Results of the National Proficiency Test for 16S rRNA Gene Sequencing

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National Molecular Group* [NMG] *Meeting

Hospital for Sick Children, Toronto, ON Oct. 23, 2012

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[Disclosures: NONE]

Objectives

- Background for 16S proficiency Test
- Review methods used to create a participant's list
- Criteria used to select bacteria for panel
- Review Results
- Comments provided by Participants
- Suggestions for other possible proficiency tests by NMG
- MALDI USER GROUP update

2006 - 2011

- NMG had discussed possibility for holding a national proficiency test for 16S
- Such a test not readily available from commercial proficiency test organizations, such as CAP, CMPT etc
- We (Special Bact) were asked to conduct a test in ~2010
- Initiated test in November 2011 by conducting a poll of interest

Establishing a Participant's List

- Surveyed NMG members for interest in participating in 2 tests:
  - 16S rRNA gene sequencing
  - *Bordetella pertussis* detection

- Used the 'Survey Monkey' tool to poll, with relatively tight (7d) deadline for response
- Survey Monkey poll sent to every member of the NMG list, plus several additional lab contacts, deemed to be possibly interested in participating= totalled surveyed ~ 205

Information asked for in Survey

- Basic demographics of participants
- 'Possible' or 'Definite' interest in participating in 1 or both proficiency tests
- If definite, would be asked to provide courier account no. closer to shipping time
- Suggestions for additional tests to be funded by NMG, to be funded at a future date

Results of Survey

- 16 respondents (~8%)
- 13 participant-sites for 16SrRNA sequencing proficiency test (only 1-2 for Bord pert)
  - 5 from hospitals (ON, 2; SK, 1; MB, 1; NB, 1)
  - 4 from PHLs (BC, AB, MB, SK)
  - 5 from 3 NML labs: SB, Enterics, BADD (note: Spec Bact staff blinded to test process; 1st (not counted here) performed 'pretest assay'; 2 rec'd strains at same time as everyone else)
- Participant numbers lower than expected
- Survey Monkey good tool for collecting info

Selecting Strains for Panel

- Due to anticipated mix of capacities/ skill sets, selected strains which were:
  - RL 1 or 2 (PT for RL3 agents done by NML's BADD group)
  - Good growers in 24-48h, should strain be subcultured upon receipt (eg did not send strict anaerobes; slow growing nocardias etc)
  - Should be relatively easy to extract DNA
  - Should be unambiguously identified by use of 16S rRNA gene sequencing alone.

What does Unambiguous MEAN exactly?

- Various philosophies on this in literature dating back to the first days of labs doing 16S rRNA with modern sequencing methods, dated back to mid to late 1990s

- Here, implied that after BLASTing, sequence for test strain was sufficiently discernable to be unequivocally differentiated from species / taxa which were closest relatives…

AVOIDED sending out Strain like this:
NML 100241-ID, Identified as a *Bacillus cereus*

Accession	Description	Max ident
AB295053.1	Bacillus thuringiensis gene for 16S rRNA, NK2	99%
AE017194.1	Bacillus cereus ATCC 10987, complete genome	99%
CP001407.1	Bacillus cereus 03BB102, complete genome	99%
CP000485.1	Bacillus thuringiensis Al Hakam, complete genome	99%
CP001746.1	Bacillus anthracis str. CI, complete genome	99%
GU826153.1	Bacillus anthracis strain Q23	99%
GU826150.1	Bacillus anthracis strain H13	99%
CP001598.1	Bacillus anthracis str. A0248	99%
CP001215.1	Bacillus anthracis str. CDC 684	99%
EU429664.1	Bacillus thuringiensis sv ostriniae	99%
AE017334.2	Bacillus anthracis str. 'Ames Ancestor' c. genome	99%
AE017225.1	Bacillus anthracis str. Sterne, complete genome	99%
AY425946.1	Bacillus cereus str G9241 16S rRNA	99%

B. mycoides &*B weihenstephensis* +species above, ≥99.3% to each other
[Blast edited to fit slide]

Info Requested from participants

- Demographics
- No. bps obtained
- 'Blast Scores Obtained'
- Bacterium identifed as (Interpretation based on BLAST obtained)
- Databases used when doing BLAST (eg RIDOM, Genbank, RDP etc)
- Other comments

