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Original Article

CRISPR Typing of Clinical Strains of *Salmonella* spp. Isolated in Tehran, Iran

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Abstract

Background: *Salmonella* is one of the most leading causes of food-born infection and death among infants and people with the poor immunity system. Because *Salmonella* spp. have diversity in the genome composition and pathogenicity, access to rapid identification and genotyping is necessary to control of salmonellosis. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) typing is a genotyping method that checks these variable sequences in the bacterial genome in a specific species. This study aimed to differentiate *Salmonella* strains using CRISPR region.

Methods: *Salmonella* isolates, previously identified via standard microbiological and molecular tests, were subjected to the study. Bacterial DNA was extracted and PCR was done using specific primers. The different PCR products were sequenced and the repeats patterns were used to identify additional or degenerate repeat clusters in the CRISPR region. All different sequences were analyzed using CRISPRtionary tool for dendrogram generation using the binary file.

Results: Overall, 119 strains of various *Salmonella* serovars were used. The result showed unique CRISPR and diversity in spacer both in sequence and the number. Analysis of the extracted sequence and band patterns illustrated that, except for *S. infantis,* both *S. enteritidis* and *S. typhimurium* isolates were classified as a separate cluster.

Conclusion: CRISPR genotyping could provide serotype/spacers dictionary and it is performed at low cost and high speed in comparison to the other typing methods. Therefore, the assessment of CRISPR and spacer content can be considered as a powerful and practical discriminatory method for subtyping of *Salmonella* isolates.

Keywords: *Salmonella* spp.; Genotyping; CRISPR-Cas Systems

Introduction

Salmonella, as a gram-negative bacterium, is one of the major leading causes of food-born infection and death in the human population worldwide (1). Access to a rapid, efficient and accurate method for subtyping of Salmonella is essential to track specific strains of this organism throughout sporadic or epidemic salmonellosis (1). The traditional method, such as culture and serotyping, not only have low sensitivity and specificity but also their results are not reliable and time-consuming too. The molecular subtyp-

ing methods provide powerful tools for epidemiological studies (2).

There are several molecular typing methods include RFLP (3), Ribotyping (4), PFGE, MLST (5), VNTR, PCR-based methods such as (RAPD), and CRISPR (6).

The CRISPR arrays are a small and variable region of the genome that comprised of three main parts: (a) 24-47 bp invariable direct repeats and 21-72 bp variable spacers that incorporate direct repeats. (b) cas genes that encode proteins involved in adaptation and interference. (c) A-T rich leader sequences that supposed are involved in transcription of the CRISPR arrays (7, 8). The whole of these three component form CRISPR-cas system in some eubacteria is known as a prokaryotic adaptive immune system that provides resistance against viruses, plasmid, and transposons and is found in the genomes of 45% bacteria and 99% of archaea (9). The first part (direct repeat and variable spacer) is a good choice for molecular genotyping since this fragment is heterogeneous in sequence among bacterial strains. Several molecular typing studies using CRISPER analysis have been performed on pathogenic bacteria such as *Mycobacterium tuberculosis*, *Yersinia pestis*, *Streptococcus pneumoniae*, *Lactobacillus buchneri*, *Salmonella enterica*, *Escherichia coli*, *Klebsiella pneumoniae*, *Escherichia coli*, *Listeria monocytogenes*, that reviewed in (8).

Analysis of *Salmonella* genome has revealed two CRISPR regions in the chromosomal genome which have variation in sequence and number of the spacer in different strains and isolates of Salmonella spp. (1). We aimed to determine the genotype of clinical isolates of *Salmonella* spp. using CRISPR detection and sequencing.

Materials and Methods

Bacterial isolates and DNA extraction

Overall, 119 strains of various *Salmonella* serovars obtained from the microbial collection of Molecular Biology Research Center of Baqiyatallah University, Tehran, Iran were used in this study. *Salmonella* strains have been isolated from patients admitted to the major hospitals in Tehran, Iran.

