Pontifícia Universidade Católica do Rio Grande do Sul
Faculdade de Biociências
Programa de Pós-Graduação em Biologia Celular e Molecular

Diversidade genética de isolados de *Salmonella* Enteritidis avaliada por FAFLP (Análise de fragmentos polimórficos amplificados e fluorescentes) e MLST (Tipificação por sequenciamento de múltiplos *loci*)

Tese apresentada ao Programa de Pós-graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul – PUCRS, como requisito para obtenção do grau de Doutor.

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Porto Alegre, RS
Janeiro, 2010

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RESUMO

A *Salmonella* Enteritidis é uma das principais bactérias causadoras de gastrenterite, também podendo ser responsável por doenças sistêmicas. A adequada caracterização deste microrganismo é essencial nos estudos epidemiológicos. Neste contexto, este estudo avaliou a utilização de dois métodos moleculares, Tipificação por Sequenciamento de Múltiplos *loci* (MLST) e Análise de Fragmentos Polimórficos Amplificados e Fluorescentes (FAFLP), para a diferenciação de 32 isolados de *S.* Enteritidis oriundos de diferentes fontes do sul do Brasil, bem como de cinco isolados de *S*. Enteritidis provenientes de outras áreas geográficas. Também foram incluídos neste estudo quatro isolados pertencentes a outros sorovares (*S*. Panama, *S*. Senftenberg, *S*. Typhimurium e *Salmonella* [4,5:-:1,2]. As duas técnicas escolhidas para este trabalho já foram empregadas concomitantemente para analisar diferentes sorotipos de *Salmonella*, mas este estudo é o primeiro a utilizá-las para a diferenciação de um mesmo grupo de isolados de *S*. Enteritidis. O esquema desenvolvido para o MLST incluiu a amplificação de fragmentos de dois genes constitutivos (*hem*D e *mdh*) combinado com dois genes de virulência (*ssa*Q e *sly*A), aumentando, assim, a capacidade discriminatória do método. Ambas as técnicas apresentaram altos índices de poder discriminatório, calculados pelo índice de diversidade de Simpson, 0,99 e 0,88 para FAFLP e MLST, respectivamente. Além disso, os métodos avaliados também mostraram-se eficientes na discriminação de isolados de diferentes sorovares de *Salmonella*. Entretanto, a FAFLP e a MLST não foram capazes de diferenciar isolados provavelmente não relacionados epidemiologicamente, bem como não agruparam isolados pertencentes a um mesmo fagotipo. Desta forma, os resultados obtidos sugerem que ambas as técnicas podem ser ferramentas úteis para a análise epidemiológica molecular de isolados de *Salmonella*, inclusive para um sorovar com grande homogeneidade genética como a *S*. Enteritidis.

Palavras-chave: *Salmonella* Enteritidis, FAFLP, MLST, caracterização molecular.

ABSTRACT

Salmonella Enteritidis is a common foodborne pathogen that causes gastroenteritis and systemic infections in humans. The characterization of this bacterium is essential for epidemiological studies. In this context, two molecular methods, MLST and FAFLP, were tested for characterization of 32 *S*. Enteritidis isolates obtained from South of Brazil, as well as five isolates obtained from other geographical areas. Four isolates of different serovars of *Salmonella* (*S*. Panama, *S*. Senftenberg, *S*. Typhimurium and *Salmonella* [4,5:-:1,2]) were included as outgroup. These two techniques were already used to analyze different *Salmonella* serovars, but this study is the first to use them to discriminate the same *S*. Enteritidis isolates. The MLST scheme was performed with two housekeeping genes (*hem*D and *mdh*) combined with two virulence genes (*ssa*Q and *sly*A) to improve the discriminatory power of method. Both methods presented high discriminatory indexes calculated by Simpson's index of diversity, 0.99 and 0.88 for FAFLP and MLST, respectively. These methods were efficient to differentiate isolates of distinct *Salmonella* serovars, but did not distinguish isolates probable epidemiologically non-related, and also did not group isolates of same phage type. These results suggested that these two techniques can be used as a tool for the epidemiological molecular characterization of *S*. Enteritidis isolates.

Keywords: *Salmonella* Enteritidis, FAFLP, MLST, molecular characterization.

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1. Capitulo 1 INTRODUÇÃO E OBJETIVOS

1.1 INTRODUÇÃO

A *Salmonella* é uma bactéria de distribuição global, transmitida através de alimentos contaminados, que pode provocar diversas doenças em humanos, tais como enterocolites, bacteremia, febre entérica e infecções sistêmicas.

As bactérias do gênero *Salmonella* são bastonetes Gram negativos, normalmente produtoras de H_2S e não fermentadoras de lactose, comumente encontradas no trato intestinal de animais domésticos e selvagens. Estas bactérias são oxidase negativas, catalase positivas, utilizam o citrato de sódio como única fonte de carbono e descarboxilam a lisina e a ornitina[1]. Clinicamente, são classificadas em dois grupos distintos: tifóides (*S.* Typhi e *S.* Paratyphi), que causam febre tifóide, e não tifóides (diferentes sorovares de *S. enterica*), causadores de gastrenterites e outras infecções associadas[2]. A infecção por *Salmonella* sp. tende a ter consequências mais graves em crianças com menos de 5 anos de idade e em pacientes imunocomprometidos, como os portadores de HIV [3]. Alguns sorovares de *S. enterica* são adaptados a um hospedeiro específico; no entanto, a maioria pode colonizar e causar doença em uma grande variedade de espécies[4,5,6], tanto causando surtos epidêmicos, como estando associada com infecções esporádicas.

Todos os anos são reportados 40.000 casos de salmonelose nos Estados Unidos. Entretanto, como nem todos os casos são diagnosticados ou reportados aos órgãos de saúde, estima-se que ocorram aproximadamente 1,4 milhões de casos por ano, ocasionando cerca de 400 mortes por salmonelose aguda[7]. Desde a década de 80, tem-se observado um significativo aumento de *S*. Enteritidis relacionada com infecções humanas em pelo menos três continentes (América, Europa e Ásia) [8]. Nos Estados Unidos, a incidência de *S*. Enteritidis aumentou de 2,38 por 100.000 habitantes em 1985 para 3,9 por 100.000 habitantes em 1995, e apresentou um declínio de 49% em 1999 [9], fato que parecia ser uma tendência, apesar deste sorovar continuar sendo responsável por mortes relacionadas a surtos, mas que estabilizou em 2001 [10]. Dentre os casos de gastrenterites registrados na América do Sul e Central, cerca de 39% estão relacionados à *S*. Enteritidis [11].

No Brasil, a partir de 1993, a *S*. Enteritidis emergiu como um grande problema para a indústria avícola e para a saúde pública, causado provavelmente pela importação de matrizes de aves contaminadas dos países da Europa [12]. A *S*. Enteritidis é descrita como prevalente em várias regiões do Brasil em amostras isoladas de humanos e de outras fontes [13,14,15,16,17,18]. Aproximadamente 90% dos surtos de salmonelose ocorridos no Paraná, entre 1999 e 2002, foram causados pelo sorovar Enteriditis [19] e durante o período de 1999 a 2008 foram notificados 2974 surtos de toxi-infecções alimentares no Brasil, sendo o gênero *Salmonella* responsável por 1275 (42,9%) destes. Durante o mesmo período, 119 destes surtos foram atribuídos à *S*. Enteritidis[20].

A contaminação com *S*. Enteritidis normalmente ocorre pela ingestão de alimentos contaminados ou processados inadequadamente, especialmente aqueles de origem animal. Os principais veículos são os ovos e a carne de frango, embora a bactéria possa ser encontrada também em carne de outros animais, em embutidos e no leite [21].

Geralmente, o agente etiológico responsável por um surto de infecção é originado de uma única célula cuja progênie é geneticamente idêntica ou amplamente relacionada ao organismo de origem, veiculado a partir de uma fonte comum [22]. Entretanto, pode existir diversidade suficiente ao nível de espécie para que os organismos isolados de diferentes fontes em diferentes períodos e em diferentes regiões geográficas possam ser diferenciados.

Os métodos convencionais para a identificação de *Salmonella* incluem o cultivo em meio seletivo e a caracterização bioquímica e sorológica [23,24]. Já, a classificação da *Salmonella* em sorovares é realizada de acordo com o esquema Kauffman-White, que é baseado nas propriedades antigênicas das proteínas flagelares (H1 e H2), do lipopolissacarídeo de superfície celular (antígeno O) e do antígeno capsular (Vi) [25]. O esquema Kauffman-White reconhece 46 sorogrupos O e 114 antígenos H, resultando na caracterização de 2541 sorotipos [26]. Este esquema tem sido muito válido no diagnóstico clínico e epidemiológico dos sorotipos envolvidos em Samoneloses [27], mas requer a combinação com outros métodos quando o objetivo é uma análise

epidemiológica dos isolados, já que poucos sorovares são responsáveis pela grande maioria dos surtos em humanos [28,29].

