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Potential Use of Molecular-Typing Methods for the Identification and Characterization of Salmonella Enterica Serotypes Isolated in the Poultry Production Chain

■Author(s)

Baratto CM
Gelinski JMLN
Bernardi AZ
Marafon A
Braun F

■Mail Adress

Correponding author: César Milton Baratto. Universidade do Oeste de Santa Catarina – Molecular Biology Laboratory - building K. 198, Paese Street. CEP.89560-000 Videira-SC-Brazil. E-mail: cesar.baratto@unoesc.edu.br
Phone number: +55 49 35334479 Fax: +55 49 35334422

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ABSTRACT

Salmonella is widespread in nature and can be found in all links of the poultry production chain. Due to its high impact on meat processing, techniques for the rapid detection and reproducible characterization of *Salmonella* serotypes in foods are needed. The present study investigated the potential of molecular profiling to identify and differentiate 15 *Salmonella* serotypes isolated from the poultry production chain, based on 5 primers by random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC-PCR), amplification of rDNA internal spacer analysis (RISA), and amplified ribosomal DNA restriction analysis (ARDRA) of 16S-23S rRNA internal spacer region (ISR) cleaved with Alu I and Hha I restriction enzymes. Three isolates of each serotype were analyzed for the identification of similar and different profiles. Dendrograms were constructed from molecular profiles using the UPGMA method (unweighted pair-group method for the arithmetic averages) and the software program WinBoot. The present study indicates the usefulness of RISA and ARDRA of the 16S-23S rRNA intergenic spacer region (ISR) for systematic, epidemiological, and diagnostic purposes. Since these techniques can be used for the differentiation of serotypes, they are highly promising for the characterization of *Salmonella* serotypes and intra-serotypes. Data indicate that these techniques may be used to produce more consistent, reliable, and reproducible results in the identification and epidemiological study (traceability) of *Salmonella* in the poultry industry.

INTRODUCTION

Salmonellosis is one of the most common infectious diseases in the world and affects both animals and humans. *Salmonella* infections may cause gastroenteritis, involving an abrupt onset of nausea, fever, vomiting, and diarrhea, with several virulence factors involved (Baumler *et al*., 1998; Schaechter *et al*., 2001). Some outbreaks affecting the population are caused by specific *Salmonella* serovars (serotypes), and the difficulty in detecting carriers makes these a potential source of contamination, particularly due to detection limitations of culture techniques, which are also time-consuming (Robinson *et al*., 1995; Aspinall *et al*., 1992).

Moreover, about 2,600 *Salmonella* serovars are known and new serovars, which may potentially be foodborne pathogens, have been discovered (Guibourdenche *et al*., 2010). This high number and wide diversity of serovars causes the nomenclature of the *Salmonella* genus, species and serovars to be very complex (Smith *et al*., 2011).

There is a high incidence of *S.* Enteritidis in broiler breeder (57.5%) and broiler flocks (84.0%), and it is the serovar most frequently responsible for foodborne outbreaks and sporadic cases of salmonellosis in humans (Kanashiro *et al*., 2005).

rev_v14_03_3_SA_638.indd 173 05/11/12 16:18

When dealing with the epidemiology of *Salmonella* infections, determining how humans acquire the infection is essential (Dhillon *et al*., 2001). Therefore, when monitoring the health quality of poultry meat used for human consumption, it is important to identify which *Salmonella* serotypes are present in the production chain. Aiming at developing a more robust assessment of intraspecific diversity within *Salmonella* species using genetic markers, some techniques based on PCR were devised (Tindall *et al*., 2005; Wang *et al*., 2009).

Among the methods used to identify *Salmonella* isolates are random amplified polymorphic DNA (RAPD) analysis (Betancor *et al*., 2004; Smith *et al*., 2011), which is based on the amplification of repetitive elements present in several copies in the chromosome; enterobacterial repetitive intergenic consensus (ERIC-PCR) (Rasschaert *et al*., 2005; Anderson *et al*., 2010); repetitive extragenic palindrome (REP-PCR) sequences (Merino *et al*., 2003; Woo & Lee, 2006); *Salmonella enteritidis* repetitive extragenic (SERE) sequences (Rajashekara *et al*., 1998); and BOX elements (Woo & Lee, 2006; Suh & Song, 2006). Previous studies have shown PCR fingerprinting techniques to be strain specific and highly useful in *Salmonella* strain typing (Woo & Lee, 2006; Suh & Song, 2006; Merino *et al*., 2003; Smith *et al*., 2011; Kumao *et al*., 2002).

