROGGENTIN, P.; MIKOLEIT, M.; GUIBOURDENCHE, M.; PINNA, E.; NAIR, S.; FIELDS, P.I.; WEILL, F. Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme. **Research in Microbiology**, v. 165, p.526-530, 2014.

KAGKLI, D.; FOLLONI, S.; BARBAU-PIEDNOIR, E.; EEDE, G.V.D.; BULCKE, M.V.D. Towards a pathogenic *Escherichia coli* detection platform using multiplex SYBr Green real-time PCR methods and high resolution melting analysis. **PLoS ONE**, v. 7, p.1-12, 2012.

LIN, L.H.; TSA, C. Y.; HUNG M.H.; FANG, Y.T.; LING, Q.D. Rectal swab sampling followed by an enrichment culture-based real-time PCR assay to detect *Salmonella enterocolitis* in children. **Clinical Microbiology and Infection**, v. 17, p.1421-1425, 2011.

LÖFSTRÖM, C.; HANSEN, F.; HOORFAR, J. Validation of a 20-h real-time PCR method for screening of *Salmonella* in poultry faecal samples. **Veterinary Microbiology**, v. 144, p.511-514, 2010.

MA, K.; DENG, Y.; BAI, Y.; XU, D.; CHEN, E.; WU, H.; LI, B.; GAO, L. Rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork using a multiplex real-time PCR assay based on immunomagnetic separation. **Food Control**, v. 42, p.87-93, 2014.

MACKAY, I.M. Real-time PCR in the microbiology laboratory. **Clinical Microbiology and Infection**, v. 10, p.190-212, 2004.

MALORNY, B.; BUNGE, C.; HELMUTH, R. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. **Journal of Microbiological Methods**, v. 70, p.245-251, 2007.

MALORNY, B.; LÖFSTRÖM, C.; WAGNER, M.; KRÄMER, N.; HOORFAR, J. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for

quantitative microbial risk assessment. **Applied and Environmental Microbiology**, v. 74, p.1299-1304, 2008.

MCGUINNESS, S.; MCCABE, E.; O'REGAN, E.; DOLAN, A.; DUFFY. G.; BURGESS, C.; FANNING, S.; BARRY, T.; O'GRADY, J. Development and validation of a rapid real-time PCR based method for the specific detection of *Salmonella* on fresh meat. **Meat Science**, v. 83, p.555-562, 2009.

MYINT, M.S.; JOHNSON, Y.J.; TABLANTE, N.L.; HECKERT, R.A. The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated Salmonella in raw poultry compared to conventional culture. **Food Microbiology**, v. 23, n. 6, p.599-604, 2006. Access in: <https://doi.org/10.1016/j.fm.2005.09.002>

O'REGAN, E.; MCCABE, E.; BURGESS, C.; MCGUINNESS, S.; BARRY, T.; DUFFY, G.; WHYTE, P.; FANNING, S. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. **BMC Microbiology**, v. 8, p.1-11, 2008.

PARK, S.H.; HANNING, I.; JARQUIN, R.; MOORE, P.; DONOGHUE, D.J.; DONOGHUE, A.M.; RICKE, S.C. Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. **FEMS Microbiology Letters**, v. 316, p.7-15, 2011.

PARK, M.; WEERAKOON, K.A.; OH, J.; CHIN, B.A. The analytical comparison of phage-based magnetoelastic biosensor with TaqMan-based quantitative PCR method to detect *Salmonella* Typhimurium on cantaloupes. **Food Control**, v. 33, p.330-336, 2013.

PLETTIG, R.; NOWAK, A.; BALAU, V.; HAHNENKAMP, K.; USICHENKO T. Prospective comparison of a PCR assay and a microbiological culture technique for identification of pathogens from blood and non-blood samples in septic patients. **Journal of Intensive Care**, v. 3, n. 51, p.1-8, 2015.

PONCHEL, F.; TOOMES, C.; BRANSFIELD, K.; LEONG, F.T.; DOUGLAS, S.H.; FIELD, S.L.; BELL, S.M.; COMBARET, V.; PUISIEUX, A.; MIGHELL, A.J.; ROBINSON, P.A.; INGLEHEARN, C.F.; ISAACS, J.D.; MARKHAM, A.F. Real-time PCR based on SYBr-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. **BMC Biotechnology**, v. 3, p.1-13, 2003.

POSTOLLEC, F.; FALENTIN, H.; PAVAN, S.; COMBRISSON, J.; SOHIER, D. Recent advances in quantitative PCR (qPCR) applications in food microbiology. **Food Microbiology**, v. 28, n. 5, p.848-861, 2011.

RAJTAK, U.; LEONARD, N.; BOLTON, D.; FANNING, S. A real-time multiplex SYBr Green I polymerase chain reaction assay for rapid screening of *Salmonella* serotypes

prevalent in the European Union. **Foodborne Pathogens and Disease**, v. 8, p.769-779, 2011.