A tipificação bacteriana tem sido realizada há décadas por vários métodos e, quanto mais métodos de tipificação forem empregados conjuntamente, maior será a probabilidade de confiar que o organismo é clonal [30]. Os métodos de tipificação podem ser divididos em dois grupos com base no tipo de características ou macromoléculas utilizadas como alvos para tipificação: métodos fenotípicos e genotípicos. Os métodos fenotípicos são aqueles que detectam características expressas pelo organismo e os métodos genotípicos são aqueles que envolvem a análise direta de DNA cromossômico e/ou extracromossômico [31,32,33].

A fagotipificação, utilizando padrões de lise obtidos com um conjunto definido de bacteriófagos, é um método clássico usado desde 1950 para diferenciação de sorovares bacterianos. Em 1987, foi descrito um esquema de fagotipificação para *S*. Enteritidis, diferenciando 27 fagotipos (PT) [34]. Desde então, este método têm sido usado como ferramenta em estudos epidemiológicos envolvendo surtos de *S*. Enteritidis, e têm a vantagem de ser rápido e de fácil execução quando comparado com algumas técnicas moleculares [18,35]. Entretanto, a fagotipificação tem poder discriminatório limitado e requer uma coleção especializada de fagos, o que dificulta a sua realização em muitos laboratórios [36,37].

Na Europa ocidental e em alguns países da América do Sul, desde 1987, o aumento de casos de infecções por *S*. Enteritidis está relacionado com o PT 4 [8]. A partir de 2001, observa-se uma mudança com o predomínio do PT14b na Suécia a também na Inglaterra [38]. Nos Estados Unidos, os fagotipos mais comuns são PT8 e PT13a[9]. O PT 13 foi ocasionalmente associado à sepse e ao aumento de mortalidade em frangos no Canadá [39]. No Brasil, observou-se um aumento do número de isolados oriundos de humanos e não humanos pertencentes ao PT4, fago tido como altamente clonal. No Estado de São Paulo, no período de 1975 a 1995, na análise de 574 isolados de *S*. Enteritidis, foi observada a predominância do PT 8 (80,9%) até 1992 e, após 1993, a grande maioria dos isolados pertenceu ao PT 4 [15]. Já, no Paraná, o fagotipo mais prevalente foi o PT4 até 2000[19], seguido do PT4a [40] e do PT9 em 2008[41].

Os métodos genotípicos empregados para o estudo de *Salmonella* incluem análise de perfis plasmidiais e padrões de restrição plasmidial [30], ribotipificação [18,42], RAPD (*Random Amplified Polymorphic DNA*) [43], RFLP (*Restriction Fragment Length Polymorphism*) [30], MLEE (*Multilocus Enzyme Electrophoresis*) [44,] PFGE (*Pulsed-field Gel Electrophoresis*) [36,45], repPCR [46] AFLP (*Amplified Fragment Length Polymorphism*) [47,48] e MLST (*Multilocus Sequence Typing*) [49,50]. Para *S*. Enteritidis, o uso de uma única técnica molecular normalmente não é suficiente para a diferenciação de isolados. O uso conjunto de técnicas fenotípicas e genotípicas pode prover informações importantes na evolução, ecologia e epidemiologia de subtipos de *S*. Enteritidis associados a diferentes hospedeiros [51,52,53,54].

A ribotipificação é um método que pode identificar e classificar bactérias baseando-se nas diferenças do RNAr. O operon ribossômico em bactérias é composto principalmente de genes constitutivos e qualquer variação nestes genes resulta em ribotipos polimórficos [55]. Em *Salmonella*, este método é utilizado em conjunto com a sorotipificação [56].

O uso de uma combinação de enzimas de restrição, como *Pst*I e *Sph*I, parece aumentar o poder discriminatório da técnica quando comparada a outros métodos, principalmente quando o objetivo é a tipificação de *S*. Enteritidis PT4 e PT8[57]. A utilização da ribotipificação para a análise de isolados de *S*. Enteritidis não tem mostrado capacidade em discriminar isolados deste sorovar, o que foi atribuído a possível endemicidade de um ribotipo nas áreas geográficas analisadas ou a uma grande homogeneidade clonal [18,56]. Além disso, a ribotipificação manual é bastante trabalhosa para uso de rotina em laboratórios e de difícil reprodutibilidade, enquanto que a ribotipificação automatizada ainda é bastante onerosa [58].

A análise de perfil plasmidial, que determina o número e o tamanho de plasmídeos, tem sido utilizada para discriminar isolados de *Salmonella* de variados sorotipos[59]. Este método pode ser complementar à fagotipificação[35], podendo diferenciar isolados de um mesmo fagotipo, como descrito para a *S*. Enteritidis PT8 [60]. Este método apresenta a limitação da possibilidade de mobilidade dos plasmídeos, o que pode ser minimizado pela utilização de outros métodos complementares de análise[61].

A PFGE, considerada como “padrão ouro” para a avaliação epidemiológica de sorovares de *Salmonella*, está baseada na análise de grandes fragmentos de DNA gerados pela digestão do DNA genômico com enzimas de restrição[61,62]. Esta técnica também pode indicar diversidade genética entre isolados [30], além de ser altamente reprodutível [63,64].

A diversidade genética entre isolados bacterianos também tem sido avaliada pela técnica AFLP. Esta técnica é baseada na digestão do DNA cromossômico normalmente com duas enzimas de restrição, ligação a oligonucleotídeos adaptadores específicos que impedem a reconstituição dos sítios de clivagem enzimática e posterior amplificação dos fragmentos por PCR utilizando oligonucleotídeos iniciadores seletivos ou não seletivos [65,66]. A observação dos resultados normalmente é realizada em gel de agarose [66]. A AFLP tem sido usada para genotipificação de várias espécies de bactérias [67,68], incluindo *Salmonella* [69,70]. Uma modificação do método de AFLP, o SE-AFLP, que, emprega apenas uma enzima de restrição para clivagem do DNA [71], demonstrou eficiência e maior poder discriminatório do que rep-PCR para onze isolados de *S*. Enteritidis de suínos da região sul do Brasil e nove isolados de outras áreas geográficas [54]. Quando são utilizados oligonucleotídeos iniciadores marcados com fluorescência, a técnica é denominada de FAFLP [72,73,74] e os fragmentos marcados obtidos podem ser detectados diretamente em sequenciador automático[48], facilitando a identificação de picos polimórficos em um grande número de isolados [48,75]. O poder discriminatório da técnica pode ainda aumentar se forem acrescentados nucleotídeos específicos aos oligonucleotídeos iniciadores, devido ao aumento de estringência destes oligonucleotídeos [76].

A FAFLP tem sido eficiente para a diferenciação de sub-espécies [73] e para a discriminação de sorotipos de *Salmonella* [69,77]. A comparação entre PFGE e FAFLP tem gerado resultados contrastantes. Alguns autores descrevem estas duas técnicas com poder de discriminação semelhante [77,78], enquanto Desai e colaboradres[48] mostram que FAFLP tem um maior índice de discriminação do que PFGE para *S*. Enteritidis PT4, e Scott e colaboradores[47] relataram que culturas de referência de *S*. Enteritidis pertencentes a 25 diferentes fagotipos originaram 25 perfis de FAFLP distintos.

Outra técnica empregada para a análise epidemiológica de *Salmonella* sp. foi a MLEE [79,80,81]. Esta técnica é baseada na identificação de proteínas variáveis, devido à substituição de aminoácidos, visualizadas em eletroforese em gel. Na MLEE, as variações observadas na motilidade eletroforética correspondem às variações no *locus* que codifica para a enzima. Se forem utilizadas enzimas metabólicas, não submetidas a pressões seletivas, podem ser detectadas variações neutras que definem linhas clonais relativamente estáveis na população analisada [98]. A MLEE é uma técnica de difícil padronização entre laboratórios e não representa um método de subtipificação eficiente para utilização na rotina [82]. Desta forma, os métodos baseados na análise de DNA, especialmente os automatizados, são mais rápidos e apresentam resultados mais acurados do que a MLEE [33].

A técnica da MLST, que utiliza a amplificação por PCR de vários genes constitutivos, que codificam proteínas necessárias para funções celulares básicas, tem sido utilizada para determinar diversidade genética entre isolados, podendo ser empregada em estudos epidemiológicos. Esta técnica foi desenvolvida para tipificação de várias bactérias, tais como *Streptococcus pneumoniae* [83], *Staphylococcus aureus* [84], *Listeria monocytogenes*[85], *Haemophilus influenzae*[86] e *Salmonella* [49,50]. O alto poder discriminatório e a facilidade no compartilhamento de dados entre diferentes laboratórios, inclusive utilizando a *Web*[53], são atributos importantes para que esta técnica possa ser considerada uma ferramenta adequada para análise epidemiológica global [33,87,88]. O lento acúmulo de variação dentro dos *loci* dos genes constitutivos fornece informações fidedignas sobre as relações evolutivas dos isolados, mas também pode ser um limitador do método, impedindo a diferenciação de linhagens e isolados amplamente relacionados [89,50], destacando a importância da correta escolha dos genes alvo a serem utilizados neste método. Uma maneira de contornar a dificuldade de discriminação entre isolados de espécies com grande homogeneidade genética é a inclusão de genes com maior variabilidade, tais como aqueles que codificam para fatores de virulência [52,90]. Além disso, outra limitação do método pode ser a dificuldade em amplificar fragmentos dos genes escolhidos para todos os isolados investigados [49,51], ou a amplificação de fragmentos oriundos de mais de uma cópia do gene, como

descrito para o *man*B [51], além da limitada capacidade para a discriminação intra-sorotipos de *Salmonella*, o que também é descrito para PFGE e para fagotipificação [51].

