ACTA MEDICA LITUANICA. 2009. Vol. 16. No. 3–4. P. 98–102
DOI: 10.2478/v10140-009-0014-8

The use of high-resolution melting analysis for *Salmonella* spp. CRISPR sequence genotyping

Maksim Bratčikov,

Mykolas Mauricas

Institute of Immunology,
Vilnius University,
Vilnius, Lithuania

Background. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) of *Salmonella* spp. and of other bacteria continuously evolve due to the rearrangements of fragments. This phenomenon forms a high degree of strain polymorphism. Real-time PCR and high-resolution DNA melting (HRM) analysis of CRISPR regions provide a possibility for a rapid evaluation of strain polymorphism. Our intent was to apply the HRM technique for *Salmonella* spp. genotyping.

Materials and methods. *Salmonella* spp. strains DNA were amplified and analyzed with HRM using Rotor-Gene 65H0-100.

Results. Forty-nine *Salmonella* spp. strains were analyzed, and 23 genotypes were identified for each CRISPR (CRISPR1 and CRISPR2). We have found the genotypes of both CRISPRs to be related to the same *Salmonella* spp. serotypes.

Conclusions. The CRISPRs HRM method is a valuable tool for *Salmonella* spp. genotyping.

Key words: CRISPR, HRM, *Salmonella* spp. genotyping, Rotor-Gene 6000, Syto9

INTRODUCTION

Salmonellosis is one of the most common and widely spread foodborne diseases. It is caused by the *Salmonella* bacterium.

Today, there are over 2500 known *Salmonella* spp. serotypes. All serotypes are defined by O and H antigens. These antigens are determined by some genomic areas that occupy a relatively small part of the *Salmonella* spp. genome. Thus, we can suspect that other genome sequences for the same serotype can be different. There are many genotyping methods for genome study. The results obtained are called genovars.

One of the most interesting areas for bacterial genotyping is Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs). The CASS system (a CRISPR and several *cas* genes) has been identified in a broad range of prokaryotic species in almost all archaea and in 40% of bacteria. A CRISPR is a succession of 21–47 bp sequences, called Direct Repeat (DR), separated by unique sequences of a similar length (spacers). The nature of the unique sequences is still largely unknown, but several recent studies have identified some of them as fragments of foreign DNA, mostly of viral origin (1–3). Sometimes, at one end of the CRISPR, the DR is not totally conserved: it is called degenerate DR. The CRISPR locus is generally flanked on one side by a common AT-rich leader sequence of 200–350 bp. Different observations suggest that CRISPR loci are transcribed into small RNA possibly from the leader acting as a promoter, and that these might play a role of siRNA to block the entry of foreign sequences (4, 5). CRISPR-associated genes are *cas* genes always found closely linked to the repetitive sequences. In a given strain, several CRISPRs can be found with a single or different DR sequences, but only one is associated with a group of four to six *cas* genes (6).

The CRISPR structure is continuously evolving, either through the addition of new motifs (a DR and a spacer) or by interstitial deletion of one or several motifs through recombination between two DRs. New motifs are added to the CRISPR in a polarized manner by duplicating the DR next to the leader and adding a new fragment of DNA (3, 7). Those CRISPRs rearrangements form a high degree of polymorphism from strain to strain.

In the CRISPRs Database (http://crispr.u-psud.fr/crispr/) there are a lot of bacterial strains already discovered for CRISPRs. All *Salmonella* spp. serotypes included in this database contain 1, 2 or 3 CRISPRs motives. They are formed of 31 bp, 30 bp, 29 bp, 28 bp or 27 bp long repeats. The sequence of those repeats is the same and only differs in the length. Developing a new CRISPR typing scheme may thus be a good addition to the classical typing techniques applicable

Correspondence to: Maksim Bratčikov, Institute of Immunology, Vilnius University, Molėtų pl. 29, LT-08409, Vilnius, Lithuania.
E-mail: bratchikov@gmail.com

for strain differentiation, epidemiological investigations, and phylogenetic reconstruction.

