Use of MALDI-TOF Mass Spectrometry, and DNA Sequencing-based SLST and MLST Analysis for the Identification of *Cronobacter* spp. Isolated from Environmental Samples

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Abstract

Cronobacter **spp. are emerging opportunistic foodborne bacteria that can cause acute meningitis and necrotizing enterocolitis in neonates and immunocompromised individuals. While little is known about its reservoirs or transmission routes, it has been primarily linked with various foodborne outbreaks associated with powdered infant formula (PIF) and related products, worldwide. The distinctive biology of *Cronobacter* having a higher resistance to desiccation, osmotic stress, and thermotolerance as compared to other members of Enterobacteriaceae, is considered for the post-process contamination in these products. Nevertheless, it has been isolated from a variety of foods, mostly from plant-based and dairy products and a few from meat products; and from a wide range of environment that include domestic household, manufacturing plants of milk powder, and infant formula. Three *Cronobacter* spp. (*Cronobacter sakazakii*, *Cronobacter malonaticus* and *Cronobacter turicensis*) have been described as more virulent, and isolated frequently from infant meningitis cases. The estimated mortality rates are as high as 80% in infants. Thus, surveillance and typing of *Cronobacter* spp. isolated from food and environmental samples is essential to prevent contamination and spread of this pathogen. In this study, a total of 83 *Cronobacter* isolates were recovered from various environmental samples by conventional microbiologic protocols and analyzed. Initial species identification was completed using the VITEK 2 system and real-time PCR analysis. Consequently, these isolates were analyzed by the VITEK MALDI-TOF MS system. Single locus sequence typing (SLST) was performed by sequence characterization of the regions of 16S rRNA and *rpo*B genes. Multilocus sequence typing (MLST) was accomplished by sequencing the region of seven housekeeping genes (*atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, and *pps*) on the ABI 3500XL Genetic Analyzer. MALDI-TOF mass spectrometry identified majority of the isolates as *Cronobacter sakazakii* with a high confidence value (99.9%). MLST analysis ascertained 12 distinct clonal complexes (cc1, cc4, cc8, cc13, cc17, cc21, cc31, cc40, cc52, cc64, cc73, and cc83) for the recovered *Cronobacter sakazakii* isolates. Results of this study suggest that the MALDI-TOF MS is a reliable diagnostic tool for rapid species identification, whereas the 7-loci MLST is a powerful technique that can differentiate *Cronobacter* spp. isolates of public health importance.**

Disclaimer

- **The findings and conclusions made in this presentation are those of the presenter and do not necessarily represent the views or official position of U.S. Food and Drug Administration (FDA), U.S. Centers for Disease Control and Prevention (CDC) or the U.S. Department of Health and Human Services (DHHS).**
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Materials and Methods

- **MALDI-TOF MS Identification**
  - bioMérieux VITEK® MS System
  - bioMérieux VITEK® MS Database
- **DNA Extraction**
  - Qiagen DNeasy Blood and Tissue DNA Extraction Kit
- **Polymerase Chain Reaction**
  - Qiagen HotStarTaq Master Mix
  - 16S rRNA, *rpo*B specific PCR primers
  - 7-loci MLST specific PCR primers
- **Nucleotide Sequencing**
  - ABI Big dye 3.1
  - ABI 3500 XL Genetic Analyzer
  - 16S rRNA and *rpo*B specific sequencing primers
  - MLST specific sequencing primers
- **Multiple Alignment and Genetic Analysis**
  - BioEdit, Geneious,
  - CLC Genomics Workbench 12.0.2 programs
  - https://pubmlst.org/organisms/cronobacter-spp/

Introduction

- ***Cronobacter sakazakii*** **is a Gram-negative human-pathogenic bacterium known to cause life-threatening meningitis and necrotizing enterocolitis in neonates.**
- **It has been linked to contaminate the powdered infant formula (PIF) worldwide.**
- **Several DNA fingerprinting techniques such as PFGE, AFLP, MLVA and Ribotyping have been applied to identify the *Cronobacter* strains.**
- **Nucleotide sequencing of 16S rRNA has been most widely used genetic marker to determine phylogenetic relationships between distantly related members of Enterobacteriaeceae. However, it is often less discriminatory beyond species level.**
- **Additionally, the *rpo*B gene has also been described as a suitable target for bacterial identification and phylogenetic studies.**
- **Application of 7-loci based MLST enables identification of *Cronobacter sakazakii* sequence types (STs) and clonal complexes (CCs).**
- **MLST based clonal lineage has been effective to understand the *Cronobacter* species contamination of PIF and the epidemiology of neonatal meningitis cases.**
- **MALDI-TOF mass spectrometry is currently recognized as a rapid, cost-effective, and reproducible method for the identification of human pathogenic Gram-positive, Gram-negative, fastidious, and anaerobic bacteria isolated from foods, clinical and environmental samples.**
- **In this study, we have used the MALDI-TOF MS, the SLST (16S rRNA, *rpo*B) and the MLST (7-loci) techniques, for the species identification of 83 *Cronobacter*-like isolates, recovered from environmental surveillance samples.**