Vários autores têm destacado o uso da MLST para discriminação de linhagens e para tipificação de isolados de *Salmonella* [33,49,52,63,88], procurando refazer um histórico da evolução deste microrganismo. A análise filogenética de cinco sorovares clinicamente importantes de *Salmonella* através de MLST demonstrou que a maioria dos sorotipos desta bactéria representa linhagens monofiléticas [51]. A utilização da MLST empregando três genes alvos, o *fim*A, que é um gene de virulência, e dois genes constitutivos (*man*B e *mdh*), em vez dos seis genes constitutivos preconizados na metodologia original [51], resultou em 56 sequências tipo (ST) para isolados oriundos de humanos e 6 ST para isolados bovinos, além de 10 ST comuns a humanos e bovinos[52]. Desta maneira, foi obtido um poder discriminatório semelhante ao esquema usando 6 genes constitutivos, mas com a redução do custo do procedimento [52].

Diversos trabalhos vêm sendo realizados com o objetivo de comparar a MLST com a PFGE, apresentando resultados contrastantes. A MLST foi descrita com menor poder discriminatório do que a PFGE para diferentes sorovares de *Salmonella* e para isolados de *S*. Typhimurium [50,91], bem como existiram relatos afirmando o contrário, onde a MLST proporcionou a inferência de relações genéticas entre vários sorotipos de *Salmonella* [49,52].

A caracterização por PFGE, MLST e AFLP de 110 isolados de diferentes sorotipos de *S. enterica* subespécie *enterica* oiundos de humanos e de origem veterinária mostrou PFGE e AFLP com poder discriminatório semelhante, e as duas técnicas com habilidade discriminatória maior que a MLST [91]. A combinação das técnicas de MLST e FAFLP para caracterização de isolados de *S.*Enteritidis não foi relatada até o presente momento.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Avaliar a diversidade genética de isolados de *Salmonella* Enteritidis através das técnicas de FAFLP e MLST, procurando estabelecer métodos que possam permitir o entendimento da dinâmica populacional e auxiliem nos estudos de levantamento epidemiológico das doenças causadas por este microrganismo.

1.2.2. Objetivos Específicos

1- Avaliar a capacidade do FAFLP em tipificar isolados de *S*. Enteritidis oriundos de carcaças de frango, aves, suínos, alimentos e humanos envolvidos em surtos de salmonelose.

2- Avaliar a capacidade do MLST em tipificar isolados de *S*. Enteritidis oriundos de carcaças de frango, aves, suínos, alimentos e humanos envolvidos em surtos de salmonelose.

3- Comparar o poder discriminatório das técnicas de FAFLP e MLST para a tipificação de isolados de *S*. Enteritidis.

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2. Capítulo 2 ARTIGO CIENTÍFICO 1

Differentiation of *Salmonella* Enteritidis Isolates by FAFLP

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Differentiation of *Salmonella* Enteritidis Isolates by FAFLP

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Running title: Typing of *Salmonella* Enteritidis by FAFLP

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Abstract

Salmonella Enteritidis is responsible for human gastroenteritis outbreaks worldwide and the molecular characterization of isolates is an important tool for epidemiological studies. Fluorescent amplified fragment length polymorphism (FAFLP) analysis was performed on thirty-two *S*. Enteritidis strains from South Brazil isolated from human, foods, swine, broiler carcasses and other poultry-related samples to subtype isolates. Five strains of *S*. Enteritidis from different geographical regions and four isolates of different *Salmonella* serovars were also tested. Among the 41 isolates tested, 96 polymorphic AFs were obtained, producing 40 distinct profiles with a Simpson's index of diversity of 0.99. Nine FAFLP clusters could be inferred based in Dice similarity coefficient. FAFLP clustering readily identified different serotypes of *Salmonella*, but did not distinguish isolates non-related epidemiologically or distinct phage types. Therefore, these results indicate that FAFLP is a rapid method for epidemiological investigations of *Salmonella* outbreaks, presenting a high discriminatory power and providing an efficient differentiation of distinct serotypes of *Salmonella*.

Key words: FAFLP, *Samonella* Enteritidis, molecular subtyping, genetic diversity.

Introduction

Salmonella is a pathogen that may infect a wide range of animal species and is able to survive in water, soil and food for extended periods of time (Velge *et al.*, 2005; Crum-Cianflone, 2008). *Salmonella* spp transmission to humans commonly occurs through the food chain, mainly by the consumption of contaminated eggs and poultry meat as well as foods that contain raw eggs and/or contaminated poultry products (Tavechio *et al.*, 1996). Infections by non-typhoid *Salmonella* serovars usually are responsible for gastroenteritis, but these bacteria can also spread beyond the intestine and cause systemic infections (Hensel, 2004; Crum-Cianflone, 2008). *Salmonella* Enteritidis is a non-typhoid serovar and can colonize both humans and chicken. This serovar have been the main cause of human gastroenteritis outbreaks worldwide (Rodrigue *et al.*, 1990; CDC, 2008) and, therefore, is responsible for significant economical losses derived from food waste and health treatments (Sockett, 1991; Tavechio *et al.*, 1996; Mead *et al.*, 1999).

For outbreak investigation and epidemiological surveillance, a correct identification and characterization of the causal bacteria is essential. Serotyping and phage typing are classical phenotypic typing methods used to differentiate isolates of *Salmonella* (Ward *et al.*, 1987, Olsen *et al.*, 1994), but these methods alone are many times not enough for an accurate identification (Liebana *et al.*, 2004). Thus, molecular characterization becomes an important tool for epidemiological studies. However, the investigation of food-borne outbreaks caused by *S.* Enteritidis by molecular methods have been proven difficult, as this serovar displays great genetic homogeneity and the techniques most used for subtyping *S.* Enteritidis shows limited discriminatory power (Boxrud *et al.*, 2007). Therefore, it is crucial that a higher discriminatory molecular typing

technique be developed in a way to differentiate *S.* Enteritidis strains (Foley *et al.*, 2007), and the use of multiple methods often is required for an effective discrimination (Liebana *et al.*, 2001; Oliveira *et al.*, 2007).

Several molecular epidemiological techniques, such as PFGE (Ridley *et al.*, 1998; Fernandes *et al.*, 2003), plasmid profiling (Olsen *et al.*, 1994; Miljkovic-Selimovic *et al.*, 2008; Kalender *et al.*, 2009), RAPD (Betancor *et al.*, 2004), AFLP (Desai *et al.*, 2001; Scott *et al.*, 2001;) and MLST (Kotetishvili *et al.*, 2002; Fakhr *et al.*, 2005), have been performed together with phenotypic techniques in an attempt to improve the reproducibility and discriminatory ability of *S.* Enteritidis genetic typing. The implementation of these techniques has led to an enhanced surveillance of outbreaks worldwide (Winocur *et al.*, 2003; Sukhnanand *et al.*, 2005).

Fluorescent amplified-fragment length polymorphism (FAFLP) is an accurate, rapid and reproducible method based in fragmentation of genomic DNA with restriction endonucleases, followed by ligation of adapters and latter amplification with selective or non-selective fluorescent labeled primers (Scott *et al.*, 2001). The fragments generated by a large number of isolates can be analyzed directly by automated sequencing in a short period of time (Desai *et al.*, 2001; Foley *et al.*, 2009). In this sense, the aim of this study was to characterize *S.* Enteritidis isolates from different sources by FAFLP in order to evaluate its discriminatory potential to this genetically homogeneous serovar.

Materials and Methods

Bacterial strains and culture conditions

Thirty-two *S.* Enteritidis strains isolated from human, foods, pigs, broiler carcasses and poultry-related samples collected over the period 1995-2001 were

evaluated by FAFLP. These strains were isolated from the Rio Grande do Sul and Santa Catarina States, Brazil, and fagotyped in preview studies (Santos *et al.*, 2003; Oliveira *et al.*, 2005). Five epidemiologically unrelated *S.* Enteritidis strains isolated from other countries and four isolates of other serovars (*S.* Senftenberg, *S.* Typhimurium, *S.* Panama and *Salmonella* [4,5:-:1,2]) were also included (Table 1). Isolates were grown overnight in TSB (Trypticase Soy Broth) at 37℃.

DNA extraction

An 1 mL aliquot of cultures in TSB were centrifuged at 12,000 *g* for 5 min. Cells were washed twice in 1 mL of 1 M NaCl and pelleted by centrifugation at 12000 *g* for 3 min. Cells were resuspended in 100 µL of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Genomic DNA was extracted according to the method described by Rademaker and de Bruijn (1997). Briefly, bacterial cells were lysed with 500 µL of 5 M guanidine thiocyanate, 0.03 M N-lauryl sarkosine and 0.1 M EDTA for 5 min at 4ºC. After, 250 µL of cold 7.5 M ammonium acetate were added; tubes were gently shaken and incubated for 5 min at 4ºC. An aliquot of 500 µL of chloroform/iso-amyl-alcohol (24:1) was added and the mixture was vortexed vigorously. After centrifugation at 16,000 *g* for 10 min, the DNA-containing pellet was further washed with isopropyl alcohol. The DNA samples were stored at - 20ºC. The DNA quantification was performed in a spectrophotometer.

