Full Length Research Paper

Molecular identification of four *Salmonella* serovars isolated from food in Tunisia based on the sequence of the ribosomal RNA genes

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In this study, the 5′-end of the 16S-23S intergenic spacer regions and domains II and III of the 23S ribosomal DNA (rDNA) of six *Salmonella* strains were analyzed. These strains belong to four serovars: Agona, Amesterdam, Corvallis and Enteritidis, and were isolated from food samples. Polymerase chain reaction (PCR) products were then sequenced on both strands and the sequences obtained were aligned using the “Clustal W” program. The comparative sequence analysis revealed that the 5′-end of the 16S-23S spacer region rDNA is highly conserved among the four *Salmonella* serovars tested. All of these sequenced spacer regions contained a tRNAIle gene. For the domains II and III of the 23S rDNA, the sequence analysis showed the presence of both conservative and variable regions. Some mutations, substitutions and deletions were also detected. This work supports the hypothesis that the 23S rRNA genes are useful for the definition of serovar-specific probes for the detection of *Salmonella* in food.

Key words: *Salmonella*, spacer region, rDNA, 23S, identification.

INTRODUCTION

Members of the genus *Salmonella* have been recognized as a major cause of gastrointestinal disease in both humans and animals. Infection with *Salmonella* can lead to serious and potentially fatal infections, including bacteremia, septic arthritis, meningitis, and pneumonia (Asai et al., 2010). Fecal–oral transmission of *Salmonella* occurs most commonly through the consumption of contaminated food. In fact, salmonellosis is still one of the most widespread foodborne bacterial illnesses in humans (Magistrali et al., 2008). The most frequently isolated serovars from food-borne outbreaks are *Salmonella enterica* serovars Typhimurium and Enteritidis (Settanni and Corsetti, 2007). The conventional methods for the detection of *Salmonella* require multiple subculture steps followed by biochemical and serological confirmation, which may take up to 4 - 6 days. Up to now, more than 2500 serovars have been identified (Wise et al., 2009). Thus, rapid and efficient methods for the detection of *Salmonella* serovars are required. During the last decade, biologists have employed a variety of molecular techniques based mainly on the ribosomal DNA (rDNA) genes to identify microorganisms.

The major product of all cellular transcriptions in both prokaryotes and eukaryotes is the ribosomal RNA (rRNA). In most prokaryotes, rDNA forms an operon with the order 16S-23S-5S and is co-transcribed in a single polycistronic RNA that has to be processed to generate the RNA species present in the mature ribosome (Luz et al., 1998). Comparative sequence analysis of rDNA has
been widely used to infer phylogenetic relationships. In addition to the phylogenetic aspects, structural studies on rRNAs are of fundamental importance for understanding the topology and function of ribosomes (Woese, 1987, 1992). Furthermore, rDNA molecules contain signature structures, which are unique for groups of organisms and render the rDNA ideal targets for specific nucleic acid probes (Nour, 1999). Many studies based on the sequence of the 16S and the 23S rDNA (Lin and Tsen, 1995; Pabbaraju et al., 2000; Pabbaraju and Sanderson, 2000) have been carried out. However, due to the less selection pressure, the 16S–23S rDNA intergenic spacer region sequence seems to be more genetically variable and species specific than that of 16S rDNA and 23S rDNA (Lin and Tsen, 1995; Tsai et al., 2008). In addition, the variation in lengths and sequences of these spacer regions suggest their usefulness for designing genus or species specific DNA probes (Tsai et al., 2008). It has been reported that the helices 45 and 54 (Escherichia coli numbering) are the most variable regions in the 23S rDNA (Nour, 1999).

The aim of the present study was to investigate the variability of these two stem loop structures located, respectively in domains II and III of the 23S RNA and the 5′-end of the 16S-23S spacer region of four Salmonella serovars isolated from chicken and eggs, and to define, if possible, serovar-specific oligonucleotide probes. These probes, based on the sequence of ribosomal RNA genes, could provide a precise and a more rapid method for Salmonella identification in food in less time than usually required with the current biochemical test systems. For this purpose, we have amplified and sequenced these two genes.

Comparative sequence analysis and secondary structure models served to assess the variability of 23S rRNA and 16S-23S intergenic spacer region genes among the studied Salmonella serovars.