Other Info Requested from each Participant

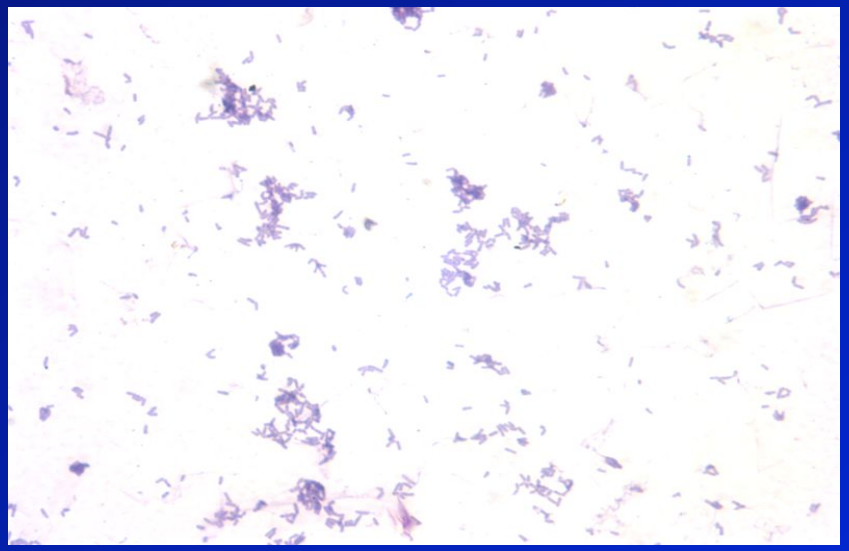
- Brief outline of Methods used
- Regions targeted
- Typical no. bps expected from method (as opposed to no. bps ACTUALLY obtained)
- Discuss concept of 'percent cut off' and other 'quality indices' to assign BLAST result to a specific genus and species [cite literature reference for decision]
- Other relevant info

Results: Bacteria selected for Panel

External Proficiency Test (EPT)-01, *Corynebacterium kroppenstedtii* CCUG 35721T

- Described by Collins et al 1998 IJSEM 48:1449-1454; somewhat lipophilic
- Associated with granulomatous mastitis
- <95% identity to other Coryne spp (using GB refseq)

***C. kroppenstedtii,* 48h, SBA enriched with 1% Tween 80, Google Image**

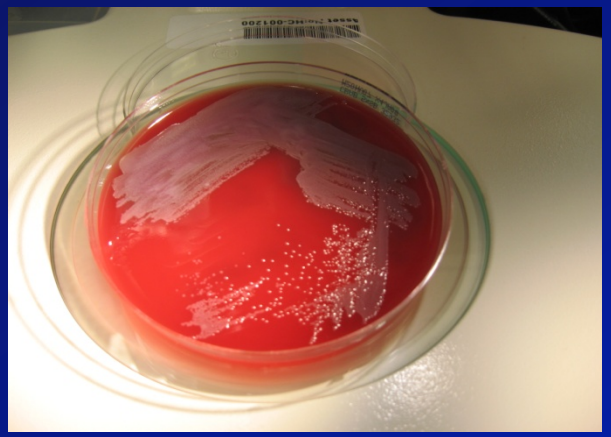


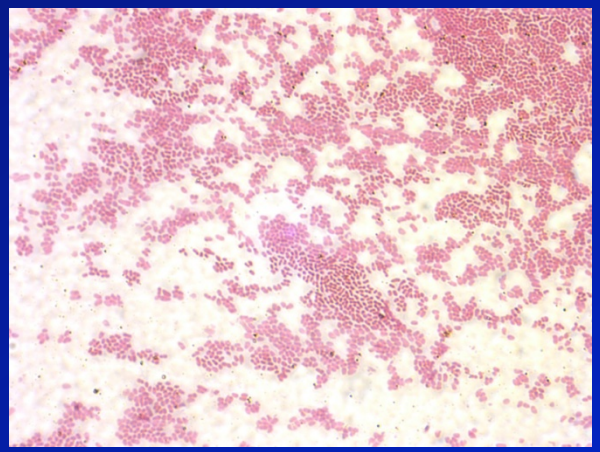
***C. kroppenstedtii* CCUG 35717T 48h, SBA, NML photo**