FAFLP typing

FAFLP was performed using approximately 400 ng of genomic DNA from each isolate and simultaneously digested with 2.5 U of *Eco*RI and *Mse*I restriction endonucleases (Invitrogen), in a total volume of 25 µL. The reaction mixtures were incubated at 37°C for 2 h and then at 70°C for 15 min to heat inactivate the enzymes. Restriction fragments were ligated to double stranded adapters in a reaction containing 5 ρmol of *Eco*RI adapter and 50 ρmol of *Mse*I adapter, 1 U T4 DNA ligase and 0.2 µL of 10X T4 ligase DNA buffer (Fermentas Life Sciences). The reaction mixture was incubated at 12°C for 17 h and heated at 65°C for 10 min to inactive the ligase (Desai et al., 1998).

The forward primer, a non-selective *Eco*RI primer (5'GACTGCGTACCAATTC3'), was labeled with a 6-carboxyfluorescein. The reverse primer, a non-labeled *Mse*I primer, had an extra selective base C at the 3' (5'GATGAGTCCTGAGTAA<u>C</u>3'). Primers and adapters were supplied by Integrated DNA Technologies Inc.

Amplification reactions were performed in a volume of 25 µL, containing 1 µL of ligated DNA, 20 µM of 6-FAM labeled *Eco*RI primer, 100 µM of *Mse*I primer, 0.2 U *Taq* DNA polymerase (Photoneutria Biotecnologia e Serviços), 2.5 µL of 10X PCR buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl), 1.5 mM $MgCl_2$ and 0.2 mM of desoxinucleotides (GE Healthcare, USA). Touchdown PCR was performed as follows: initial denaturation at 94°C for 2 min, followed by 9 cycles of denaturation at 94°C for 20 s, annealing at 66°C for 30 s and extension at 72°C for 2 min. The annealing temperature for the first cycle was 66°C and for the next eight cycles the temperature was decrease by 1°C at each cycle. The remaining 20 cycles were performed using a denaturation at 94°C for 20 s,

annealing at 56℃ for 30 s and extension at 72℃ for 2 min with a final extension at 60℃ for 30 min. Amplifications were carried out in a Thermocycler (MiniCyclerTM, MJ Research). FAFLP products were separated on a MegaBace 1000 automatic sequencer (GE Healthcare, USA). Each FAFLP reaction mixture was loaded with an internal size marker ET-550R (GE Helthcare) and the electrophoresis injection conditions were 3 Kv at 150 s and 10 Kv for 75 min. The amplified fragments (AFs) detected by the MegaBACE™ Fragment Profiler software were screened for detection of polymorphisms, verifying the presence or absence of fragments from 90 to 510 bp.

Data analysis

Data were scored in a binary matrix format in Microsoft Excel (Microsoft Inc.) and exported to SPSS program (PASW Statistics 17) for the analysis of the similarity index between pairs of isolates. Cluster analysis was performed using neighbor-joining method based in Dice coefficient of similarity from which a dendrogram was generated. The isolates with > 89.8% of similarity were grouped in same cluster. The discriminatory power was measured by the Simpson's index of diversity (*D*) (Hunter, 1990).

Results

The FAFLP analysis of 37 *S.* Enteritidis strains and four strains of other *Salmonella* serovars consisted of 147 AFs in the size range of 61 bp to 510 bp. Only fragments with sizes between 90 and 510 bp were included in the final analysis based on a minimal sizing accuracy estimation of approximately 0.5 bp. The number of AF differences between individual profiles ranged from 1 to 116. Nine FAFLP clusters were generated based in Dice similarity coefficient, which can be visualized in the neighbor-

joining dendrogram (Fig. 1 and Table 1). Among the 40 profiles obtained, 96 AFs were shown to be polymorphic, resulting in a Simpson's index of diversity of 0.99.

Cluster A was formed by the *S*. Enteritidis ATCC 13076 and 18 strains isolated from human, food, swine, broiler carcasses and poultry-related samples, and which belonged to phage types PT4, PT4a, PT6, PT6a, PT7, PT9 and PT11. Cluster B included 11 isolates originated from poultry, human, food, broiler carcasses and swine, with corresponding phage types PT4, PT4a, and one isolate of PT6a.

The strains 879 (PT4a) and 9 (PT4) isolated from broiler carcasses and poultry, respectively, shared one FAFLP cluster (cluster C) with 19 different AFs.

The outgroup, constituted by four isolates of distinct serovars, was discriminated by FAFLP. The *S*. Typhimurium and *Salmonella* [4,5:-:1,2] isolates were grouped in cluster F, differing from each other by one AF (137 bp). *S*. Panama (cluster D) and *S*. Senftenberg (cluster E) were discriminated from the other isolates and formed single clusters.

Three strains of *S*. Enteritidis from different geographic locations (Albania, Tanzania and Italy) were clustered together (cluster A). Isolate 1978 from Egypt constituted cluster B and isolate 1610 from Zimbabwe formed a group with the isolate 883 (cluster I).

Isolates obtained from poultry (20) and swine (23SB) formed cluster G, presenting 92.8% of similarity and 12 divergent AFs. The two isolates of cluster H (674 and 715) from food and human, belongs to phage type 4a and presented 91.2% of similarity, differing for 15 AFs one from each other. These two clusters showed around 88% similarity with each other.

Discussion

FAFLP is based on the detection of genomic restriction fragments amplified by PCR and has been shown to be efficient for the discrimination of subspecies of *Salmonella* (Scott *et al*., 2002) and between *Salmonella* serovars (Aarts *et al*., 1998; Lindstedt *et al*., 2000). This method was used in this study for the genotypic characterization of 37 isolates of *S*. Enteritidis from different sources, as well as four other *Salmonella* serovar isolates, revealing nine clusters and an index of discrimination of 0.99. These results were in agreement to those found by Desai *et al*. (2001) using the same primers and adapters, which obtained a discriminatory index of 0.98 to subtype *S*. Enteritidis PT4. These authors found association between the FAFLP analysis and epidemiological data. Conversely, our data shows that probably non-related isolates from different geographical areas (Albania, Tanzania, Italy, Egypt and Zimbabwe) were grouped in a same cluster, on the contrary that the epidemiological data could suggest. Indeed, clusters A and B harbor most isolates tested (43.9% and 26.8%, respectively), grouping together isolates obtained from different sources and displaying various phage types, including type strain ATCC 13076. This result could be compared with data from Torpdahl *et al*. (2005), which using three different molecular techniques (PFGE, MLST and AFLP) did not find significant diversity on isolates of *S. enterica* from human and veterinary sources, as well as with Lawson *et al.* (2004) that obtained 93% of outbreak associated isolates and 82% of sporadic isolates of *S*. Typhimurium in a same FAFLP profile. So, as *S*. Enteritidis displays great genetic similarity (Olsen *et al*., 1994; Saeed *et al*., 2006; Boxrud *et al*., 2007), little discrimination between isolates could be expected, since the same isolates used in this study, when analyzed by rep-PCR or RAPD,

showed high genetic homogeneity and low discriminatory power (Oliveira *et al.*, 2007; Santos *et al.*, 2008). However, the FAFLP results presented in this work showed a higher discrimination index than the other techniques, showing unique profiles for almost all isolates.

Although FAFLP clustering was not able to discriminate different phage types, it was already described that FAFLP was able to discriminate 25 PT reference strains of *S.* Enteritidis into distinct profiles, being six phage types also analyzed in this study (Scott *et al.*, 2001). Therefore, as phage typing still corresponds to an important tool for epidemiological typing of *S.* Enteritidis, its association with molecular methods may improve greatly the discriminatory power between isolates of this serovar. Also, as isolates sharing the same phage type may present highly divergent FAFLP profiles, and, therefore present considerable genomic diversity, it should be taken with caution clustering analyses based entirely on single phenotypic markers.

This study did not intend to subtype different *Salmonella* serovars by FAFLP, but four different serovars were included as outgroup. Among them, *S.* Senftenberg and *S.* Panama were grouped in distinct clusters, E and D, respectively. *S.* Typhimurium and *Salmonella* [4,5:-:1,2] isolates showed 99.5 % similarity, and were grouped in cluster F. Possibly, *Salmonella* [4,5:-:1,2] can be a monophasic variant originated from serovar Typhimurium (4,5,12:i:1,2), similar to what was suggested by others studies using FAFLP and other typing methods in relation to *Salmonella* [4,5,12:i:-] (Guerra *et al.*, 2000; de la Torre *et al.*, 2003; Alcaine *et al.*, 2006; Soyer *et al.*, 2009).

The context described here indicates that besides FAFLP presents a high discriminatory power, this method also shows to be less laborious and to possess a higher resolving power than PFGE (Desai *et al.*, 2001; Scott *et al.*, 2002), considered to

be the gold standard for use in epidemiological studies of *Salmonella*. Furthermore, FAFLP is easily reproducible (Torpdahl *et al*., 2004).