RUBIO, M.S.; PENHA FILHO, R.A.C.; ALMEIDA, A.M.; BERCHIERI JUNIOR, A. Development of a multiplex qPCR in real time for quantification and differential diagnosis of *Salmonella* Gallinarum and *Salmonella* Pullorum. **Avian Pathology**, v. 2017, p.2-9, 2017. Access in: < http://dx.doi.org/10.1080/03079457.2017.1339866>

SAMBROOK, J. and RUSSEL, D. W. **Molecular Cloning**. New York: Cold Spring Harbor Laboratory Press, 2001. Cap. 6, p. 6.1-6.62.

SORIA, M.C.; SORIA, M.A.; BUENO, D.J.; TERZOLO, H.R. Comparison of 3 culture methods and PCR assays for *Salmonella gallinarum* and *Salmonella pullorum* detection in poultry feed. **Poultry Science**, v. 92, p.1505-1515, 2013.

TA, Y.T.; NGUYEN, T.T.; TO, P.B.; PHAM, D.X.; LE, H.T.H.; THI, G.N.; ALALI, W.Q.; WALLS, I.; DOYLE, M.P. Quantification, serovars, and antibiotic resistance of *Salmonella* isolated from retail raw chicken meat in Vietnam. **Journal of Food Protection**, v. 77, n. 1, p.57-66, 2014. Access in: <doi:10.4315/0362-028X.JFP-13-221>

ZANCAN, F.B.; BERCHIERI JUNIOR, A.; FERNANDES, S.A.; GAMA, N.M.S. Q. *Salmonella* spp. investigation in transport box of day old birds. **Brazilian Journal of Microbiology**, São Paulo, v. 31, n. 3, p.230-232, 2000. Access in: <http://dx.doi.org/10.1590/S1517-83822000000300016>

WOLFFS, P.F.G.; GLENCROSS, K.; THIBAUDEAU, R.; GRIFFITHS, M.W. Direct quantitation and detection of Salmonellae in biological samples without enrichment, using two-step filtration and real-time PCR. **Applied and Environmental Microbiology**, v. 72, n. 6, p.3896-3900, 2006.

WRIGHT, A.C.; DANYLUK, M.D.; OTWELL, W.S. Pathogens in raw foods: what the salad bar can learn from the raw bar. **Current Opinion in Biotechnology**, v. 20, n. 2, p.172-177, 2009.

CAPITULO 5 – Considerações finais

O diagnóstico laboratorial de bactérias do gênero *Salmonella* spp. tem se destacado cada vez mais no meio cientifico, pois além dos prejuízos gerados à produção animal e a saúde humana, novos sorovares são identificados anualmente, o que dificulta a implementação de medidas de controle e erradicação desse importante patógeno.

O isolamento microbiológico e a caracterização bioquímica, que são metodologias de diagnóstico preconizadas pelos órgãos governamentais, já possuem reconhecido destaque dentre os exames de rotina laboratorial. No entanto, o tempo gasto para a obtenção do resultado final pode ocasionar a disseminação do patógeno para todo o lote acometido e em lotes adjacentes, por se tratar de uma bactéria de fácil propagação.

As provas moleculares tem se tornado alvo de pesquisas microbiológicas, em razão da alta sensibilidade e especificidade da técnica, além de reduzir significativamente o tempo designado para realização do teste. O principal fator limitante da técnica é o custo, não apenas com a reação, mas também com a adaptação tecnológica do laboratório, ao exigir equipamentos específicos para sua execução.

A obtenção de duas reações multiplex qPCR padronizadas no presente estudo para diferenciação de quatro estirpes de *Salmonella* spp., reduz consideravelmente os custos dessa técnica, além de diminuir o período demandado para sua realização. Tal fato torna atrativo a adesão dessa metodologia de diagnóstico para levantamentos epidemiológicos e na rotina laboratorial, ao aliar resultados mais rápidos, com custo final por reação competitivo. Além disso, a técnica padronizada e aqui esplanada possibilita a quantificação bacteriana, ferramenta importante quando da necessidade em se entender a epidemiologia de um determinado patógeno e o seu curso de disseminação no ambiente. A limitação observada para a técnica da qPCR foi em obter amostras de DNA com qualidade e quantidade suficiente, principalmente quando da utilização de amostras biológicas, como fígado e conteúdo cecal, que com a inclusão da etapa de pré-enriquecimento

ao protocolo de extração de DNA proposto pelo fabricante, foi possível contornar tal adversidade.

A comparação de duas técnicas de diagnóstico para *Salmonella* spp. não deve delimitar a utilização de uma ou de outra e sim favorecer confiabilidade e aplicabilidade da obtenção do resultado diagnóstico, com a adequação as necessidades observadas nos laboratórios e na produção animal.