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Comparative analysis of using CRISPR-1 locus, 16S rRNA and rrnH as identification systems for clinical strains of Salmonella enterica species

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SUMMARY

Salmonella enterica species are major global foodborne pathogensthat are divided into six distinct subspecies. Proper identification of *Salmonella* species is a challenge for many laboratories. In *Salmonella*, CRISPR loci were found to be conserved in all *Salmonella* species and also very diverse. Therefore, it could be potentially used as a promising target region for *Salmonella* identification. Henceforth,we aimed tointroduce a new subtyping genetic system for genotyping someclinical Zoonotic *Salmonella* isolates using a PCR assay that is targeting the CRISPR-1 region. About total 30 isolates were analysed in this study (human isolates n=10, camel isolates n=10 and poultry isolates n=10). All these isolates were previously identified at the genus and species level following an analysis of DNA sequences of 16S rRNA and rrnH.

At PCR amplification level, the system was able to amplifyall 10 human isolates (33.3%) and 7 camel-isolates (23.3%) 10 chicken isolates (33.3%).

Finally, due to the fact that the tested isolates were previously identified and serotyped by two different systems (16S rRNA and *rrnH*), this identification and serotyping was also validated by our new CRISPR-1 sequence system. The validation showed non-confirmative results as CRISPR-1 system identified the human and camel isolates as *Salmonella enterica sp. enterica* serovar Enteritidis and the chicken isolate as *Salmonella enterica sp. enterica* serovar Anatum. On the other hand, 16S rRNA identified the chicken isolate as *Salmonella enterica sp. enterica* serovar Typhimurium while the *rrnH* system identified the camel isolate as *Salmonella enterica sp. enterica* serovar Indiana. Therefore, these results showed that a definitive identification requires using multiple genomic regions instead of sequencing a one region for bacteria.

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Introduction

Salmonella enterica is a major global foodborne pathogen *(Ehuwa et al., 2021; Jiang et al., 2021; Pan et al., 2018). S. enterica* is divided into six distinct subspecies: *enterica, salamae, arizonae, diarizonae, houtenae, andindica* (*Le Minor &*Popo*ff, 1987*) . The *S.enterica* subsp. enterica consists of more than 1,500 serotypes.

CRISPR system is currently a hotspot technology for genome editing in eukaryotes, with huge potential for translational medicine and next-generation gene therapy. CRISPR-*Cas* systems, on the other hand, provide adaptive immunity in bacteria and hold great promise for a variety of applications in food bacteria, including high-resolution pathogen typing for *Mycobacterium tuberculosis, Salmonella enterica, Clostridium difficile, Escherichia coli,* and a variety of other pathogens, including food-borne pathogens *(Selle & Barrangou, 2015)* .

There are now other uses for CRISPR-Cas systems, such as DNA repair and virulence control. Additional roles, including those in DNA repair and virulence regulation, have been identified through the characterisation of CRISPR-Cas systems (Westra et al., 2014).

Despite the development of a few practical bioinformatics tools to aid in the identification of CRISPR repeats and spacers, there is still room for large-scale discovery and comparison (Biswas et al., 2016; Grissa et al., 2007). These technologies are mostly used for CRISPR-Cas detection and single-organism-level analysis of associated properties.

According to Nethery and Barrangou (2019), the CRISPR locus, which is characterized by a variety of repeat and spacer elements, is a genetic record of the ongoing conflict between bacteria and viruses and, consequently, the genomic integration of spacers obtained from invasive DNA into bacterial chromosomes. Particularly, alternate spacer acquisitions reveal different evolutionary histories and are commonly employed for high-resolution bacterial genotyping, including comparing clinical isolates, clonal lineages, and closely related species.

Materials and Methods

Bacterial Isolates

About 30 bacterial isolates were isolated and identified as Salmonella by previous researchers at Department of Veterinary Microbiology, Faculty of Veterinary Medicine, University of Kufa and were generously donated for this research. These 30 isolates of *Salmonella* were belonging to three species: *Salmonellaenteritidis* (Human source, N=10),*Salmonellaindiana* (Camel source, N=10) and Salmonella typhimurium (Poultry source, N=10). The isolates were incubated overnight at 37 ◦C in Brain Heart infusion (BHI) medium.