Conclusion

We conclude that FAFLP is a rapid method for epidemiological investigations of *Salmonella* outbreaks, presenting a high discriminatory power and providing efficient discrimination of different serotypes of *Salmonella*, although it was not able to distinguish in the clustering analysis putative epidemiologically non-related isolates.

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Table 1. SOURCE, PHAGE TYPE AND FAFLP PATTERN OF *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS STRAINS STUDIED

Isolate nº	*Source*	*Phage type*	*FAFLP pattern*
890	Broiler carcasses	4a	A
883	Broiler carcasses	4	I
879	Broiler carcasses	4a	C
362	Broiler carcasses	4a	A
192	Broiler carcasses	4	B
840	Broiler carcasses	4	A
2	Poultry	6a	A
24	Poultry	7	A
12	Poultry	4a	B
20	Poultry	4	G
9	Poultry	4	C
15	Poultry	4a	A
346	Food	6a	A
1561	Food	4	B
674	Food	4a	H
152	Food	4	B
355	Food	4a	B
1125	Food	4	A
1666	Human	4a	B
715	Human	4a	H
393	Human	4	A
1650	Human	4	B
1671	Human	4	A
720	Human	4a	A
1662	Human	4	B
30SL	Swine	ND[1]	B
23SB	Swine	7a	G
24SC	Swine	6	A
40SE	Swine	6a	A
42SE	Swine	6a	A
1SD	Swine	6a	B
2883	Tanzania	9	A
2090	Italy	11	A
2048	Albania	6	A
1610	Zimbabwe	4	I
1978	Egypt	4	B
ATCC 13076	-	ND	A
1S	*S.* Senftenberg	-	E
2S	*S.* Typhimurium	-	F
3S	*S.* Panama	-	D
4S	*Salmonella*.[4,5:-:1,2]	-	F

[1]Not determined

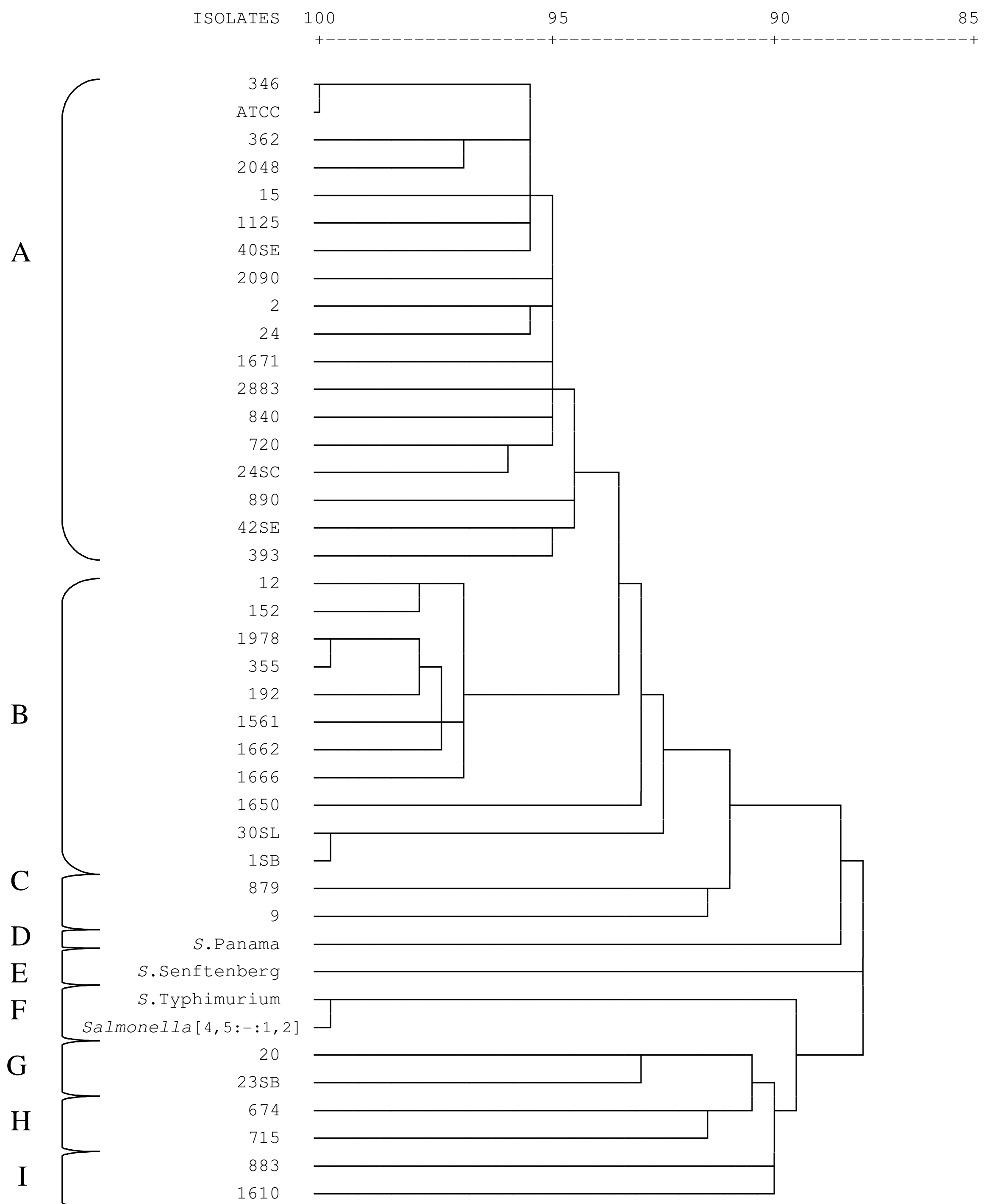

FIG.1. Dendrogram showing the genetic relationships between 37 isolates of *Salmonella* Enteritidis and 4 isolates of other *Salmonella* serovars. Similarity was determined by Dice coefficient and isolates clustered using neighbor-joining method on the basis of FAFLP profiles. Isolates exhibiting > 89.8% similarity were grouped in same cluster. The bar represents percentage of similarity.

3. Capítulo 3 ARTIGO CIENTÍFICO 2

Genetic diversity of *Salmonella* Enteritidis isolates evaluated by MLST (Multilocus Sequence Typing)

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Genetic diversity of *Salmonella* Enteritidis isolates evaluated by MLST (Multilocus Sequence Typing)

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Abstract

Salmonella Enteritidis is a common foodborne pathogen that causes gastroenteritis or systemic infections in humans, what turns its identification essential for epidemiological surveillance. A collection of thirty-two *S*. Enteritidis isolates obtained from human, foods, pigs, broiler carcasses and poultry-related samples from south Brazil and four isolates of other serovars and five isolates of other countries were characterized using a multilocus sequence typing (MLST) scheme based on the sequence of two housekeeping genes, *hem*D and *mdh*, in combination with two virulence genes, *ssa*Q and *sly*A. A total of 18 sequence types (STs) were identified between 41 isolates tested, showing that relationships between genotypic profile and place and/ or source of isolation and phage type could not to be established. The Simpson's index of diversity was 0.88. The results obtained showed that MSLT was efficient in separated isolates of *Salmonella* from distinct serovars, but not to distinguish isolates not-related epidemiologically. The MLST scheme used showed a high discriminatory power in the analysis of *S*. Enteritidis highly genetically similar isolates, suggesting its use as a useful tool for the epidemiological molecular characterization

Keywords: Multilocus sequence typing, *Salmonella,* genetic diversity, *ssa*Q, *sly*A, *mdh*, *hem*D

1 INTRODUCTION

Salmonellosis constitutes a global public health problem, as *Salmonella* species, although can reside as common commensals in the gastrointestinal tract of animals, are the cause of different diseases, including enterocolitis, bacteremia, enteric fever and focal infections (CDC, 2001; Crum-Cianflone, 2008). *Salmonella* Enteritidis represent the most frequent serovar causing human gastroenteritis worldwide (Rodrigue *et al*., 1990; CDC, 2009) and can be often transmitted through the food chain. Contaminated foods, including beef, pork, poultry and poultry products are frequent vehicles responsible for the transmission of these organisms to humans (Tavechio *et al*. 1996; de Jong and Ekdahl, 2006; Foley *et al*., 2006). Enterocolitis, the most common form of salmonellosis (CDC, 2001), cause large economical impacts due mainly to labor temporary incapability, medical care and even human death (Sockett, 1991, Mead *et al*., 1999).