Another PCR-based method that enables the study of the biodiversity of *Salmonella* isolates is amplification followed by product separation of the spacer between the 16S and 23S rRNA genes (intergenic spacer region - ISR) (Baudart *et al*., 2000; Luz *et al*., 1998; Lagatolla *et al*., 1996). This method, identified by the acronym RISA (rDNA internal spacer analysis), provides products with a highly variable size because of its hypervariable nature (García-Martínez *et al.*, 1999).

In addition, the amplification of this region followed by restriction digestion and analyses of its products is another potential use of ISR. This technique, called ARDRA (amplified ribosomal DNA restriction analysis), is easy, fast and accurate to identify and characterize *Lactobacillus* sp. and *Bradyrhizobium* sp. isolates (Moreira *et al*., 2005; Han *et al*., 2005; Mohania *et al*., 2008; Vinuesa *et al*., 1998). However, despite its great potential, ISR-ARDRA has not yet been used for the identification or characterization of *Salmonella* serotypes.

The purpose of the present study was to evaluate the usefulness and the potential application of different techniques based on PCR analysis (RAPD, ERIC, RISA and ARDRA) for the differentiation of 15 *Salmonella enterica* subsp. *enterica* serotypes isolated from the poultry meat production chain.

MATERIAL AND METHODS

Salmonella strains

The *Salmonella* isolates used in this study were obtained from poultry production chain environments, including hatcheries, broiler breeder farms, broiler farms and slaughterhouses located in the states of Santa Catarina and Rio Grande do Sul, between 2006 and 2010 (data not shown). *Salmonella* sp. were isolated and identified following the recommendations of Oliveira *et al*. (2006). The colonies suspected of *Salmonella* were collected for presumptive identification by biochemistry tests and the positive isolates were submitted to serologic tests using polyvalent serum against *Salmonella* O antigens. The positive isolates were submitted to reference official laboratories (Oswaldo Cruz Institute Foundation; FIOCRUZ, Rio de Janeiro, Brazil) for complete identification and serotyping. A total of 15 different serotypes of *Salmonella enterica* subsp. *enterica* were chosen for the study and isolated during that period, including *S*. Infantis, *S*. Tennessee, *S*. Bredeney, *S*. Schwarzengrund, *S*. Ohio, *S*. Montevideo, *S*. Newport, *S*. Sandiego, *S*. Panama, *S*. Hadar, *S*. Rissen, *S*. Anatum, *S*. Muenchen, *S*. Typhimurium and *S*. Saintpaul. Three isolates of each serotype were randomly selected and analyzed for the determination of common profiles.

DNA extraction and characterization by RAPD

For the molecular characterization of *Salmonella* serotypes, DNA extractions were performed using the extraction kit Whatman FTA® Classic Card (Whatman, USA), as specified by the manufacturer.

The RAPD profiles of *Salmonella* isolates were generated from five different primers: P1254 (5'-CCGCAGCCAA-3'), 784 (5'-GCGGAAATAG-3'), 23L (5'-CCGAAGCTGC-3'), OPA-4 (5'-AATCGGGCTG-3') and OPB-15 (5'-CCAGGGTGTT-3') (Woo & Lee, 2006; Malorny *et al*., 2001). The PCR mixture contained 2 mM $MgCl_2$, 0.25 mM dNTP, 2.5 U of *Taq* polymerase (Invitrogen), PCR buffer (Invitrogen), 50 ρmol of primer and DNA template. Amplification was performed as previously described with minor modifications (Betancor *et al*., 2004), using the following program: 4 cycles of 94°C for 4 min, 37°C for 4 min and 72°C for 4 min; then 35 cycles of 94°C for 30 s, 37°C for 1 min, and 72°C for 2 min, followed by final 10 min at 72°C

rev_v14_03_3_SA_638.indd 174 05/11/12 16:18

with PCR thermal cycler HBSP02110 (Thermo Electron Corp.). The PCR products (15 μl of each sample) were loaded on 1.5% agarose gel with 0.5 μg/ml of ethidium bromide in the electrophoresis tank, using 0.5 X TBE buffer, at 3 Vcm^{-1} for 2 hours. A DNA molecular weight marker, 100-bp DNA ladder (Ludwig Biotec, Brazil), was used as standard. Gels were observed under UV light and a digital image was captured (Photo Capt Software version 12.5 for Windows - Vilber Lourmat) for analysis. In order too confirm the reproducibility of the method, each experiment was repeated three times.

