Monday-162

A Retrospective Analysis of Bacterial DNA Identified from Clinical Isolates Using 16S rRNA Gene PCR and Sequence Analysis: 2009-2015

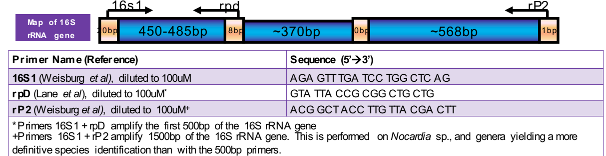
M. Dickinson, T. Passaretti, L. Thompson, D. Wroblewski, T. Quinlan, L. Mingle, K. Mitchell, A. Kidney, T. Halse, E. Nazarian, K. Musser

Overview

The 16S rRNA gene is essential for the survival of all bacteria and is highly conserved. The characterization of the 16S rRNA gene is accepted as a standard method for the identification of families, genera, and species of bacteria. As a reference laboratory for New York State (NYS), we routinely perform 16S rDNA sequence analysis to identify bacterial isolates that are difficult to classify by phenotypic methods alone. Our laboratory has been NYS-approved to report 16S rDNA PCR and sequencing results for clinical isolates since 2009. To assess the ability of this method to provide a definitive identification, a retrospective analysis was performed on 3438 clinical isolates that were tested and analyzed from 2009-2015. In addition, the impact of the implementation of Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) on both 16S rDNA PCR workload and clinical isolates identified to the species label is explored.

Methods

- Clinical isolates were received by the Bacteriology Laboratory
- From **2009-2012,** isolates underwent subculture, Gram stain and biochemical testing before being submitted for 16S rDNA PCR + sequence analysis.
- From **2013-2015**, isolates underwent subculture, Gram stain, and **MALDI-TOF MS** testing before being submitted for 16S rDNA PCR + sequence analysis.
  - Isolates undergo two different MALDI-TOF MS testing methods: formic acid smear, and formic acid-acetonitrile extraction (if necessary). All isolates are tested on the **microflex™ LT MALDI-TOF MS** instrument. Protein fingerprints are identified using the **MALDI Biotyper3 Software for Microorganism Identification and Classification** (Bruker Daltronics, Billerica, MA).
    1. **Formic Acid Smear**: an isolated colony from a fresh pure culture isolate is transferred to an polished steel target plate. One microliter of 70% formic acid is added to the smear, followed by 1ul HCCA matrix.
    2. **Formic Acid-Acetonitrile (FA-ACN) Extraction**: fresh pure culture isolates are suspended in 300ul of LC-MS water & 900ul of 100% EtOH. The suspension is centrifuged for two minutes at 13K rpm. Supernatant is decanted, centrifugation is repeated. Residual EtOH is decanted and pellet dries for 15 minutes. The bacterial pellet is suspended in 25ul of 70% formic acid, followed by 25ul of acetonitrile. After a two minute centrifugation at 13K rpm, 1ul of the supernatant is pipetted onto the polished steel target, followed by 1ul HCCA matrix.
- If an identification cannot be made based on biochemicals, real-time PCR and/or MALDI-TOF MS, **16S rDNA PCR + sequence analysis** is performed (adapted from CLSI MM18-A).



Primer Name (Reference)	Sequence (5'→3')
16S1 (Weisburg *et al*), diluted to 100uM	AGA GTT TGA TCC TGG CTC AG
rpD (Lane *et al*), diluted to 100uM*	GTA TTA CCG CGG CTG CTG
rP2 (Weisburg *et al*), diluted to 100uM+	ACG GCT ACC TTG TTA CGA CTT

*Primers 16S1 + rpD amplify the first 500bp of the 16S rRNA gene
+Primers 16S1 + rP2 amplify 1500bp of the 16S rRNA gene. This is performed on *Nocardia* sp., and genera yielding a more definitive species identification than with the 500bp primers.