Genomic DNA extraction

The pellets of recent cultures of all isolates in BHI broth were prepared by centrifugation of about 2 ml at 14,000 rpm for 2 min. The bacterial genomic DNA was extracted using a commercial DNA extraction kit (AddPrep Genomic DNA Extraction Kit, Cat.No.:10023, [AddBio, Korea]) following the manufacturer's instructions. Quality and quantity of the extracted DNA were assessed using NanoDrop® spectrophotometer (NanoDrop-1000, Wilmington, DE).

Amplification and DNA sequencing of CRISPR-1 region in *Salmonella* isolates

Novel primers were specifically designed to amplify the CRISPR-1 region in Salmonella species. These primers were forward primer (5′-CGGAGTCGGAAACGTAGTAATG-3′), and a reverse primer (5′-GAATGAGTGACGCTGAGAAGAA-3′) (Figure 1). The PCR reactions were performed using Applied Biosystem thermal Cycler (USA) under the following conditions: 95 ◦C for 5 min; 34 cycles of 95◦C for 30s, 56◦C for 30s, and72◦C for 5 min; and finally, 72 ◦C for 5 min. The PCR products were separated using 1% agarose gel electrophoresis. Then, the PCRs were cleaned using a specific Kit for PCR products cleaning(Wizard® SV Gel and PCR Clean-Up System, Cat.No.:A9281, [Promega, USA]). Finally, all PCR products were DNA sequenced using Sanger DNA sequencing method (Macrogen, Korea).

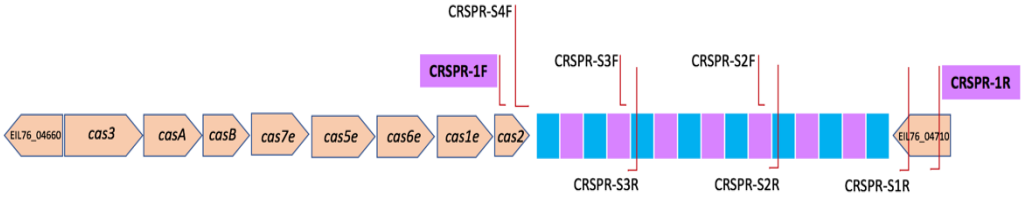

Figure 1: Genetic map of CRISPR-1 locus and primers used for this experiment.

CRIPSR-1 locus is located between gene number EIL76_04660 and gene EIL76_04710 in the *Salmonella enterica sp. enterica* serovar Typhimurium ATCC14028 (accession number: CP034230). Primers used for amplification of CRISPR-1 locus are highlighted in two lavender boxes.

Validation of primers for amplification of CRIPSR-1 region

Due to the fact that the primers used in this study werenovel, therefore, these primers were validated using a DNA extracted from a reference strain of *Salmonella enterica sp. enterica* serovar Typhimurium ATCC14028 (Registered in the NCBI data base under accession number: CP034230). Three annealing temperatures were applied (Ta= 52 ºC, 54 ºC & 56 ºC) for this purpose. The primers were successfully amplified the expected size of PCR band for CRISPR-1 region related to this strain (2213 bp) (Figure 2).

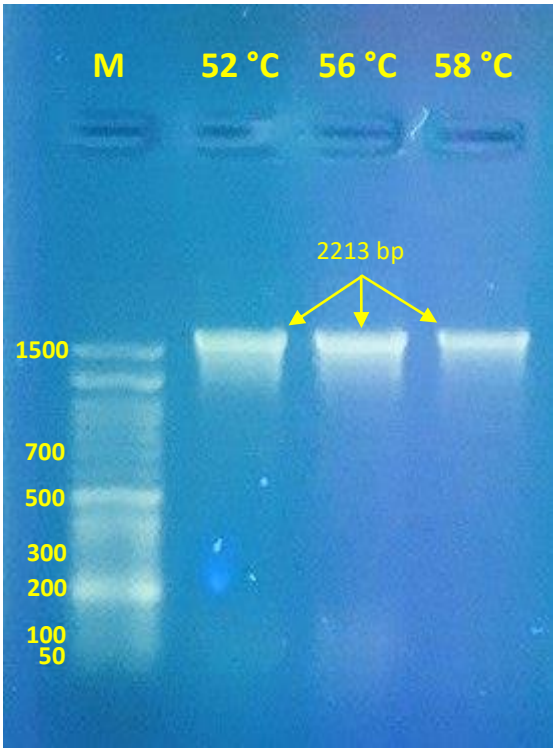

Figure 2: PCR products of CRISPR1 region of *Salmonella enterica sp. enterica* serovar Typhimurium ATCC14028 using primers of set-PCR.