ERIC-PCR analysis

For the amplification of ERIC motifs, the PCR mixture and reaction programs were utilized according to similar conditions reported on previous studies (Suh & Song, 2006), using 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and 2R (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers. The gel and digital image were produced as previously described.

Molecular analysis by RISA and ARDRA methods

The amplification of the 16S-23S rRNA intergenic spacer region (ISR) was performed using a 50 µL mix (10X PCR buffer with $MgCl_2$ 1.5mM, 0.25mM dNTP, 2.5U of Taq polymerase (Invitrogen), 50 ρmol of each primer and DNA template) and the following universal primers: P1 (5'-TTGTACACACCGCCCGTCA-5') and P2 (5'-GGTACTTAGATGTTTCAGTTC-3') (Lagatolla *et al*., 1996). Samples were submitted to the following program: 35 cycles of 94ºC for 1 min, 53ºC for 1 min and 72ºC for 1 min and 30 sec, and the reaction products were analyzed by electrophoresis as described. For the ARDRA method, 14 microliters of the amplification product of each *Salmonella* isolate were digested with restriction endonucleases. Ten units of enzymes - Alu I and Hha I (Invitrogen) - were added to each reaction and the mixture was incubated at 37ºC for 2 hours. The restriction fragments were separated by electrophoresis on 3% agarose gel at 2 Vcm^{-1} for 4 hours.

Phylogenetic data analysis

Dendrograms were constructed by UPGMA (unweighted pair-group method for the arithmetic average) based on Jaccard's similarity coefficient from a matrix based on the binary code of molecular profile data for bootstrapping. Each phenogram was reconstructed 2000 times by repeated sampling with replacement using computer program WinBoot (Yap & Nelson, 1996).

RESULTS AND DISCUSSION

A total of 15 different serotypes of *Salmonella enterica* subsp. *enterica* isolated from the poultry production chain were typed using different molecular methods for the purpose of obtaining a common molecular profile. Three isolates of each serotype were used.

Salmonellosis is a worldwide problem in the poultry industry, affecting both animal and public health. Studies in several regions of Brazil have shown that *S.* Enteritidis, *S*. Typhimurium, *S*. Panama, *S.* Newport, *S*. Infantis, *S*. Senftenberg, *S*. Heidelberg, *S*. Saintpaul, *S*. Indiana, *S*. Agona, and *S*. London are the most common *Salmonella* serovars found in poultry commercial breeders and broiler flocks (Ribeiro *et al*., 2006; Oliveira *et al*., 2006; Kanashiro *et al*., 2005). As expected, these serovars were found in the present study and were submitted to molecular analyses. However, the most prevalent *Salmonella* serotypes causing human toxic infections are *S*. Enteritidis and *S*. Typhimurium (Robeson *et al*., 2008; Oliveira *et al*., 2006).

Because of the need of detecting and identifying infections to prevent disease and their dissemination, methods are required for the epidemiological study of salmonellosis (Dhillon *et al*., 2001). The genetic characteristics of *Salmonella* serotypes must be accurately and objectively analyzed using an efficient, reliable, and discriminatory genetic analysis method (Christensen *et al*., 1998). Thus far, several genetic analysis methods for the genotyping of *Salmonella* serotypes have become available (Lagatolla *et al*., 1996; Baudart *et al*., 2000).