Results and Discussion

- **A total of 83 *Cronobacter*-like bacterial isolates were recovered from various environmental samples following conventional microbiological methods.**
- **All recovered *Cronobacter* spp. isolates were identified by the VITEK 2 system (as *Cronobacter sakazakii* group) and real-time PCR analysis (as *Cronobacter* species).**
- **The VITEK MS system was able to provide species level identification for 80 isolates as the *Cronobacter sakazakii* with a high confidence value (99.9%), and genus level identification for the remaining three isolates.**
- **A distinct *Cronobacter sakazakii* species-specific genetic polymorphism was observed among the 83 recovered *Cronobacter*-like isolates, when the regions of 16S rRNA as well as *rpo*B loci were sequenced.**
- **MLST is a DNA sequence-based technique that involves the identification and clustering of bacterial isolates focused on the sequencing of a locus of multiple housekeeping genes of the organism. The sequences can be either attained individually or predicted in silico from the whole genome sequences.**
- **MLST technique defines the sequence types (ST) based on typically seven allelic profiles, and the clonal complexes (CC) based on the relatedness of the sequence types (1–3 loci differences).**
- **MLST was performed based on the seven recognized *Cronobacter*-specific genes (*atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, and *pps*), to better understand the genetic diversity among the recovered *Cronobacter sakazakii* isolates.**
- **MLST analysis revealed twelve distinct clonal complexes (cc1, cc4, cc8, cc13, cc17, cc21, cc31, cc40, cc52, cc64, cc73, and cc83) for the 83 environmental *Cronobacter sakazakii* isolates investigated.**
- **Seven loci *Cronobacter* MLST method has been successful in assessing the extent of genetic polymorphism of *Cronobacter sakazakii* strains recovered from the PIF, the ingredients of PIF, and the PIF production facilities.**
- **Several of these *Cronobacter sakazakii* clonal complexes have high public health significance as these clonal complexes been previously recovered from the Powdered Infant Formula, the Infant Formula Production Factory, and from the patients with Neonatal Meningitis (this study).**

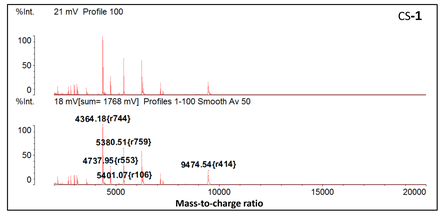

Figure 1. Spectral signature pattern for the *Cronobacter sakazakii* isolate recovered from the environmental surveillance sample using the VITEK MS system with SARAMIS RUO (Research Use Only) database

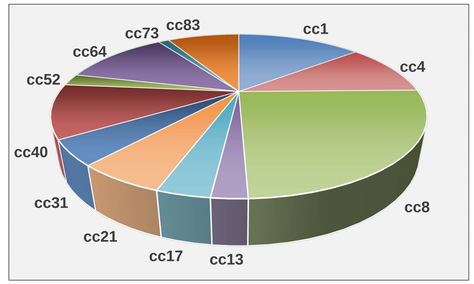

Figure 2. Distribution of twelve distinct *Cronobacter sakazakii* clonal complex among 83 recovered isolates from environmental samples.

References

- Baldwin *et al.* 2009. *BMC Microbiology* 9:22.
- Fei et al. 2015. *Applied and Environmental Microbiology* 81: 5430-5439.
- Forsythe *et al.* 2014. *BMC Genomics* 15:1121.
- Forsythe 2018. *Annual Review of Food Science and Technology* 9:23-44. Review.
- Lehner *et al.* 2004. *BMC Microbiology* 4:43.
- Segars et al. 2016. Current Microbiology 72:190–197.
- Sonbol et al. 2013. *International Dairy Journal* 30: 1-7.
- Stoop *et al.* 2009. *International Journal of Food Microbiology* 136: 165–168.
- Sulaiman et al. 2015. *IAFP Annual Meeting*.
- Sulaiman et al. 2016. *ASM Microbe Meeting*.
- Sulaiman *et al.* 2016. *Current Microbiology* 73:273-279.
- Sulaiman et al. 2018. *ASM Microbe Meeting*.
- Sulaiman *et al.* 2017. *Journal of Food Protection* 80: 963-968.
- Sulaiman *et al.* 2018. *Journal of AOAC International* 101:1135-1143.
- Sulaiman *et al.* 2019. *Journal of AOAC International* 102:975-975.

Conclusion

- **The VITEK MS system appears to be a powerful tool under our laboratory conditions.**
- **The VITEK MS system can provide rapid species identification for the *Cronobacter sakazakii* isolates recovered from the environmental samples.**
- **The VITEK MS based ID was comparable to the data attained by the SLST and the MLST techniques.**
- **SLST based on the sequence characterization of 16S rRNA and *rpo*B genes provided *Cronobacter sakazakii* specific ID.**
- **MLST technique showed higher discriminatory capability. Thus, MLST can be more useful to conduct epidemiologic studies to better understand the transmission dynamics of these pathogens causing foodborne disease.**