The size of the PCR product is 2213 bp. The gel was 1% and the DNA dye is RedSafe (Intron, Korea). Electrophoresis conditions: V: 90, Time: 42 minutes. M: DNA ladder.

Results of PCR amplification of CRISPR-1 region

Total 30 samples of *Salmonella entericasp. enterica* serovars Enteritidis (n=10), Typhimurium (n=10) and Indiana/Kentucky (n=10) isolated from humans, chicken and camels respectively were tested for amplification of CRISPR-1 region. The CRISPR-1 region was successfully amplified in 27(90%) samples out of total 30 samples.

These samples were 10 (33.3%) from a human-source, 7 (23.3%) samples were from a camel-source and 10 (33.3%) form a chicken-source (Table 1 & Figure 3). Furthermore, the PCR amplification showed different sizes of PCR bands for the CRISPR-1 region in the positive samples (Figure 3).

Table 1: Results of amplification of CRISPR-1 PCR system for tested strains of *Salmonella enterica sp. enterica*.

Number of isolates	Source of isolate	Successful PCR amplification	
		CRISPR-1 region	16S rRNA region
10	Human	10 (33.3%)	10 (33.3%)
10	Camel	7 (23.3%)	10 (33.3%)
10	Poultry	10 (33.3%)	10 (33.3%)

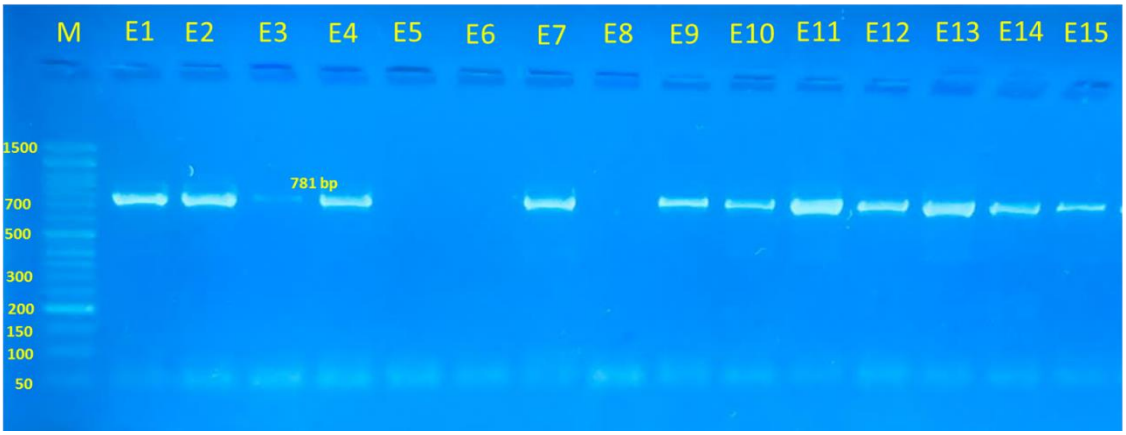

Figure 3: Amplification of CRISPR1 region of *Salmonella enterica sp. enterica* serovars *Enteritidis, Typhimurium and Indiana.*

Results of analysis of the sequences of CRISPR-1

Total 27 samples were sequenced and a strict criterion for considering a sequence as a "valid result" was applied. Briefly,the analysis is applied only when the obtained sequences are covered the entire region of CRISPR-1 locus which is located in-between the 3' prime of gene *EIL76_04705* (codes for *cas2*) toward 3' prime of gene *EIL76_04710* (codes for aminopeptidase) in the genome of *Salmonella enterica sp. enterica* serovar *Typhimurium* ATCC14028 (Figure 1).

As a result, only 5 sequences from a human-source (HSE01, HSE02, HSE03, HSE04 and HSE05), 3 sequences from camels (CSI01, CSI02 and CSI03), and 1 sequence from chicken (PST01) were considered as complete sequences of CRISPR-1 region. Other sequences were excluded and not considered in this study because it failed to cover the entire CRISPR-1 region.

BLASTn of CRISPR-1 locus was definitive for identification of *Salmonella*

At last, we blast the sequences of CRISPR-1 locus of all 9 isolates using the BALSTn server in the NCBI. The results were interesting as it seems that CRISPR-1 loci of clinical *Salmonella* isolates are very restricted to *Salmonella enterica species*(Table 2). For *Salmonella* isolates of human-source, the BALSTn server clarified that all 5 isolates were closely related to *Salmonellaentericaspp*. *enterica* serovar *Enteritidis*(Table 2). This result was compatible with the previous identification of these strains as *Salmonellaentericaspp*. *enterica* serovar *Enteritidis*.