Because of it is easy to use and it has discriminatory power, RAPD-PCR has become an important tool to fingerprint bacteria involved in disease outbreaks and to determine the sources, vectors and vehicles of transmission (Betancor *et al*., 2004). For RAPD analysis, five known primers used for *Salmonella* characterization were selected, as well as the best simplification conditions to differentiate each serotype based on their DNA amplification profiles. The use of RAPD enabled the differentiation of most genotypes (Betancor *et al*., 2000). Primers P1254, 784, 23L, OPA-4 and OPB-15 were effective for the differentiation of most *Salmonella* serotypes isolated from the broiler production chain (Figure 1).

rev_v14_03_3_SA_638.indd 175 05/11/12 16:18

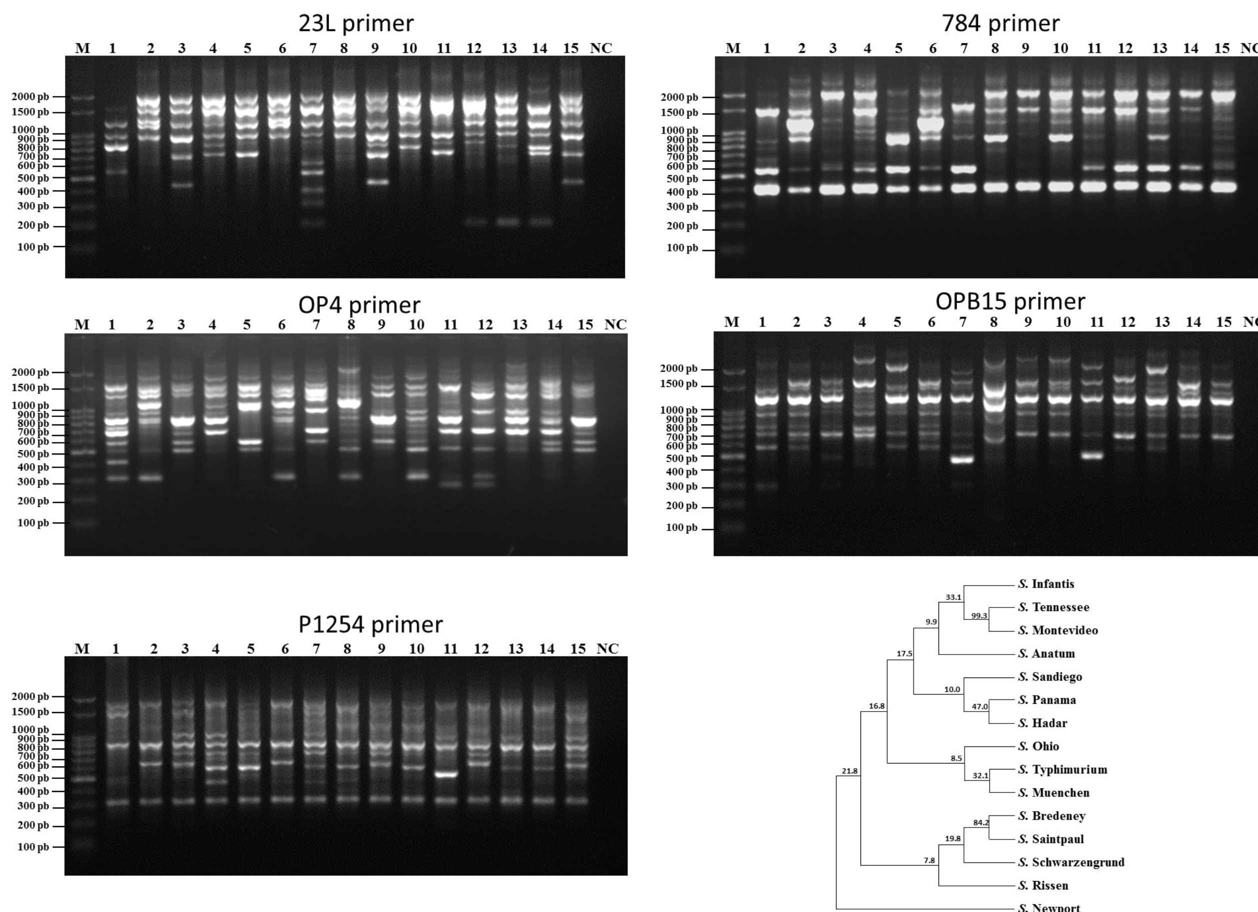

Figure 1 – Representative molecular profiles of 15 *Salmonella* serotypes obtained by RAPD. Electrophoretic analysis of amplification profile of *Salmonella* isolates obtained by RAPD using 5 primers (identified over each gel). Where: M identifies the molecular weight marker 100 bp DNA ladder, NC - negative control and the lines 1 – 15, respectively, *Salmonella* serotypes *S.* Infantis, *S.* Tenesse, *S.* Bredeney, *S.* Schwarzengrund, *S.* Ohio, *S.* Montevideo, *S.* Newport, *S.* Sandiego, *S.* Panama, *S.* Hadar, *S.* Rissen, *S.* Anatum, *S.* Muenchen, *S.* Typhimurium, *S.* Saintpaul. Below and right: Dendrogram constructed by UPGMA using Jaccard's coefficient based on RAPD patterns. The values found in the groups indicate the percentage recorded for a particular branch.