Numerous typing methods have been developed to characterize isolates of *Salmonella* involved in outbreaks in order to improve the epidemiological surveillance. Usually, strains of *Salmonella* sp. are classified into serovars according to the Kauffmann–White scheme (Popoff *et al*., 2001). Methods based on phenotypic characterization, such as phage typing, biotyping and antimicrobial resistance traditionally have also been used for epidemiological studies (Ward *et al*., 1987, Olsen *et al*., 1994; Santos *et al*., 2003). However, these methods alone are not sufficient for an accurate typing of *S*. Enteritidis isolates (Liebana *et al*., 2004), since this serovar displays great genetic homogeneity (Stanley *et al*., 1992, Olsen *et al*., 1994). Therefore, the investigation of food-borne outbreaks can be difficult and alternative molecular typing techniques, such as PFGE (pulse-field gel electrophoresis) (Ridley *et al*., 1998;

Fernandes *et al*., 2003), plasmid profiling (Olsen *et al*., 1994; Miljkovic-Selimovic *et al*., 2008; Kalender *et al*., 2009), RAPD (Random Amplified Polymorphic DNA) (Betancor *et al*., 2004), AFLP (Amplified Fragment Length Polymorphism) (Desai *et al*., 2001; Scott *et al*., 2001;) and MLST (Multilocus Sequence Typing) (Kotetishvili *et al*., 2002; Fakhr *et al*., 2005) are required for an appropriate differentiation between isolates (Olsen *et al*., 1993; Bennasar, 2000; Foley *et al*., 2007). As the reproducibility and discriminatory ability of these methods can vary (Olsen *et al*., 1993), it would be appropriate to use an association of different methods for an effective discrimination (Liebana *et a*l., 2001; Oliveira *et al*. 2007) as well as for a better epidemiological investigation of outbreaks worldwide (Winokur, 2003, Sukhnanand *et al*., 2005).

MLST is a method based on the sequences of gene fragments from several housekeeping *loci* and has been used to subtype and explore the evolutionary relationships of several bacterial pathogens, such as *Neisseria meningitidis* (Maiden *et al*., 1998), *Streptococcus pneumoniae* (Desai et al., 1998), *Listeria monocytogenes* (Salcedo *et al*., 2003), *Escherichia coli* (Adiri *et al*., 2003) and *Salmonella* sp. (Kotetishvili *et al*, 2002; Torpdahl *et al*., 2005). The scarce variability found within housekeeping *loci* becomes global studies feasible and turns the information concerning evolutionary relationships more reliable, but it also limits the discrimination between very closely related strains or isolates (Cooper and Feil, 2004). Therefore, serovar-specific differences have been studied by a variation of this method, using virulence associated genes (presenting higher degrees of variability) together with housekeeping genes (Fakhr *et al*., 2005; Alcaine *et al*., 2006; Tankouo-Sandjong *et al*., 2007). Various studies have described the use of MLST for strain discrimination and typing of *Salmonella* sp.

isolates, with data that can be accurately shared between laboratories (Kotetishvili *et al.*, 2002; Winocur, 2003; Alcaine *et al.*, 2006; Harbottle *et al.*, 2006; Foley *et al.*, 2007).

The purpose of this study was to characterize isolates of *S.* Enteritidis from different sources by MLST, using housekeeping and virulence genes, aiming to obtain a higher discriminatory power than conventional schemes.

2 MATERIALS AND METHODS

2.1. Bacterial isolates and culture conditions

A total of forty-one *Salmonella* isolates, including thirty-two *Salmonella* Enteritidis isolated from the Rio Grande do Sul and Santa Catarina States (Brazil) collected over the period 1995-2001, four isolates of other serovars (*S.* Senftenberg, *S.* Typhimurium, *S.* Panama and *Salmonella* [4,5:-:1,2]), and one outgroup formed by five *S.* Enteritidis strains isolated from other countries were included in this study (Table 1). The *S.* Enteritidis from Brazil were isolated from human, foods, swine, broiler carcasses and poultry-related samples and fagotyped in preview studies (Santos *et al.*, 2003; Oliveira *et al.*, 2005) (Table 1). All isolates were grown overnight in TSB (Trypticase Soy Broth) at 37℃ and an aliquot of each was stored in TSB containing 15% glycerol at -80ºC.

2.2. DNA Extraction

After 1 mL of cultures had been centrifuged at 12,000 g for 5 min, washed twice with NaCl, the cells were resuspended in 100 µL of TE (10 mM Tris HCl pH 8.0,1 mM EDTA), and genomic DNA was extracted according to the method described by

Rademaker and de Bruijn (1997). Briefly, this extraction is based in initial bacterial lise with 500 µL of 5 M guanidine thiocyanate, 0.03 M N-lauroyl sarkosine and 0.1 M EDTA for 5 min at 4ºC. After incubation, it was added 250 µL of cold 7.5 M ammonium acetate, and the tubes were kept, after gently mixing, at 4ºC for 5 min. An aliquot of 500 µL of chloroform/iso-amyl-alcohol (24:1) was added, the mixture was vortexed vigorously and centrifuged at 9000 *g* for 10min. The 700 µL of upper phase containing DNA was transferring to tubes containing 378 µL of isopropyl alcohol. After centrifugation with ethanol, DNA pellet was resuspended in 200 µL of TE. The quantification and quality of DNA was determined in a UV spectrophotometer using A(260):A(280) absorbance ratios.

2.3 MLST

2.3.1 *Loci* and oligonucleotide primers

Four genes were chosen for the MLST scheme: two housekeeping and two virulence genes. The housekeeping genes used were *mdh* (malate dehydrogenase) and *hem*D (uroporphyrinogen III cosynthase), required for growth on nonfermentable carbon sources (Xu *et al*., 1992). The virulence genes used were *ssa*Q and *sly*A (salmolysin) chosen to intent to increase discrimination power this method. The *ssa*Q gene, belong to SPI2 (*Salmonella* Pathogenicity Island 2), is required for survival of *Salmonella* in macrophages (Hensel *et al*., 1997) and the *slyA* gene codify a transcriptional regulator of virulence genes required for survival within macrophages, but not resides in SPIs (Soto *et al*., 2006). Primers used are described in Table 2.

2.3.2 Polymerase Chain Reaction

PCR was performed in a 25 µL final volume containing 2.5 µL of 10X PCR buffer (10 mM Tris HCl pH 8.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide (Amerscham), 20 ρmoles of each primer (Integrated DNA Technologies, Inc and Invitrogen Life Technologies), 0.2 U *Taq* DNA polymerase (Invitrogen Life Technologies) and 1 µL genomic DNA template (20 ng).

The PCR conditions for *sly*A and *ssa*Q genes were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. For *hem*D, the amplification conditions were initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 1 min, with a final extension of 72°C for 10 min. For *mdh*, the cycles were the same used for *hem*D except for the annealing temperature at 61°C. Amplifications were carried out in a Thermocycler (MiniCycler™, MJ Research). Amplicons were separated on 0.8 % agarose gel electrophoresis at 5 V/cm. Gels were stained with ethidium bromide (0.5 µg/mL) and visualized on a UV transiluminator. Sizes of amplification products were determined by comparison with a concurrently run DNA molecular size marker (100-bp DNA ladder) (Fermentas, São Paulo, Brazil).

2.3.3 Sequencing

Amplicons were treated with an equal volume of PEG solution (20% PEG 8000 in 2.5 M NaCl) and washed twice: first with 125 µL of 80% ethanol followed by 125 µL of absolute ethanol. After incubation at 37°C, the DNA fragments were ressuspended in 12

µL Milli-Q water and quantified in 0.8% agarose gel electrophoresis. The PCR products were then submitted to automated sequencing with either the forward PCR primer in MegaBace 1000 (GE Healthcare, USA) or in an ABI 3130 XL Genetic Analyzer (Applied Biosystems).

2.3.4 Data analysis

Sequences were analyzed with Chromas software and fragments were aligned using Molecular Evolution Genetics Analysis software (MEGA, version 2.1). Dendrograms for each of the four genes were construct with MEGA using 1000 bootstrap replicates and Sequence Type Analysis and Recombinational Tests (START 1.0.5) program (http://pubmlst.org/software/analysis/start) were used to determine proportion of polymorphic sites, number of alleles and to construct an unweighted pair group method with arithmetic (UPGMA) phylogenetic tree with all genes tested (Jolley *et al.*, 2001). Sequence types (ST) were determined from a concatenated code of alleles assignment for individual genes and ST numbers were assigned using arbitrary numbers. The discriminatory power was measured by the Simpson's index of diversity (*D*) (Hunter, 1990). All gene sequences were subjected to a BLAST search in Gene Bank and *hem*D sequences were compared with sequences deposited in MLST website database (http://mlst.ucc.ie) to verify allele types.

3 RESULTS AND DISCUSSION

The characterization of isolates of genetically homogenous species normally needs the use of several phenotypic and genotypic typing methods. MLST was chosen as a genotypic typing method in this work due to its ability to differentiate serotypes and provide information about the genetic relationship between isolates (Sukhnanand *et al.* 2005). On the other hand, the low level of nucleotide variation obtained with classical MLST schemes, based in housekeeping *loci*, limits the discriminatory power of highly homogenous isolates (Cooper and Feil, 2004), what justifies the inclusion of genes that present higher levels of genetic variability, such as virulence genes (Tankouo-Sandjong *et al.*, 2007).

A total of 37 *Salmonella* Enteritidis isolates from human, foods, pigs, broiler carcasses and poultry-related samples, as well as five *S*. Enteritidis isolates from other geographical areas and four isolated from other *Salmonella* serovars were characterized. The MLST scheme developed in this study included a partial DNA sequence of two housekeeping genes (*hem*D and *mdh*) already used as targets for MLST to subtype *Salmonella* sp. (Kidgell *et al.* 2002, Alcaine *et al.*, 2006), and two virulence genes (*sly*A and *ssa*Q), whose proteins are involved in the bacterial survival mechanisms within macrophages.