A recent development in real-time PCR technology is high-resolution DNA melting (HRM) analysis (8, 9). Melt curves are predominantly used to determine the melting temperature (Tm) of amplified double-stranded DNA. The basis of HRM analysis is to discriminate melt curve shape, irrespective of whether amplicons share the same Tm. Accurate melting curves are derived using very small temperature increments (as low as 0.01 °C). Normalization and comparison of melting curves allow the user to determine whether two similar melt curves differ from one another (8). Following these advantages, the potential resolving power of this approach is much greater than the conventional melting curve analysis.

Generally, HRM is applied to a wide range mutation screening within small DNA fragments. Usually, it is about one hundred base length. Nevertheless, this method is implemented for differentiation of larger DNA fragments. Recently, for the first time, HRM has been used for CRISPRs fragment differentiation of *Campylobacter jejuni* (10) and *spa* Repeat Region of *Staphylococcus aureus* (11). The aim of the present investigation was to evaluate the possibility to use the HRM method for genotyping two separate CRISPR regions of the *Salmonella* spp. Also, we tried to compare the variability of targeted sequences.

MATERIALS AND METHODS

***Salmonella* spp. isolates.** All *Salmonella* spp. strains were isolated and differentiated to serotype at the National Public Health Surveillance Laboratory.

Preparation of genomic DNA. DNA template was extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions from an overnight culture. Extracted DNA concentrations were measured with the Rotor-Gene 6000 5-plex HRM model (Corbett research). Extracted DNA samples were stored at –20 °C until use.

Primer pairs. Two primer pairs were designed. One pair was designed for the first and another pair for the second CRISPR region. All *Salmonella* sequences were obtained from the National Center for Biotechnology Information data base and aligned with Vector NTI Advance™ (Invitrogen). The selected primers were blasted using the nucleotide blast program for mispriming at the National Center for Biotechnology Information.

Real-time PCR and HRM. Real-time PCR and HRM assays were performed using the Rotor-Gene 6000 5-plex HRM model (Corbett research). Rotor-Gene 1.7.87 software version was used for the process and data analysis. Each 15 µl of reaction contained 200 nM of each primer (Integrated DNA technologies), 2–10 ng *Salmonella* spp. DNA, 200 µM dNTP (Fermentas), 3 mM $MgCl_2$ (Fermentas), 1.5 µM Syto9 (Invitrogen-Molecular Probes) and 0.04 U/µl TrueStart™ *Taq* DNA Polymerase and TrueStart™ *Taq* buffer (Fermentas). Cycle conditions were the following: 95 °C for 4 min, followed by 35 cycles of 95 °C for 20 s; annealing at 45 °C for 60 s and extension at 72 °C for 120 s) and 50 °C for 1 minute. HRM dissociation curves spanning at 75–95 °C by 0.1 °C wait for 4 s for each step. CRISPRs HRM data were normalized by two linear normalization regions. For the first CRISPR they were within 82.28–82.67 °C and 94–95 °C, and for the second CRISPR they were within 87–87.5 °C and 93–93.5 °C regions. All samples were tested in duplicate to ensure the reproducibility of the melt curves and to determine appropriate cutoffs for the "same" and "different" genotypes. CRISPR HRM genotypes were determined as the same by a cutoff of $\leq \pm 4$ U in the difference graph relative to a control sample of interest and did not display reproducible differences such as double peaks or crossing the x-axis more than twice in both replicates.

RESULTS

In the National Center for Biotechnology Information (NCBI) data base we found 15 sequences of different *Salmonella* strains for two CRISPRs motives (Table 1). Primers were taken from flanked conservative CRISPRs sequences. For these strains, the first CRISPR (CRISPR1) amplification product from 318 bp to 1830 bp and the second CRISPR (CRISPR2) amplification product from 293 bp to 2185 bp. Following the alignment data, we determined that CRISPR1 was more variable than CRISPR2. This sequence variability excludes the *S. Paratyphi* B SPB7 strain. It was not possible to select the common reverse primer for it.