DNA fragments ranging from approximately 2.5 to 0.3 kbp were generated, providing the molecular profile from which a dendrogram was constructed using UPGMA by profile numerical analysis to group isolates by similarity. This approach allowed molecular analyses because, due to the high number of molecular markers developed over the last decades, it has been difficult to associate genetic diversity or molecular profiles with classification and phylogeny, especially of closely-related *Salmonella* isolates (Yap & Nelson, 1996; Baudart *et al*., 2000).

Similarly to the RAPD results, ERIC-PCR results (Figure 2A) provided evidences of clear molecular diversity among the *Salmonella* serotypes isolated in this study. As suggested earlier, the molecular profile obtained by ERIC, associated with the dendrogram (Figure 2B), proved to be a convenient method for fingerprinting and therefore, a good typing tool.

According to Rasschaert *et al*. (2005), it is possible to use the ERIC1R–ERIC2 primer set to differentiate *Salmonella* serotypes and to improve the reproducibility and resolving power of the method by using appropriate annealing temperatures in order to obtain a correlation between the molecular pattern (footprinting) and the specific serotype.

The results obtained by RISA (rDNA internal spacer analysis) demonstrated that 16S-23S rRNA intergenic spacer regions (ISR) are highly polymorphic in *Salmonella* isolates, and therefore, it is possible to use this method to detect variability . Amplification generated DNA fragments of approximately 500 to 1000 pb, and it was possible to differentiate most of the isolates analyzed (Figure 3A-RISA). The high polymorphism of the DNA spacer in the regions between 16S and 23S is due to the fact that sp., as well as , has seven ribosomal operons, and they are usually not identical (intercistronic heterogeneity) (García-Martínez *et al*.,