The total length of sequences obtained was 1709 bp, with 459, 457, 325 and 468 bp for *hem*D, *mdh*, *sly*A and *ssa*Q, respectively (Table 2). The target genes presented four to twelve allelic types. The number of polymorphic sites for each gene ranged from 7 to 12 (1.5 to 3.7%) with the *ssa*Q gene displaying the highest variability (3.7%), but showing only five allelic types. *mdh* and *hem*D genes demonstrated less variable

sequences than the virulence genes, showing indexes of 1.5% and 1.7%, respectively (Table 2). Although *hem*D and *mdh* are widely accepted as reliable gene markers in MLST schemes, mainly for highly similar isolates, as they show higher variability levels than other housekeeping genes (Harbottle *et al*., 2006; Alcaine *et al*., 2006), the results presented here shows that the inclusion of the virulence genes *ssa*Q and *sly*A led to a higher discriminatory power, specially *sly*A, which data grouped the isolates in 12 different clusters (data not shown). The *mdh* and *hem*D sequences obtained in other MLST schemes presented higher indexes of polymorphic sites (Sukhnanand *et al*., 2005; Torpdahl *et al*., 2005; Alcaine *et al*., 2006; Harbottle *et al*., 2006), however these studies were performed using isolates from different *Salmonella* serovars, which may explain the presence of a higher polymorphism. Therefore, the inclusion of virulence genes in the MLST scheme improved its discriminatory power, as already described in other studies using other virulence genes, such as *fli*C (Tankouo-Sandjong *et al.* 2007) and *fim*A (Alcaine *et al*, 2006). However, Fakhr *et al*. (2005), the use in a MLST scheme of the housekeeping genes *man*B, *pdu*F and *gln*A with the virulence gene *spa*M, found 100% nucleotide identity between 85 *S*. Typhimurium isolates. These results corroborates the importance of the identification of genes with adequate sequence variation indexes in MLST schemes, in order to elucidate the relationship between isolates that present a high genetic similarity, like *S*. Enteritidis, specially those not recognized by serotyping.

A simple MLST scheme has also been described using genes *fim*A, *man*B and *mdh* and used to genetically type 41 *Salmonella* isolates of various serotypes, allowed discrimination of 25 sequence types among 66 isolates and showed good correlation between STs and serotypes tested (Sukhnanand *et al*., 2005).

The comparison of the *hem*D sequences obtained in this study with allele types describe on the *S. enterica* MLST website database (http://mlst.ucc.ie) showed high similarity between the sequences of 35 isolates with hemD91, *S*. Panama isolate with *hem*D 25, and the another 5 isolates with hemD12, including *Salmonella* [4,5:-:1,2], *S*. Senftenberg and *S*. Typhimurium isolates.

The use of MLST for *Salmonella* has revealed difficulties in the amplification of gene targets from isolates investigated (Kotetishvili *et al*., 2002). Another challenge for some MLST schemes is the occurrence of more than one allelic type for the same gene, as the two distinct *man*B alleles within three avian *S*. Montevideo isolates (Sukhnanand *et al*., 2005) or the two copies of *man*B found in 33 isolates of a total 335 isolates from bovine and humans (Alcaine *et al*., 2006). However, in this study all amplifications were successful and neither of the genes analyzed displayed any evidence of multiple genomic copies.

A dendrogram was constructed by UPGMA using the concatenated sequences of all four genes with the START program displaying the clustering of 18 MLST Sequence Types (STs) between the 41 isolates tested (Fig. 1). The overall discriminatory abilities for MLST, determinated by Simpson's index of diversity test (Hunter ,1990), was 0.88, what may be considered a high value when compared with the 0.61 obtained with MLST in *S*. Newport isolates (Harbottle *et al*., 2006) and 0.92 encountered for a collection of 355 clinical isolates of *Salmonella* sp. (Alcaine *et al*., 2006). Among the clusters, ST 1 (cluster A) harbored 29.3% of isolates from poultry, broiler carcasses, food, swine, human and the *S*. Enteritidis ATCC 13076, which belongs to PT4, PT4a, PT6 and PT6a phage types. Another clusters, named C and F, showed STs with 5 and 6 isolates, respectively, from broiler carcasses, human, swine and poultry. Cluster I (ST 9)

presented three isolates, corresponding to the isolate from Tanzania (PT6) and two isolates from poultry and broiler carcasses (PT4). The other clusters presented one or two isolate sequences (Fig. 1). These results did not show any direct association between genotypic profiles, place of isolation/source of infection and phage type. Indeed, the putative epidemiologically non-related isolates from other geographical areas formed independent clusters with isolates of *S*. Enteritidis from different sources, further indicating that the epidemiological data did not reflect genetic divergence. Therefore, it may be assumed that different outbreaks, even in different geographical areas and from distinct sources, can be caused by bacteria of a common origin. Similar results were obtained in the typing of the same isolates by FAFLP (Kober *et al*., 2010, submitted). This context indicates that assumptions based on epidemiological data and/or on single phenotypic markers alone should be analyzed with caution.

All *S*. Enteritidis isolates showed a high divergence comparing to the outgroup, with the exceptions of isolates 1SB and 30SL, which grouped more closely to *Salmonella* [4,5:-:1,2]. *S*. Senftenberg, *S*. Typhimurium and *S*. Panama formed distinct, but related, clusters. These results denote the efficiency of MLST to differentiate isolates of different *Salmonella* serotypes, corroborating previous published data (Sukhnanand *et al*., 2005; Torpdahl *et al*., 2005). However, the characterization of intra-serovars has been shown that it may become a challenge, as the *S*. Enteritidis isolates tested in this study were previously typed for rep-PCR, presenting a low discriminatory power (Oliveira *et al*., 2007). Although MLST showed to possess a higher discriminatory power as compared with rep-PCR, this method was not as discriminatory as FAFLP (Kober *et al*., 2010, submitted). In this sense, Torpdahl *et al*. (2005) suggested that MLST is more suitable to analyse evolutionary relationships on a more global scale and that FAFLP

could be a possible tool in local outbreak investigation. However, MLST is easiest to interpret and to compare between laboratories (Maiden, 2006; Harbottle *et al*., 2006), providing phylogenetic-relationship inferences and no ambiguous data that are easily reproducible.

In conclusion, our results indicate that MLST present a high discriminatory power in the analysis of *S*. Enteritidis highly genetically similar isolates, suggesting its use as a useful tool for the epidemiological molecular characterization.

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Table 1 Source, phage type and characterization of *Salmonella* Enteritidis isolates by FAFLP and MLST.

Isolate nº	*Source*	*Phage type*	*FAFLP cluster[1]*	*MLST cluster*
890	Broiler carcasses	4a	A	A
883	Broiler carcasses	4	I	I
879	Broiler carcasses	4a	C	D
362	Broiler carcasses	4a	A	A
192	Broiler carcasses	4	B	A
840	Broiler carcasses	4	A	C
2	Poultry	6a	A	A
24	Poultry	7	A	E
12	Poultry	4a	B	A
20	Poultry	4	G	I
9	Poultry	4	C	A
15	Poultry	4a	A	F
346	Food	6a	A	A
1561	Food	4	B	A
674	Food	4a	H	C
152	Food	4	B	K
355	Food	4a	B	J
1125	Food	4	A	C
1666	Human	4a	B	F
715	Human	4a	H	A
393	Human	4	A	F
1650	Human	4	B	F
1671	Human	4	A	L
720	Human	4a	A	A
1662	Human	4	B	G
30SL	Swine	ND[2]	B	O
23SB	Swine	7a	G	H
24SC	Swine	6	A	F
40SE	Swine	6a	A	A
42SE	Swine	6a	A	C
1SB	Swine	6a	B	N
2883	Tanzania	9	A	I
2090	Italy	11	A	C
2048	Albania	6	A	F
1610	Zimbabwe	4	I	H
1978	Egypt	4	B	B
ATCC 13076	-	ND	A	A
1S	*S.* Senftenberg	-	E	Q
2S	*S.* Typhimurium	-	F	P
3S	*S.* Panama	-	D	R
4S	*Salmonella.* [4,5:-:1,2]	-	F	M

[1]Kober et al. (2010), submitted for publication.
[2]Not determined

Table 2 Primers used for the amplification of target genes *hem*D, *mdh*, *ssa*Q and *sly*A, size of amplicon, number of alleles and number of polymorphic sites detected for each gene.