EPT-2

EPT-2. *Chryseobacterium hominis* CCUG 52711T

- Described in 2007 [Vaneechoutte et al IJSEM 57:2623-2628]; CDC groups II-h, II-c consistent
- Rare pathogen, recovered from blood cultures
- Using GB 'refseq' option, closest relative to (97%) to *C. hispanicum* & several other species in genus
- *C. arothi* >99% in BLAST, but considered as heterotypic synonym (commented on by 2/13 participants)





NML Photos of CCUG 52711T 24h, 35C SBA

Results

- Provided demographics on results sheet: 11/13 (2/13, got info from fax)
- Length of seq obtained from method
  - Full or nearly full seq: 4/13 (>1450 bps)
  - Partial: 9/13, ranging from 450-800 bps; if described, seq from 5' end (V1-V4)
  - All labs ~ achieved anticipated/usual no. bps
- Databases used: Genbank (nr/nt; refseq; or option not described); RDP as sole or 2nd DB
- Most (11/13) described 'their' method either in brief or in some detail
- 2 labs: colony/pheno rxs must agree with 16S

Identification Results

Identified as	**EPT-1 *Corynebacterium kroppenstedtii* CCUG 35721T**	**Identified as**	**EPT-2, *Chryseo-bacterium hominis* CCUG 52711T**
C. kroppenstedtii	10/13	*C. hominis*	10/13
Corynebacterium species, most like or most closely resembling, *C. kroppenstedtii*	2/13	*Chryseobacterium* species, probably or most closely resembles, *C. hominis*	2/13
Target organism not recovered	1/13	*Chryseobacterium* species	1/13

Methods for Work

Extraction: 6/13 described method

- most (5/6) used boil prep and/or Qiagen kit esp if boil not satisfactory (2 described problem with boil alone)
- 1 used Epicentre kit; 1 started with emulsion in glass beads

Product:

- most used in-house method; 1 used 16SrDNA Bacterial ID PCR, Sequencing kit (Applied Biosystems)

Sequencing:

- Most used in-house sequencing services or did not state; 2 used external seq. services

References Cited for In-House Methods

- * Edwards, U., Rogall, T., Blocher, H. et al. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S rRNA. Nuc. Acids Res. 17: 7843-7853.
- Ehrsmann et al 1972. The determination of the primary structure of the 16S rRNA of E. coli. 2. Nucleotide sequence of products from partial enzymatic hydrolysis. Biochimie 54:901-967
- Heritz et al 1997. Detection of eubacteria in interstitial cystitis by 16S rDNA amplification. J. Urol. 158:2291-2295
- Lane et al 1985 Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses PNAS 82:6955-6959
- Petti et al 2007. Detection and Identification of microorganisms by gene amplification and sequencing CID 44:1108-1114
- * Petti et al 2008. Interpretive Criteria for Identification of bacteria and fungi by DNA Target sequencing. Approved Guideline. MM18-A CLSI Wayne PA
- Weisburg et al 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173:697-703
- Woo et al, 2009 Detection & Identification of 16S..for identification of medically important aerobic Gram-positive bacteria J. Med. Microbiol. 58:1030-1036

* **Cited by > 1 lab/participant.**

Info Inferred from Results

- EPT-1: 12/13 labs got same (correct) answer to genus (1/13 could not grow target org.).
- EPT-1: 2/13 reported difficulties with extraction, had to move to 'cleaner' (Qiagen) prep
- EPT-2, 13/13 correct to genus
- EPT-1 & -2: Several labs did not ID to species, associated with sole use of RDP OR less stringent 'cut off' value
- Results similar, in spite of use of a wide variety of source methods being cited for in-house procedures
- Results similar, whether full or partial sequencing used
- All conversant in meaning of quality indices ['Max & Total Score', 'Query Coverage', 'Max Ident']
- Ambiguous bps or other technical issues usually commented on

Spec Bact- Our Philosophy re: 'Cut Offs'