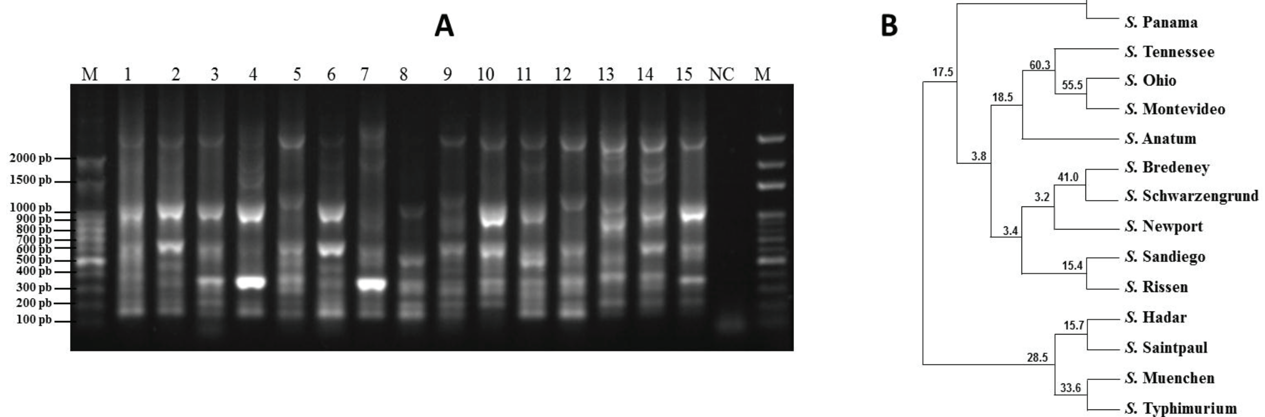

Figure 2 – Molecular profile of 15 Salmonella serotypes obtained by ERIC-PCR. (A) Electrophoretic analysis of amplification profile of Salmonella isolates obtained by ERIC. M - molecular weight marker 100 bp DNA ladder and NC - negative control. Lines 1 to 15, respectively, Salmonella serotypes sequence as described in the key to Figure 1. (B) Dendrogram constructed by UPGMA using Jaccard's coefficient based on ERIC patterns in Panel A. The values found in the groups indicate the percentage recorded for a particular branch.

1999; Jensen *et al*., 1993; Thomson *et al*., 2998). In consequence, spacer region polymorphisms have been useful in the identification of *Listeria*, *Staphylococcus*, and *Salmonella*, as well as the identification of *Salmonella* serotypes (Jensen *et al*., 1993), and the intraserovar or subtyping discrimination of *Salmonella* (Baudart *et al*., 2000; Lagatolla *et al*., 2006). From the results obtained, most of the examined serotypes could be differentiated by their profile. The length and sequence polymorphisms present in the PCR product can therefore be used for the recognition of genotypic diversity.

The most direct and rapid method to visualize the polymorphic character of 16-23S rRNA ISR is PCR amplification of the spacer regions with the use of primers from highly-conserved flanking sequences (García-Martínez *et al*., 1999).

A second approach is to use the PCR product digested with a restriction enzyme (amplified ribosomal DNA restriction analysis - ARDRA), and have