MATERIALS AND METHODS

Bacterial strains

Six Salmonella strains belonging to different serotypes were collected from the “Hygiene Department of the Regional Laboratory of Public Health of Monastir”, Tunisia. These included: Salmonella Enteritidis, Salmonella Agona, Salmonella Corvallis and three strains of Salmonella Amsterdam. These strains were isolated from chicken and eggs in Tunisia.

Identification of strains

Salmonella strains were identified according to standard bacteriological procedures: colony morphology, Gram stain reactions, oxidase test, biochemical reactions and serotyping. Salmonella Enteritidis ATCC 13076 was used as a control strain.

Genomic DNA extraction

Genomic DNA was extracted by the “Wizard Genomic DNA Purification Kit” according to the manufacturer’s instructions (Promega, Madison, WI). The total DNA was resuspended in sterile distilled water.

Polymerase chain reaction and agarose gel electrophoresis

Helices 45 and 54 of the 23S rDNA gene were amplified using a couple of appropriate primers described by Pabbaraju et al. (2000). The amplification of the 5′-end of the 16S-23S spacer region was performed as previously reported (Chiu et al., 2005). All polymerase chain reaction (PCR) products were electrophoresed on 2% agarose gels in 1X tris-borate-EDTA (TBE) buffer (containing 90 mM Tris HCl, 90 mM boric acid, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) at 8 V/cm in the presence of 0.5 μg/ml of ethidium bromide, and visualized using the Gel Doc 2000 system (Bio-Rad). Image processing and analysis of DNA bands were carried out using the Quantity One software program (Bio-Rad).

Nucleotide sequence determination

PCR products were purified using the “Wizard PCR preps DNA purification kit” according to the manufacturer’s instructions (Promega, Madison) and then sequenced. Cycle sequencing was performed using Automated Applied Biosystems (AB) sequencing and the Taq Dye Deoxy Terminator cycle sequencing kit (ABI).

Sequence analysis

Sequence alignments were done using the “Clustal W” program (Multiple Sequence Alignment; http://align.genome.jp/).

Nucleotide sequence accession numbers

The Genbank accession numbers of the 16S-23S rDNA spacer region of Salmonella studied serovars were FJ573220, FJ573221, FJ573222 and FJ573223 and those of the 23S rDNA were FJ573224, FJ573225 and FJ573226.

RESULTS

The 5′-end of the 16S-23S intergenic spacer region (ITSR) is highly conserved among Salmonella serovars

The 5′-end of the 16S-23S intergenic spacer region was amplified out of genomic DNA extracted from six Salmonella strains belonging to four different serovars: Salmonella Agona, S. Amsterdam, S. Corvallis and S. Enteritidis using two appropriate primers. All serovars studied generated PCR products with molecular weight equal to 312 nucleotides.

Amplified rDNA fragments were sequenced on both strands and the sequences obtained were aligned using the “Clustal W” program (Figure 1). The comparative sequence analysis revealed that the 5′-end of the 16S-23S rDNA spacer region is highly conserved among the four studied Salmonella serovars. All of these sequenced regions contained a tRNAle gene as shown in the Figure 2.
**Sequence determination of domains II and III of the 23S rDNA**

Using the whole genomic DNA from *Salmonella* strains as a template, we have amplified domains II and III of the 23S rRNA (containing respectively helices 45 and 54; *E. coli* numbering). The PCR product had 733 nucleotides. PCR amplicons were sequenced on both strands and then aligned using the “Clustal W” program (Figure 3). The comparative nucleotide sequence analysis of these two domains in *Salmonella* serovars showed the presence of both conservative and variable regions.
Secondary structure model of the 5′-end of the intergenic spacer region (ITSR) 16S-23S of *Salmonella*. *S*. Amesterdam sequence is designed with black color. The blue color indicates differences between *S*. Amesterdam and *S*. Agona, and the green those between *S*. Amesterdam and *S*. Corvallis. Serovars Enteritidis and Amesterdam have the same ITSR sequence. For the variable nucleotides, International Nomenclature (UIB) was used as follows: H : A/C/U; K : G/U; M : A/C; R : A/G; S : C/G; W : A/U; Y : C/U.

Some mutations, substitutions and deletions were also detected. Based on previous published models (Höpfli et al., 1989; Nour, 1999), a secondary structure model was drawn (Figure 4); it appears that the helix 54 is strongly conserved among these serovars, whereas the helix 45 seems to be serovar-specific.