Interestingly, the situation in the isolates of camels and chicken sources was different. Isolates of camels were identified as *Salmonellaenterica* sp. *enterica* serovar *Indiana* (Abbas, 2022). However, based on CRISPR-1 region analysis, CSI01, CSI02 and CSI03 were showed high identity percentage of 89%, 93% and 89% respectively towards *Salmonellaenterica* sp. *enterica* serovar *Enteritidis* but not to *Indiana* strain (Table 2).

On the other hand, the only isolate of chicken which was previously identified as *Salmonellaenterica* sp. *enterica* serovar *Typhimurium* was also found to be shared an identity percentage of 100% to *Salmonellaenterica* sp. *enterica* serovar *Anatum* strain 05-4434 CRISPR1 repeat region (accession number: JF724470.1) (Table 2).

Table 2: Analysis of the sequences of CRISPR-1 locus of the tested isolates

Strain	Source of isolation	Previous identification	According to CRISPR-1 locus
HSE01	Human	*S. enteritidis*	*S. enteritidis*
HSE02	Human	*S. enteritidis*	*S. enteritidis*
HSE03	Human	*S. enteritidis*	*S. enteritidis*
HSE04	Human	*S. enteritidis*	*S. enteritidis*
HSE05	Human	*S. enteritidis*	*S. enteritidis*
CSI01	Camels	*S. indiana*	*S. enteritidis*
CSI02	Camels	*S. indiana*	*S. enteritidis*
CSI03	Camels	*S. indiana*	*S. enteritidis*
CST01	Poultry	*S. typhimurium*	*S. anatum*

Discussion

Why CRISPR-1 locus was chosen to be analyzed in this research?

For many decades, serotyping of *Salmonellae* using specific antisera and considered as a golden reference method for identifying the possible types of *Salmonella* strains *(Gal-Mor, 2018)*. However, this method is limited to specific high-quality laboratories and it is available for a limited number of *Salmonella* strains *(Heymans et al., 2018).*This would imply a challenge for researchers who are trying to identify a wide variety of *Salmonella* strains other than the most frequent strains like Typhi, Paratyphi, Typhimurium and Enteritidis *(Wattiau et al., 2011).*

On the other hand, two putative molecular serotyping protocols were proposed. The first protocol is targeting two genes (*fliC* and *fljB*) that are involved in biosynthesis of the flagella while the second is targeting genes of rfb locus that are involved in the O-polysaccharide synthesis *(Fitzgerald et al., 2007; McQuiston et al., 2011)* From a genetic point of view, the rfb locus is about 8-23 kb long which makes it difficult to amplify using a conventional PCR protocol. Furthermore, this system is failed in identifying majority of *Salmonella* O serogroups. Additionally, this system also provides poor discrimination between the identified *Salmonella* O serogroups *(Fabre et al., 2012)*.These reasons were initiative enough to propose the CRISPR-1 locus as a promising genetic tool for studying the possible genetic-relationship among different *Salmonella* strains isolated from different sources.

Analysis of CRISPR-1 provides different identification from 16S rRNA or rrnH region

Although the results of analyzing the sequences of CRISPR-1 locus were similar to the previous identification of *Salmonella* isolates from human sources as they were identified as *Salmonellaenterica* sp. *enterica* serovar Enteritidis. However, the situation was completely different in the isolates of camels and chicken. Isolates of camels were identified previously as *Salmonellaenterica* sp. *enterica* serovar Indiana based on *rrnH* region *(Jin et al., 2022).* Surprisingly, and based on CRISPR-1 region analysis, we re-identified these isolates as *Salmonellaenterica* sp. *enterica* serovar Enteritidis. Furthermore, the chicken isolate was also re-identified as *Salmonellaenterica* sp. *enterica* serovar Anatum. These results were completely unexpected and could be used (if reproduced in future research) for re-examining the validity of 16S rRNA or rrnH systems in the purpose of identifying the serotypes of Salmonella strains. However, during the current situation, it seems that CRISPR-1 locus is challenging the two widely identification systems (16S rRNA and rrnH). Due to the fact that the 16S rRNA and rrnH systems along with CRISPR locus were all validated by peer-reviewed experts, it seems like two-edged sword as it could be a limitation in the aforementioned two systems or a limitation in the CRISPR-1 locus system itself. Therefore, further research aims to validate the three systems is strongly recommended.

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