Table 1. **Clustered regularly interspaced short palindromic repeat primers and amplicon size for the 15 sequences of different *Salmonella* spp. strains taken from NCBI data base**

Primer pairs	Amplicon length (bp)	
F_CR1 GCTGGTGAAACGTGTTTATCC R_CR1 ATTCCGGTAGATYTKGATGGAC	CRISPR 1	
F_CR2 AACGCCATGGCCTTCTCCTG R_CR2 CAAAATCAGYAAATTAGCTGTTC		CRISPR 2
Salmonella spp. strains from NCBI data base		
Enteritidis str. P125109	684	844
Typhimurium str. LT2	1692	2185
Choleraesuis str. SC-B67	440	1974
Heidelberg str. SL476	1830	1330
Agona str. SL483	1294	720
Gallinarum str. 287/91	318	844
Dublin str. CT_02021853	318	538
Newport str. SL254	1770	1390
Paratyphi A str. AKU_12601	611	415
Paratyphi A str. ATCC 9150	489	415
Typhi CT18	541	293
Typhi Ty2	576	293
Schwarzengrund str. CVM19633	916	1269
Paratyphi C str. RKS4594	806	2096
Paratyphi B str. SPB7	–	537

Table 2. ***Salmonella*** **spp. genotyping data**

Chosen *Salmonella* spp. serotypes	Number of serotypes	Number of different genotypes	
		CRISPR 1	CRISPR 2
S. enteritidis	12	4	2
S. typhimurium	8	5	5
S. agona	6	1	1
S. infantis	7	5	5
S. London	2	1	1
S. Glostrup	3	1	3
S. Abony	2	1	1
S. Hadar	3	1 (same as for *S. Glostrup*)	2 (same as for *S. Glostrup*)
S. Dublin	2	1	1
S. Mbandaka	2	2	2
S. Chester	2	2	2
Total	49	23	23

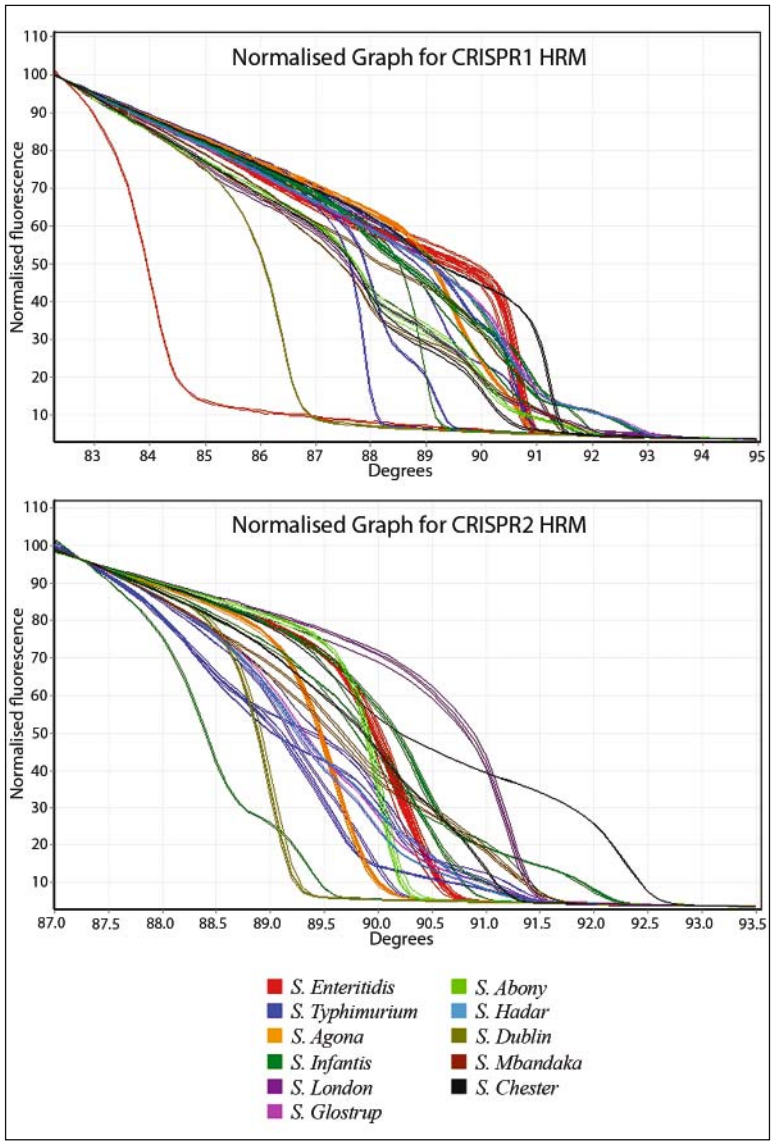

Fig. 1. High-resolution melting normalized graphs for Clustered Regularly Interspaced Short Palindromic Repeats

Another interesting fact is connected with the CRISPR2. As is shown in the CRISPRs Database (http://crispr.u-psud.fr/crispr/), S. typhi Ty2 and S. typhi CT18 serovars have no CPISPR2, but the flanked conservative sequences are present. Hence, the chosen primers must work to amplify small fragments.