**DISCUSSION**

To examine the possibility of deriving serovar-specific DNA probes for the detection of *Salmonella* in food samples, domains II and III of the 23S rDNA gene and the 16S-23S intergenic spacer rDNA were amplified and sequenced. Many investigations of bacteria have been performed on the 16S rRNA gene. However, the 23S rRNA gene is twice as large as the 16S rRNA and is consequently more laborious to analyze for the selection of oligonucleotide probes (Zhang and Arias, 2007). The amplification of domains II and III of the 23S rDNA gene (containing respectively helices 45 and 54, *E. coli* numbering) generated a PCR amplicon equal to 733 nucleotides for all studied *Salmonella* strains. This result is in full agreement with the data reported by Pabbaraju et al. (2000). On the basis of the comparative sequence analysis of these two domains of the 23S rDNA, it appears that the helix 54 is highly conserved among *Salmonella* serovars. However, the domain II of the 23S rDNA exhibits nucleotide differences at the helix 45, and thus this helix constitutes an important feature for the design of targets for oligonucleotide serovar-specific probes. It has been reported that the helix 45 is one of the most variable helices in bacterial 23S rDNA and is also at a position homologous to an expansion segment in eukaryotes (Pace and Burgin, 1990). Taking into account these results, it appears that helices 45 and 46 (*E. coli* numbering) are the most variable stem-loop structures in domain II. Through domain III, the helix 54 is highly conserved among *Salmonella* serovars. However, the helix 58 sequence is also variable. According to these findings, the stem-loop structures 45, 46 (domain II) and 58 (domain III) are useful for designing *Salmonella* oligonucleotide serovar-specific probes.

The 16S-23S intergenic spacer region appears to be more diverse than the rRNA genes themselves and so is more useful for the identification of bacteria. In fact, it has
Figure 3. Sequence alignment of Domains II and III of the 23S rDNA of *Salmonella* serotypes Amsterdam, Enteritidis, Corvallis and Agona. The “Clustal W” program was used for this alignment.
been reported that the spacer region found within rRNA loci shows significant degrees of variability in both length and sequence (Nour, 1998). This diversity is, in part, due to variations in the number and type of tRNA genes found within the spacers. However, for the studied *Salmonella* strains, a unique amplified fragment of the 5′-end of the 16S-23S spacer region was obtained and all of the amplified fragments had an identical length (312 bp). This data was also reported by Chiu et al. (2005).

Based on this result, we can confirm the presence of one form of *rrn* operon. Since we have sequenced directly a PCR product (many molecules) and a unique sequence was found for each strain, we could conclude that the seven *rrn* copies have the same sequence for a given *Salmonella* strain. Moreover, on the basis of the sequence of the 5′-end of the 16S-23S rDNA, it appears that this spacer region is conserved among *Salmonella* serovars except some substitutions. This result supports the idea that there is a strong pressure on these potentially hyper-variable regions to remain constant, which indicates a strong inter-serovars conservation of the 16S-23S spacer rDNA sequence. We can also conclude that this spacer region is conserved among the seven *rrn* copies. Nevertheless, taking into account that the number of *Salmonella* strains studied is limited, these data need to be extrapolated to some other *Salmonella* serotypes in order to confirm these findings.

**Conclusion**

This work leads to the proposal that a rapid and universal *Salmonella* identification and typing scheme is possible based on the sequence of the 23S rDNA gene. Considerable sequence heterogeneity is apparent, especially at the stem loop structures 45, 46 and 58. This finding supports the fact that the 23S rDNA is a useful tool for the definition of *Salmonella* serovar-specific probes and that the use of these variable helices should allow a precise and more rapid identification of *Salmonella* in less time than usually required with current biochemical test systems. Hence, studying a higher number of *Salmonella* serovars is clearly a challenge in the near future to confirm these results and to increase...
Figure 4. Secondary structure of Domains II and III of the 23S rRNA gene of *Salmonella* (S.). S. Amsterdan is designed with black color. The differences between S. Amsterdan and S. Corvallis were designed with rose and those between S. Amsterdan and S. Agona were indicated in green. For the variable nucleotides, International Nomenclature (UIB) was used as follows: H: A/C/U; K: G/U; M: A/C; R: A/G; S: C/G; W: A/U; Y: C/U.
our understanding concerning this recent molecular identification method.

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REFERENCES