Target gene	Primer sequences (5'→3')	Size (bp)	N°. of alleles	Nº. (percentage) of polymorphic sites	Reference
*hem*D	F: GTG GCC TGG AGT TTT CCA CT R: GAC CAA TAG CCG ACA GCG TAG	459	4	8 (1,7)	Kidgell 2002
mdh	F: TAT CCA GCA TAG CGT CCA GC R: GAT GAA AGT CGC AGT CCT CG	457	7	7 (1,5)	Alcaine, 2006
*sly*A	F: GCC AAA ACT GAA GCT ACA GGT G R: CGG CAG GTC AGC GTG TCG TGC	468	12	11 (2,4)	Guerra 2000
*ssa*Q	F: GAA TAGCGA ATG AAG AGC GTC C R: CAT CGT GTT ATC CTG TGT CAG C	325	5	12 (3,7)	Soto 2006

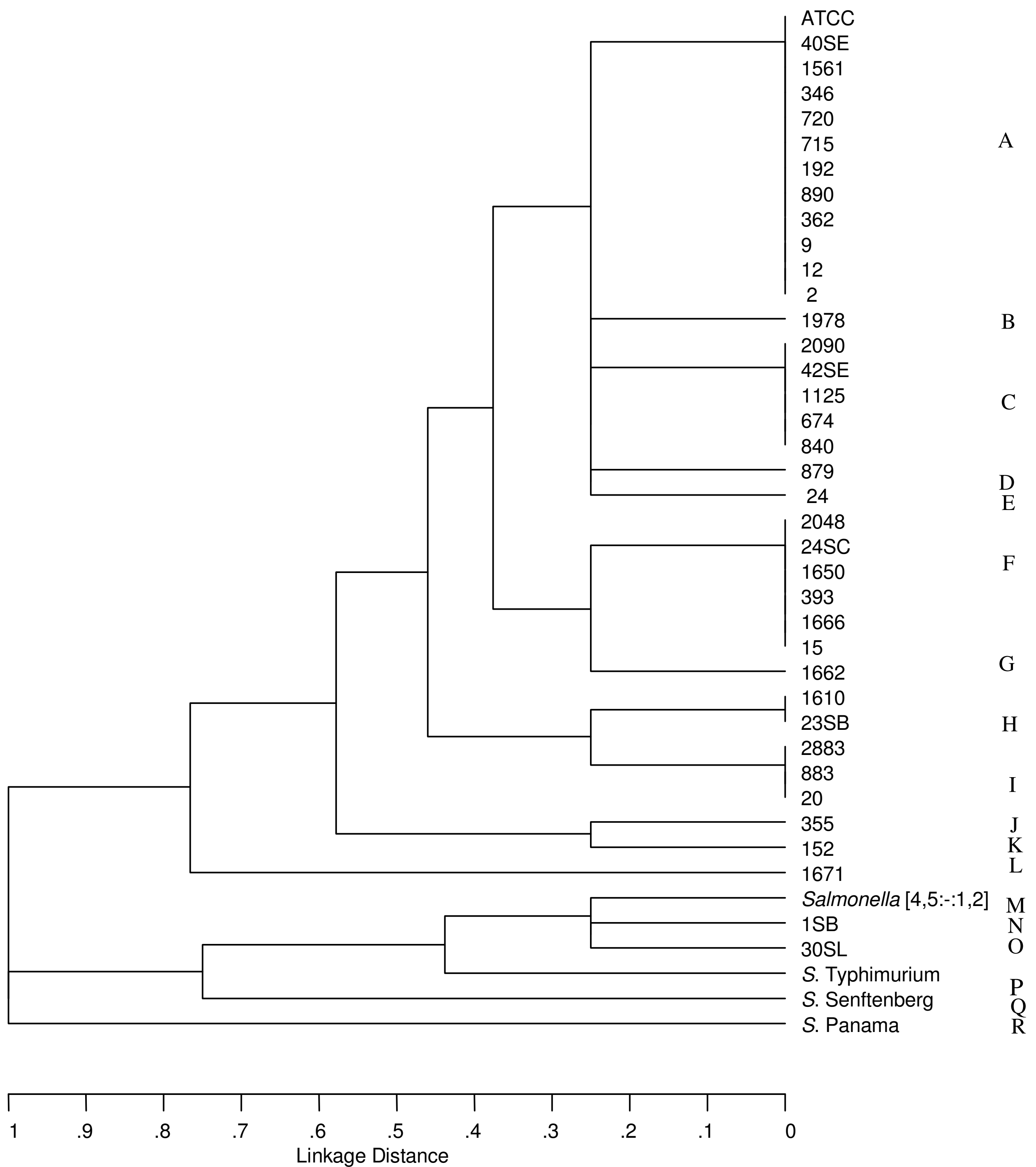

Fig. 1. Dendrogram shows the relationship of 37 *Salmonella* Enteritidis isolates and four other *Salmonella* serovars constructed by the unweighted pair-group method using with arithmetic averages (UPGMA) based on the concatenated sequences of four genes in MLST scheme. The bar represents linkage distance. The clusters are represented by letters.

4. Capitulo 4 CONSIDERAÇÕES FINAIS

A *Salmonella* Enteritidis é uma bactéria de distribuição global que representa um importante problema de saúde pública, sendo considerada uma das principais causas de gastrenterites [14,92,81] e responsável por perdas econômicas significativas com faltas ao trabalho e internações para tratamento dos infectados [93,94,14].

A correta identificação de isolados envolvidos em surtos e seu acompanhamento são essenciais para estudos epidemiológicos e torna-se bastante dificultada, uma vez que a *S*. Enteritidis apresenta grande homogeneidade genética [95,30,96, 58], sendo necessária a utilização de vários métodos fenotípicos e genotípicos de tipificação associados para uma melhor discriminação [46,97,61]. Neste contexto, este é o primeiro estudo utilizando as técnicas de FAFLP e MLST para o mesmo grupo de isolados de *S*. Enteritidis.

Dentre as técnicas genotípicas, PFGE tem sido o método de escolha, já que é de fácil reprodução e bem padronizado, para discriminar isolados de *S*. Enteritidis e tem demonstrado maior eficiência do que perfil plasmidial ou ribotipificação [37], contudo nem sempre consegue distinguir entre fagotipos [98,99] ou separar isolados não relacionados epidemiologicamente [100,101], além de ser um método demorado e bastante trabalhoso [33]. Outros métodos de tipificação, tais como FAFLP e MLST, podem ser utilizados com o objetivo de minimizar estas limitações. Os resultados obtidos neste trabalho mostraram que tanto a FAFLP como a MLST apresentaram um alto poder discriminatório para a diferenciação de *S*. Enteritidis, índices de diversidade de Simpson de 0,99 e 0,88, respectivamente, bem como capacidade para discriminar entre isolados de diferentes sorovares. A FAFLP tem sido utilizada para caracterização molecular de sorovares de *Salmonella* [69,70,48], mostrando maior poder de resolução do que PFGE, além de menos trabalhosa [48,73] e de fácil comparação entre laboratórios [82], podendo ser facilmente utilizada em estudos epidemiológicos na investigação de surtos causados por *S*. Enteritidis.

A MLST também tem sido usada para caracterização molecular de *Salmonella* [88,91,102] e, uma modificação deste método com a inclusão de genes de virulência, além dos genes constitutivos, com o objetivo do aumento do poder discriminatório e melhor

detecção de pequenas variações entre isolados, tem mostrado alta capacidade de diferenciação entre sorovares [90] e contribuído para redução de custos, uma vez que podem ser usados menos genes em um esquema de MLST [52]. Os resultados obtidos com a utilização da MLST mostraram que a inclusão dos genes de virulência *ssa*Q e *sly*A aumentou o poder discriminatório do esquema de MLST proposto. Entretanto, outros autores obtiveram variabilidade ainda maior utilizando os genes de virulência *fli*C e *fim*A, [52,90] em diferentes sorovares de *S. enterica*, o que pode ter favorecido este padrão de diferença.

Isolados de *S*. Enteritidis oriundos de quatro outros países, provavelmente epidemiologicamente não relacionados, incluídos neste estudo não foram discriminados dos demais isolados de S. Enteritidis por MLST nem por FAFLP. O uso de genes constitutivos pode não ser eficiente para distinguir entre isolados com variação recente ou linhagens com grande similaridade genética, ressaltando a importância deste método para estudos epidemiológicos mais globais e não na investigação local de surtos [75,89]. É importante também a escolha dos genes de virulência que farão parte do esquema do MLST, priorizando aqueles com maior capacidade de variação dentro do mesmo subtipo.

A MLST origina dados de mais fácil interpretação e reprodução por outros laboratórios [91]. Já o FAFLP é descrito como um método rápido, facilmente reprodutível, que necessita de pouca quantidade de DNA para amplificar fragmentos em diferentes locais no genoma [48,65]. Entretanto, esta combinação de FAFLP e MLST ainda não tinha sido relatada para investigação de isolados de *S*. Enteritidis até o presente estudo. Nossos resultados mostraram maior poder discriminatório para FAFLP, entretanto isolados de mesmo fagotipo e de mesma origem, não foram agrupados pelos dois métodos.

A correta caracterização de isolados envolvidos em surtos causados por *Salmonella* Enteritidis é de extrema importância para o acompanhamento e para desenvolvimento de estratégias para controle desta bactéria.

5. Conclusões

1- A FAFLP é um método com alto poder de discriminação para diferenciar *Salmonella* Enteritidis em estudos epidemiológicos
2- A FAFLP e a MLST foram eficientes na discriminação de diferentes sorovares, entretanto não foram capazes de agrupar isolados de mesmo fagotipo, nem distinguir aqueles provavelmente não relacionados epidemiologicamente;
3- A inclusão dos genes de virulência *sly*A e *ssa*Q no esquema de MLST aumentaram o poder discriminatório desta técnica para a tipificação de *S.* Enteritidis.

6. Anexo

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