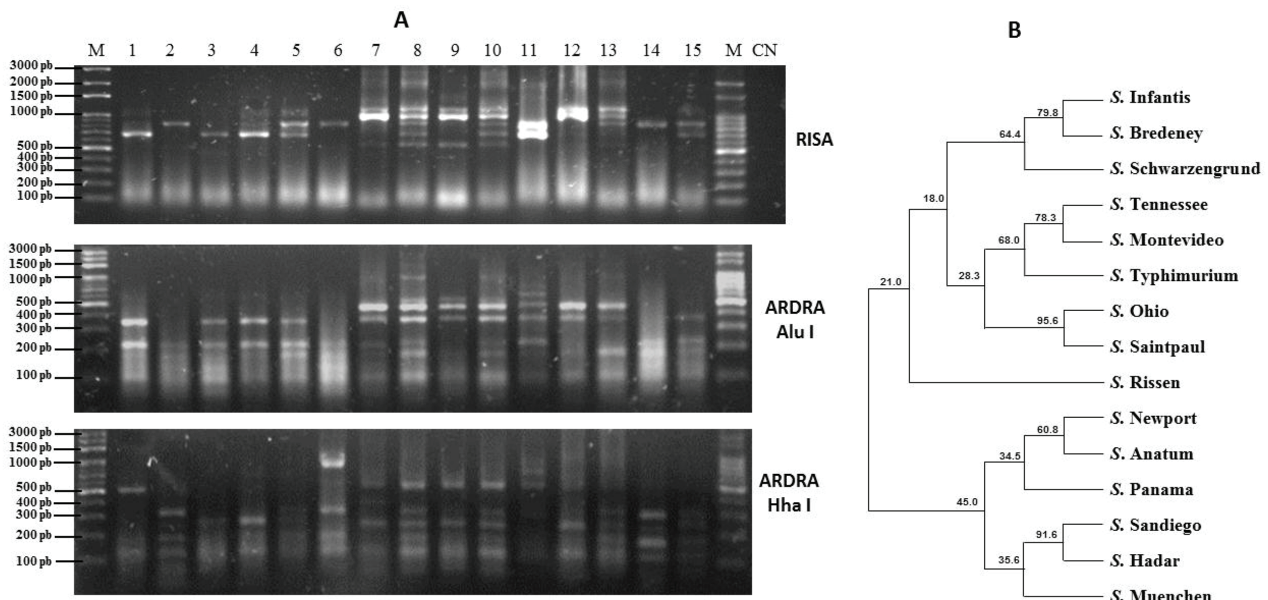

Figure 3 – Molecular profile of 15 Salmonella serotypes obtained by RISA and ARDRA. (A) Electrophoretic analysis of the amplification of the 16S-23S rRNA intergenic spacer region (RISA) by PCR and electrophoretic analysis of the restriction profile (ARDRA - Alu I and Hha I) of amplified fragments of 16S-23S rRNA intergenic spacer region (ISR). M - molecular weight marker 100 bp DNA ladder and NC - negative control. Lines 1 to 15, respectively, Salmonella serotypes sequence as described in the key to Figure 1. (B) Dendrogram constructed by UPGMA using Jaccard's coefficient based on RISA and ARDRA (Alu I and Hha I) patterns of the Panel A. The values found in the groups indicate the percentage recorded for a particular branch.

rev_v14_03_3_SA_638.indd 177 05/11/12 16:18

the resulting fragments resolved electrophoretically (Moreira *et al.*, 2005). If the PCR product contains the restriction endonuclease recognition sequence at unique or different locations, then the resultant fragment size pattern can indicate a particular profile.

In this study, the results of 16S-23S rRNA ISR – ARDRA using Alu I and Hha I restriction enzymes for the 15 serotypes analyzed showed very complex molecular profile patterns (Figure 3A-ARDRA), and no common patterns among the three isolates tested of most serotypes (data not shown). Profiles were differentiated according to band number and position, as suggested by Betancor *et al*. (2000). The number of bands per profile varied, and most fragments were below 500 pb. Additional information may be inherent in the polymorphic character of the amplified and digested products. This indicates the great potential of the technique for footprinting.

However, as those fragments were very small, possibly having small differences in length (few pb), they were not perceptible when the products were separated by agarose gel electrophoresis. In these cases the molecular profile may be obtained by other means, such as thermal gradient gel electrophoresis (TGGE) analysis (Yasuda & Shiaris, 2005), denaturing gradient gel electrophoresis (DGGE) (Anderson *et al*., 2010), or on 4-8 % polyacrylamide gel (Jensen *et al.*, 1993; Baudart *et al.*, 2000), although these methods are more expensive and time-consuming.

A dendogram was constructed by UPGMA from the molecular profiles obtained using RISA and ARDRA (Figure 3B). The clusters generated were different from those obtained by RAPD (Figure 1) and ERIC (Figure 2B), but the highest numbers on a branch, from the high bootstrap values, indicated the percentage that reflected on the concordant structure with which the nodes were supported, meaning that data were more robust than in the other dendograms.

The 16S-23S rRNA ISR (intergenic sequence region) is a hypervariable region, which is useful for the fine discrimination of operational taxonomic units, but limited work has been found focusing on *Salmonella* or other enteric pathogens. However, despite the advantages of the ISR-ARDRA method and its usefulness in the characterization of others organisms (Jensen *et al.*, 1993; Luz *et al.*, 1998), it had not been used for the identification of serotypes or the analysis of isolates, possibly due to the difficulty in visualizing the results, whereas rDNA internal spacer analysis (RISA), RAPD, repetitive extragenic palindromic-PCR (REP-PCR) and ERIC are more common (Rasschaert *et al*., 2005; Woo & Lee, 2006).

In conclusion, the main *Salmonella* serotypes found in poultry products to be sources of infection may be identified by molecular techniques such as RAPD and ERIC. Depending on the conditions of the reactions, these methods may also be used for footprinting analyses. On the other hand, RISA and ARDRA techniques, especially the 16S-23S rRNA intergenic spacer regions, are primarily intended for intra-serotype characterization, that is, the differentiation of *Salmonella* strains at the intra-serovar level. Moreover, the use of a complementary method is essential for obtaining more reliable and accurate results both in serotype determination and isolate characterization. Methods should be combined as needed. Therefore, the new approach to the ISR-ARDRA technique will possibily have important practical implications for the epidemiological analysis of *Salmonella*. The method has proven to be an important tool for *Salmonella* fingerprinting and was highly discriminatory among *Salmonella* isolates; therefore, it may potentially detect wider variability among fingerprint profiles.

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rev_v14_03_3_SA_638.indd 178 05/11/12 16:18

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rev_v14_03_3_SA_638.indd 179 05/11/12 16:18