All isolates were identified in the microbiology laboratory according to standard microbiological, biochemical, serological and PCR methods (10). Bacterial isolates were stored at -20 °C in skim milk. Out of these, we tested 56, 42 and 21 isolates of S. Enteritidis, S. Infantis and S. Typhimurium isolates resprctively. When needed, isolates were grown overnight in Luria Bertani broth (LBb) at 37 °C. For all isolates, DNA was extracted by the boiling method and then were purified by phenol-chloroform. After DNA extraction, the concentration of genomic DNA was measured by Nanodrop, which the expected OD 260/280 was between1.5-2.0 (if this index is between 1.7-2.0 shows the best quality of extracted DNA) and stored at -20 °C before use.

CRISPR Amplification and Sequencing

The primers used for the amplification of CRISPR regions were given from the article (1) designed for spacer contents. The sequence of forward and reverse primers for amplifying CRISPR1 was GTRGTRCGGATAATGCTGCC and CGTATTCCGGTAGATBTDGATGG respectively. PCR amplifications were performed using a Taq PCR master mix 2x kit (made by Bioneer Company) and a Mastercycler PCR thermocycler (Applied Biosystems, America). 25 µl of PCR reaction mixture included 12 µl of Taq PCR 2x master mix, 8 µl of PCR-grade water, 3 µl of DNA template, 1.0 µl forward primer (final concentration, 10 pmol), and 1.0 µl of revers primer (final concentration, 10 pmol). The cycling condition of CRISPR1 was performed as follow: initial denaturation step of 2 min at 94 °C; 30 cycles, with 1 cycle consisting of 1 min at 94 °C, 1 min at 58 °C, and 90 sec at 72 °C; final extension step of 8 min at 72 °C. The amplified PCR products were resolved on 1% agarose gels, stained with safe stain and visualized under a short wave length ultraviolet light source. The different PCR products were sequenced by Bioneer Company at Korea.

CRISPR Sequence Analysis

The repeats patterns were used to identify additional or degenerate repeat clusters in the CRISPR region. Spacer sequences were compared to the NCBI nonredundant database using BLASTn, with the search set limited to bacteria and viruses. All different sequences were analyzed using CRISPRtionary, a program for analyses of the spacers, in the base of those submitted genomic sequences. According to this program for each spacer allocated a number and similar spacers got a unique number and gave a graphical view of the binary file (http://crispr.i2bc.paris-saclay.fr/cgi-bin/crispr/AnnotateSpacers.cgi). Finally, a dendrogram was generated using the binary file.

Results

Salmonella CRISPR array

Salmonella spp. have two distinct CRISPR loci in their genome, CRISPR 1 and 2. In this study, CRISPR1 was used for genotyping of this organism. The Direct Repeats (DRs) of CRISPR loci is conserved and have 29 bp in length. The length of CRISPR array varies in size and 8 to 13 spacers identified in the CRISPR array. The size of PCR products, based on spacer number, were between 750 to 1150 bp, and the repeat sequences belong to the CRISPR of *Salmonella* serovars in length of 29 bp was found in all 119 isolates (Some Different size of PCR products belong to specific serovar are showed in Fig.1). All DRs had a unique sequence and the sequence logo of DRs was shown in Fig.2. Those the CRISPR sequences can discriminate the isolates within *Salmonella* serovars.

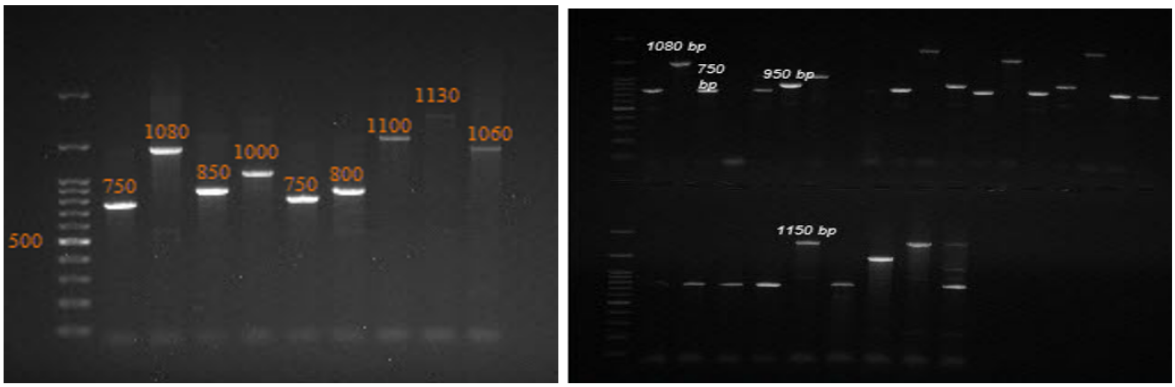

Fig.1: Gel electrophoresis showing different size of bands in different serovar of *Salmonella*