Sequence Analysis

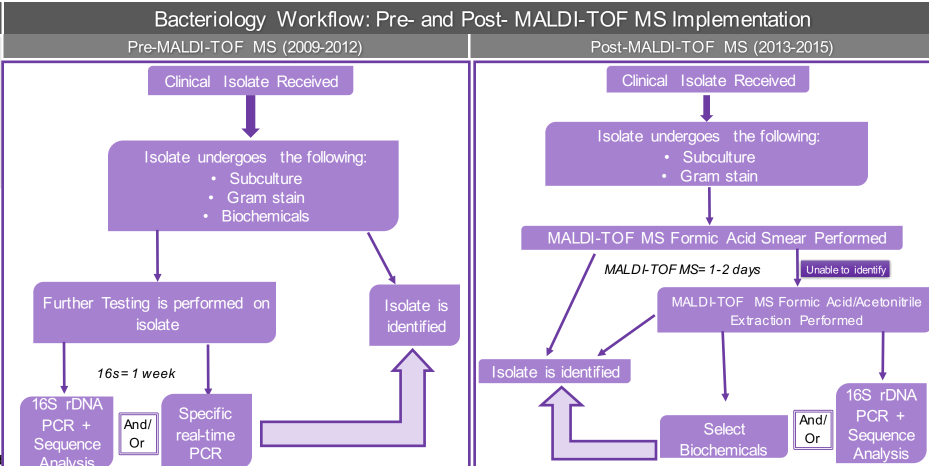
Raw sequences are generated by the Wadsworth Center Applied Technologies Genomics Core (AGTC).
Sequences are analyzed using two databases:

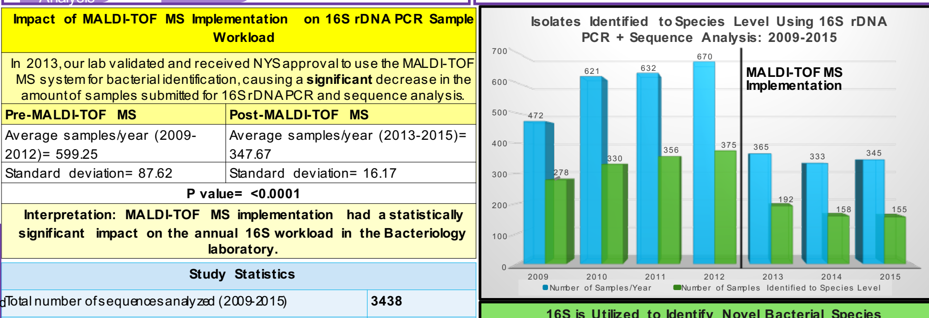
- **MicroSeq™ 16S rDNA Bacterial Identification System** (500 or Full Gene database) (Applied Biosystems)
- **EzBioCloud** (www.ezbiocloud.net/eztaxon).

Based on percent similarity of sequences from clinical isolates compared to these databases, **identifications are placed into one of five categories:**

1. **Species level identification**: >99% similarity to **one species.**
2. **Most closely resembles a species**: 98-99% **similarity to one species.**
3. **Most closely resembles a genus**: 95-98% similarity to **single or multiple organisms within the same genus.**
4. **Unable to differentiate**: >99% to **multiple species within the same genus.**
5. **Unable to identify**: <95% to any species.

Bacteriology Workflow: Pre- and Post- MALDI-TOF MS Implementation





Impact of MALDI-TOF MS Implementation on 16S rDNA PCR Sample Workload

In 2013, our lab validated and received NYS approval to use the MALDI-TOF MS system for bacterial identification, causing a **significant** decrease in the amount of samples submitted for 16S rDNA PCR and sequence analysis.

Pre-MALDI-TOF MS	Post-MALDI-TOF MS
Average samples/year (2009-2012)= 599.25	Average samples/year (2013-2015)= 347.67
Standard deviation= 87.62	Standard deviation= 16.17
P value= <0.0001	

Interpretation: MALDI-TOF MS implementation had a statistically significant impact on the annual 16S workload in the Bacteriology laboratory.

Study Statistics	
Total number of sequences analyzed (2009-2015)	**3438**
Range of sequences analyzed per year	**333-670**
Average number of sequences analyzed per year	**491**
Number of sequences identified to the **species level**	**1844 (54%)**
Number of sequences that **could not be differentiated**	**838 (24%)**
Number of sequences that **most closely resemble a genus**	**283 (8%)**
Number of sequences that **most closely resemble a species**	**162 (5%)**
Number of sequences that were **unable to be identified**	**140 (4%)**
Number of samples omitted from study (conflicting results, lack c results in LIMS, non-clinical samples)	**171 (5%)**

16S is Utilized to Identify Novel Bacterial Species

- Our vast 16S rDNA sequence collection is used to create a **Wadsworth 16S rDNA database of unknown/unclassified sequences.** This database is used to **screen for potential novel bacterial species.** Using both phenotypic and phylogenetic characterizations, we have identified and named the following species:

1. *Neisseria wadsworthii* (Wolfgang *et al.* 2011, sp. nov.)
2. *Neisseria shayeganii* (Wolfgang *et al.* 2011, sp. nov.)
3. *Psychrobacter sanguinis* (Wirth *et al.* 2012, sp. nov.)
4. *Neisseria oralis* (Wolfgang *et al.* 2013, sp. nov.)
5. *Sporosarcina newyorkensis* (Wolfgang *et al.* 2012, sp. nov.)
6. *Hazenella coriacea* (Buss *et al.* 2013, gen. nov, sp. nov) *type species for genus

Top 25 Organisms Identified to the Species level by 16S rDNA PCR Sequencing: Pre-MALDI-TOF MS and Post-MALDI-TOF MS Implementation

Pre-MALDI-TOF MS Implementation (2009-2012) (n=1354)		Post-MALDI-TOF MS Implementation (2013-2015) (n=490)	
Species	**Frequency (%)**	**Species**	**Frequency (%)**
Streptococcus intermedius	33 (2.4)	***Moraxella osloensis***	15 (3.0)
Staphylococcus epidermidis	33 (2.4)	*Capnocytophaga canimorsus*	14 (2.6)
Stenotrophomonas maltophilia	24 (1.8)	***Nocardia nova***	10 (2.0)
Neisseria elongata	22 (1.6)	***Neisseria elongata***	10 (2.0)
Aggregatibacter aphrophilus	22 (1.6)	*Gordonia bronchialis*	10 (2.0)
Pseudomonas aeruginosa	21 (1.6)	*Nocardia brasiliensis*	9 (1.8)
Nocardia cyriacigeorgica	20 (1.5)	***Nocardia cyriacigeorgica***	8 (1.6)
Nocardia farcinica	18 (1.3)	*Corynebacterium tuberculostearicum*	7 (1.4)
Moraxella osloensis	18 (1.3)	*Cardiobacterium hominis*	7 (1.4)
Streptococcus constellatus	18 (1.3)	*Sphingomonas mucosissima*	6 (1.2)
Streptococcus dysgalactiae	17 (1.3)	*Dolosigranulum pigrum*	6 (1.2)
Streptococcus anginosus	16 (1.2)	*Neisseria animaloris*^	6 (1.2)
Bacillus simplex	14 (1.0)	*Haemophilus parainfluenzae*	5 (1.0)
Lactobacillus rhamnosus	14 (1.0)	***Actinomyces europaeus***	5 (1.0)
Streptococcus mutans	14 (1.0)	*Oceanobacillus profundus*^	5 (1.0)
Eikenella corrodens	13 (1.0)	*Kroppenstedtia eburnea*^	5 (1.0)
Roseomonas mucosa	13 (1.0)	*Propionibacterium acnes*	5 (1.0)
Granulicatella adiacens	11 (0.8)	*Kerstersia gyiorum*	4 (0.8)
Nocardia nova	11 (0.8)	*Aureimonas altamirensis*	4 (0.8)
Oligella urethralis	11 (0.8)	*Psychrobacter sanguinis*	4 (0.8)
Streptococcus pyogenes	10 (0.7)	*Nocardia arthritidis*	4 (0.8)
Actinomyces europaeus	10 (0.7)	***Roseomonas mucosa***	4 (0.8)
Actinomyces neuii	10 (0.7)	***Nocardia farcinica***	4 (0.8)
Achromobacter insolitus	10 (0.7)	*Actinomyces israelii*	4 (0.8)

^Species is not represented in the Bruker database.

Conclusions

- 16S rDNA PCR and sequence analysis is an *indispensable* tool for bacterial identification in the NYS Bacteriology laboratory. Over a seven year timeframe, we have analyzed 3438 sequences, the majority of which have been identified to the species level (54%).
- The implementation of MALDI-TOF MS in our lab has had a significant impact on 16S workload, which is a great cost and time savings.
- 16S is successful in identifying isolates to the species level when MALDI-TOF MS is unable to do so, particularly in cases where the sample is not viable, there is a limited amount of bacterial growth, or the organism can only be grown in liquid media.
- 16S enables us to identify bacterial species that are not present in the Bruker database. This allows us to add these species to the Bruker database, further enhancing our MALDI-TOF MS capabilities.
- 16S has been used by our lab to identify and characterize six novel species of bacteria.

References

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., Pace, N.R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA. 82: 6955-6959.

Weisburg, W.G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. J. Bact. 173 (2): p. 697-703.

CLSI MM18-A, Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing, Approved Guideline

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S., Chun, J. (2012). Introducing EzTaxon: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* 62, 716–721.

Many thanks to the Wadsworth Center AGTC Core for providing all of the sequences used in this study.