Eleven different *Salmonella* spp. serotypes were taken for amplification and HRM (Table 2). Each serotype was represented by more than one strain. In total, 49 *Salmonella* spp. strains were analyzed.

After HRM data normalization we found very wide and robust differences between and within the chosen serotypes by discriminate melt curve shapes (Fig. 1). The total number of different genotypes for both CRISPRs is the same and equals to 23 for each of them, or 46 genotypes overall (Table 2). Some serotypes were differentiated within them, but others were not. It is not possible to differentiate between the taken *S. Glostrup* and *S. Hadar* because they have the same genotypes for both CRISPRs (Fig. 2).

All 8 samples of the *S. typhimurium* serotype were split into 5 genotypes according to CRISPR1 and 5 genotypes according to CRISPR2. We have found that the genotypes of both CRISPRs are related: each genotype of CRISPR1 is linked to a particular genotype of CRISPR2. The same kind of relation was found between CRISPRs for *S. enteritidis* in samples 2, 3, 5, 7, 9, 10 and 20 and for *S. infantis* samples in 37 and 72.

DISCUSSION

In the recent study of *Campylobacter jejuni* CRISPR fragments, the researchers encountered difficulties in interpreting results obtained with Platinum *Taq* DNA polymerase (Invitrogen), 1.5 mM $MgCl_2$ and 2.5 μM Syto9 dye (Invitrogen) due to large differences between the replicates (10). For further reactions they chose SYBR green I dye chemistry. However, it is not a suitable dye for HRM. It has been shown that SYBR green I translocates between amplicons during melt curve analysis to a higher-*Tm* product (12–14) and has a much higher inhibition to PCR than that of Syto9 (12, 15), thus suggesting not careful HRM data. The replicates obtained in our study with Syto9 dye are highly reproducible (Fig. 3), and our