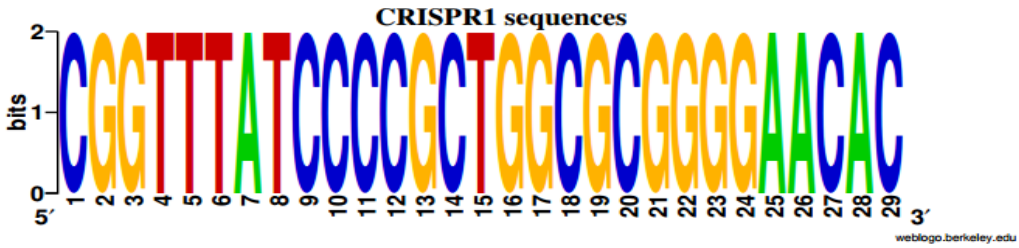

Fig.2: Sequence logo for all direct repeats in the CRISPR1 loci of *Salmonella*

Diversity in spacers

All different PCR products were sequenced by sanger sequencing method (Bioneer Company). The sequencing results showed that similar and equal band had unique DR and a constant number of spacers. The sequencing results were

analyzed using CRISPRtionary web tool to compare spacer arrangements of CRISPR. The generated binary file was used for clustering. The generated dendrogram (Fig. 3) revealed that except for *S. infantis,* both *S. enteritidis* and S. *typhimurium* isolates were classified as a separate cluster.