- Use criteria described by Stackebrandt and Ebers, based on study of 16s data applied to wide variety of phyla; reasonable coherence found between older & newer methods
- Widely used by taxonomists for describing new genera and species
- Works well for wide variety of unusual, rare pathogens which exclude enterics, mycobacteria, other [note we corroborate ID by > 1 method]
- Consider % identity after comparison only to Type Strains. If none, assign to genus or family
- If > 1 species at 98.8%, test 2$^{nd/\ additional}$ targets to discern among members of group similar by 16S

Philosophies re: "Cut Off Values"

Reference	Member of same species	Member of same genus
Stackebrandt & Ebers (2006)	≥ 98.7 %	Not delineated
MM 18 (CLSI 2008)	~ ≥99.0%	≥97.0%; some genera ≥95.0%
Tindall et al, (2010)	≥ 97 %, corrob as same or different	<95.0% can not assign to genus
Green & Janda (2010)	≥ 98.7 %	≥97.0%; <95.0% can not assign to genus

Review of 'Cut Offs' from Prof Test

- 6/13 used MM18A (SB staff, also used Stackebrandt and Ebers); do not automatically use top choice (consider Quality indices, if TS etc)
- 3/13 labs: do NOT use pre-established value, either want to hear what others do, or decide, case by case, based on taxon
- 2 labs: either 98% with ≤3 gaps for genus and species or stated '98%-99%'
- 1 lab: 99-100%, genus and species; 80-98%, Genus only
- 1 lab: > 90% for genus; >95% for species

Methods Cited for Establishing 'Cut Off' Criteria

- ★Clarridge, J. E. III. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on Clinical Microbiology and Infectious Diseases. Clin. Microbiol. Rev. 17:840-862.
- ★Drancourt et al 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. 38:3623-3630
- ★Janda and Abbott 2007 16S rRNA Gene Sequencing for Bacterial Identfication in the Diagnostic Laboratory: Pluses, Perils and Pitfalls. J. Clin. Microbiol. 45:2762-2764
- ★Patel, JB. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol. Diagn. 6:313-321
- ★★ Petti et al 2008. Interpretive Criteria for Identification of bacteria and fungi by DNA Target sequencing. Approved Guideline. MM18-A CLSI Wayne PA
- ★★ Stackebrandt, E. and Ebers, J. 2006. Taxonomic parameters revisited: Tarnished Gold Standards. Microbiol. Today. 33: 152-155.

Also cited:

- ★ Blackwood et al J. Clin. Microbiol. 42:5001-5006
- ★ Turenne et al. 2001 Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous Mycobacterium species. J. Clin. Microbiol. 39:3637-3648
- ★ Mellman et al 2003 Int. J. Med. Microbiol. 293 :

- ★ cited by 1 lab; ★★ cited by more than 1 lab; some labs cited > 1 reference.

Other Possible Proficiency Tests to consider (SM Poll)

1. Respiratory or meningeal pathogens eg *Chlamydophila/ Legionella/Mycoplasma/Strept pneumo/ H. influ*.
2. Test to differentiate: MRSA from MSSA; SCC + strains; detect *Listeria; Cronobacter*
3. Detection various respiratory viruses (human RVS-A&B), FluA, FluB, Adeno, Arbo, MPV, PIC (1-4), HRV (A, B, C); HEV; HBoV (1-4), human coronovirus 229/NL63, OC43)
4. STI, Enteric, other Viruses: Rotavirus/Adenovirusm; RT PCR Novovirus; Enterovirus; Herpes (HSV 1,2); BK viruses
5. PCR detection of *eae* of *E. coli*; *C. diphtheriae tox* gene; *van* genes of VRE; species of *Enterococcus*
6. HEP C genotyping
7. Comment: support Canadian test panels for Canadian Labs

UPDATE on MALDI

- May 2012: a Canadian MALDI-TOF User Group meeting, provisionally nicknamed 'CMUG' was held during the CACMID-AMMI meeting in Vancouver (M. Alfa, K.Bernard, co-chairs)
- ~ 40-45 people including vendors; minutes delayed but will be posted; on line discussion forum proposed, P. Legace-Wiens possible moderator;
- M-K Lee drafted first page for a CMUG website
- ?? NML host to a national database of MSPs (Bruker type), as this will be possible with next update of Bruker software

QUESTIONS:

- Interest in this? Separate org or linked to NMG? Who $$ $hould host website? Should NMG host a proficiency test for MALDI in 2013?