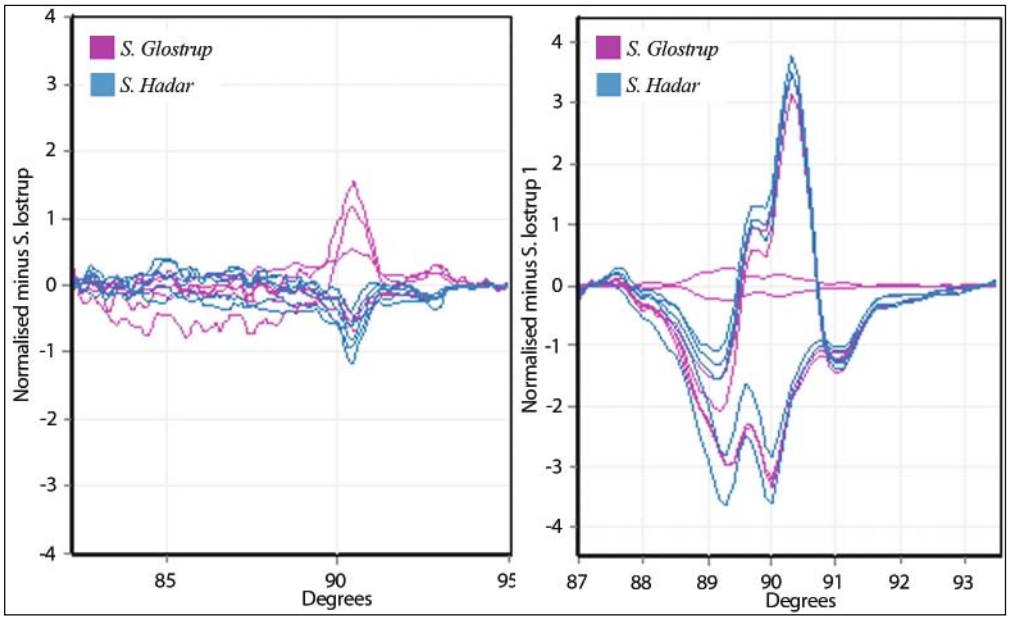

Fig. 2. *S. Glostrup* and *S. Hadar* difference graph: on the left – for Clustered Regularly Interspaced Short Palindromic Repeat 1 for *S. Glostrup* genotype and on the right – for Clustered Regularly Interspaced Short Palindromic Repeats 2 for *S. Glostrup* genotype 1

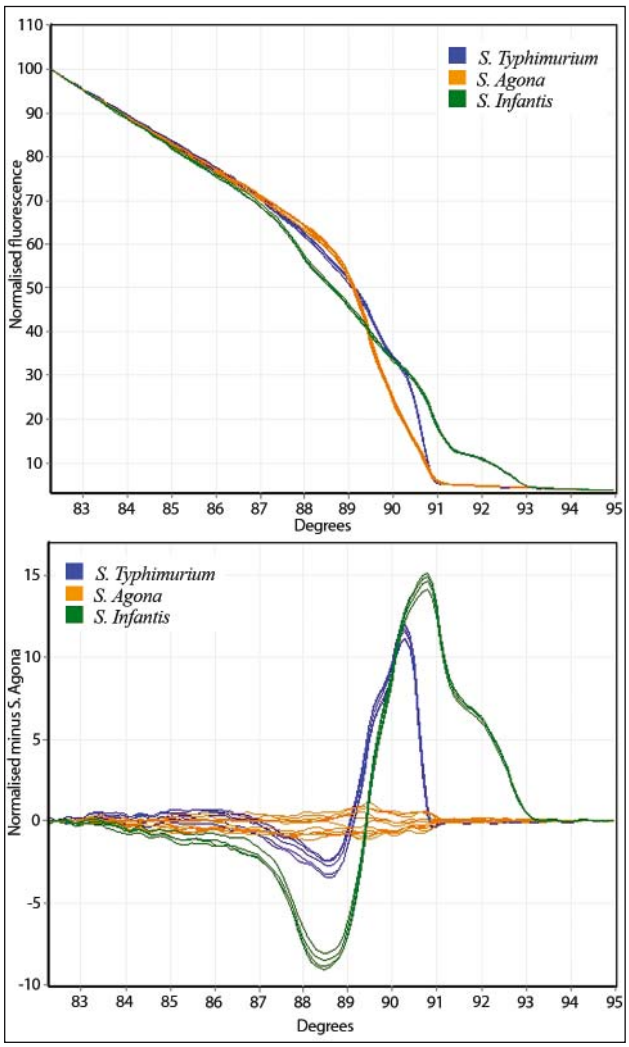

Fig. 3. Normalized high-resolution melt curve (top) and difference graph (bottom) for *S. Typhimurium* genotype 3, *S. agona* genotype 1 and *S. infantis* genotype 5 of Clustered Regularly Interspaced Short Palindromic Repeat 1

reaction mix can be used for CRISPRs DNA fragment HRM analysis.

In this investigation, the HRM method for *Salmonella* spp. CRISPRs region genotyping and comparison was used for the first time. The differences obtained between samples demonstrate a high degree of polymorphism for CRISPRs. This CRISPR typing scheme is very quick, robust, single-step, closed-tube and cost-effective and can be a good addition to classical *Salmonella* spp. typing techniques.

We did not find differences within *S. Agona*, *S. London*, *A. Abony* and *S. Dublin* serotypes, possibly for the following reasons: the number of samples taken for analysis was too small, and there was a probability that samples have been extracted from the related sources. The latter may also be true for the relation found among the *S. Typhimurium*, *S. Enteritidis* and *S. Infantis* genotypes.

Sequence alignment from the NCBI gene bank allowed us to assume quite a high variability of CRISPR1. Nevertheless, our data obtained from CRISPR1 and CRISPR2 HRM analysis didn't reveal any significant differences between them. For our samples, we discriminated the same number of genotypes for each region in the total figure. Thus, the variability comparison of CRISPR1 and CRISPR2 largely depends on sample serotype and the number of samples taken.