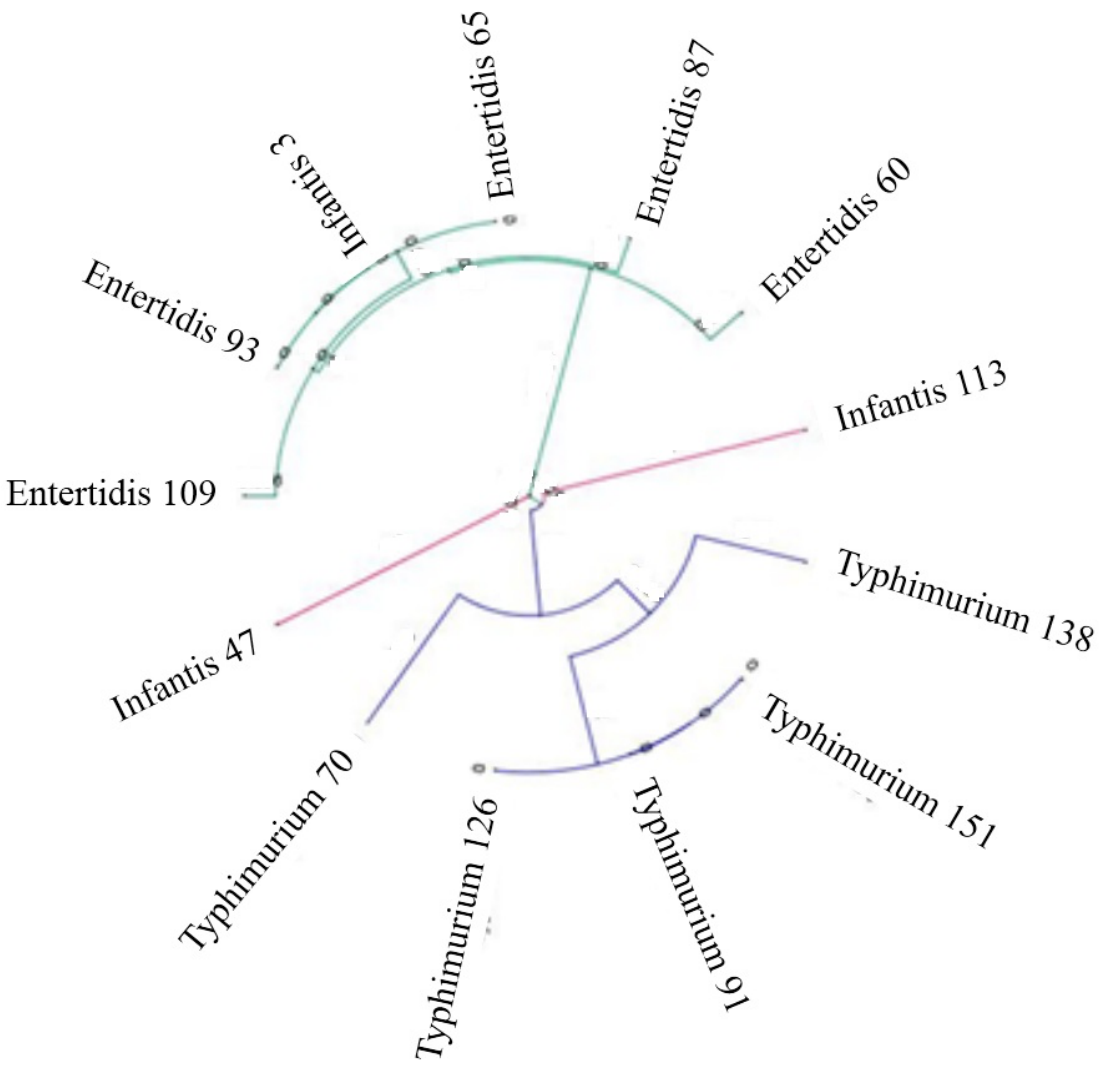

Fig. 3: The generated dendrogram of *Salmonella* based on spacers for 13 candidate sequence types

Discussion

Achieving the methods for identification and subtyping of *Salmonella* serovars is essential in order to overcome the threat caused by this organism. Several molecular subtyping methods such as PFGE have been used for subtyping of *Salmonella* (11), though this currently preferred subtyping method has known as a gold standard method, but it has some defects. Currently, subtyping of *Salmonella* using sequence-typing base on CRISPR loci might provide useful information for typing of this organism. As well as it provides a fast and cost-effective way compared to other molecular typing methods.

Different schemes about the use of CRISPR typing have emerged. Fabre et al applied CRISPR sequences for molecular typing of 783 *Salmonella* strains and found this technique as a rapid and precision typing approache due to highly poly-

morphic region of CRISPR sequences in *Salmonella* (1). The CRISPR strain characterization is a good alternative approach to both serotyping and PFGE (1).

The discriminatory power of CRISPR sequences was compared with traditional typing methods such as serotyping and found the CRISPR sequences as an appropriate target for the development of a DNA amplification assay for tracking of any strains of *Salmonella* with a particular spacer content (12).

Salmonella isolates was classified by CRISPR locus spacer pair typing (CLSPT). They developed a more convenient and efficient method, namely, CRISPR locus spacer pair typing (CLSPT), which only needs to analyze two newly incorporated spacers adjoining the leader array in the two CRISPR loci (13). A new CLSPT method presented a high level of consistency with the results of traditional serotyping, and showed it can also be used to predict serotypes of *Salmonella* spp. (13).

In the present study, we analyzed 119 clinical strains of *Salmonella* belonging to *S. enteritidis*, *S. infantis,* and *S. typhimurium serovars* previously confirmed by serological tests and PCR method. The first step for identification of *Salmonella* isolates in this study was to amplify the region with crispr and then sequencing by Sanger method. The data were analyzed using special CRISPR bioinformatics tool. The CRISPRFinder, as a web tool, was used for identification of a crispr structure based on the existence of DR and spacers. The DRs were consensus in all isolates and varied in the type and number of spacers indicating their usefulness to track the strains.

Conclusion

The CRISPR genotyping can provide serotype/spacers dictionary for tracking the serovars of *Salmonella* and this method is performed at low cost and high speed in comparison to the other typing methods. Therefore, the assessment of CRISPR and spacer content can be considered as a powerful and practical discriminatory method for subtyping of *Salmonella* isolates. The presence of identical spacers in the same CRISPR locus in distinct strains reflects shared ancestry.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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