Further investigations in this field would enable compiling the databases of CRISPRs HRM genotypes.

CONCLUSIONS

The implementation of this method as additional to the classic typing method is acceptable and not complicated for all laboratories working with PCR. We may assume that both CRISPRs are equally useful for *Salmonella* spp. strain differentiation.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Vilma Jonaitienė, Head of Microbiological Department at the National Public Health Surveillance Laboratory, for providing *Salmonella* spp. serotype total lysates.

Received 15 October 2009
Accepted 30 October 2009

References

1. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 2005; 151: 2551–61.
2. Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 2005; 60: 174–82.
3. Pourcel C, Salvignol G, Vergnaud G. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 2005; 151: 653–63.
4. Tang TH, Bachellerie JP, Rozhdestvensky T, Bortolin ML, Huber H, Drungowski M et al. Identification of 86 candidates for small non-messenger RNAs from the archaeon *Archaeoglobus fulgidus*. Proc Natl Acad Sci USA 2002; 99: 7536–41.
5. Makarova KS, Grishin NV, Shabalina SA, Wolf YI, Koonin EV. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct 2006; 1: 7.
6. Haft DH, Selengut J, Mongodin EF, Nelson KE. A guild of 45 CRISPRassociated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput Biol 2005; 1: e60.
7. Lillestol RK, Redder P, Garrett RA, Brugger K. A putative viral defence mechanism in archaeal cells. Archaea 2006; 2: 59–72.
8. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003; 49: 853–60.
9. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 2003; 49: 396–406.
10. Price EP, Smith H, Huygens F, Giffard PM. Highresolution DNA melt curve analysis of the clustered regularly interspaced short palindromic repeat locus of *Campylobacter jejuni*. Appl Environ Microbiol 2007; 73: 3431–6.
11. Stephens AJ, Inman-Bamber J, Giffard PM, Huygens F. High-Resolution Melting Analysis of the *spa* Repeat Region of *Staphylococcus aureus*. Clin Chem 2008; 54(2): 432–6.
12. Giglio S, Monis PT, Saint CP. Demonstration of referential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. Nucleic Acids Res 2003; 15: e136.
13. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clin Chem 2006; 52: 494–503.
14. Varga A, James D. Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of Plum pox virus strains C, EA, and W: effect of amplicon size, melt rate and dye translocation. J Virol Methods 2006; 132: 146–53.
15. Monis PT, Giglio S, Saint CP. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem 2005; 340: 24–34.

Maksim Bratčikov, Mykolas Mauricas

AUKŠTOS REZOLIUCIJOS LYDYMOSI TEMPERATŪRĄ NUSTATANČIO METODO PRITAIKYMAS *SALMONELLA* SPP. CRISPR SEKŲ GENOTIPAVIMUI

S a n t r a u k a

Prielaidos ir tikslas. *Salmonella* spp. ir kitų bakterijų CRISPR sekų sudėtiniai fragmentai nuolat persitvarko ir suformuoja didelį prokariotinių mikroorganizmų polimorfizmą. Realų laiką PGR ir aukštos rezoliucijos lydymosi temperatūrą nustatantis (ARLTN) metodas leidžia per trumpesnį laiką ištirti kamienų polimorfizmą. Mūsų darbo tikslas – pritaikyti šią metodiką *Salmonella* spp. genotipavimui.

Medžiagos ir metodai. *Salmonella* spp. kamienų DNR buvo pagausinta ir išanalizuota Rotor-Gene 65H0-100 prietaisu.

Rezultatai. Buvo išanalizuoti 49 *Salmonella* spp. kamienai ir nustatyti 23 genotipai kiekvienam CRISPR regionui (CRISPR1 ir CRISPR2). Išsiaiškinome, kad kai kurių ištirtų *Salmonella* spp. serotipų abiejų CRISPR regionų genotipai yra sukibę tarpusavyje.

Išvados. CRISPR sekų ARLTN metodas yra vertingas įrankis *Salmonella* spp. genotipavimui

Raktažodžiai: CRISPR, ARLTN, *Salmonella* spp., genotipavimas, Rotor-Gene 6000, Syto9