Application of metagenomics for bloodstream infections

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## Disclosure of speaker’s interests

<table>
<thead>
<tr>
<th>(Potential) conflict of interest</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potentially relevant company relationships in connection with event</td>
<td>Scientific Collaborations with Checkpoints, BioVisible, Ridom, Bioclear, Qiagen CLC bio – <strong>no personal benefits</strong></td>
</tr>
<tr>
<td>Sponsorship or research funding</td>
<td>Several National and European Grants</td>
</tr>
</tbody>
</table>
Medical Microbiology and the evolution of technology

- Classic culture → still considered the “gold standard” → laborious and time-consuming
- MALDI-ToF revolutionized the identification of microorganisms
- MDx reduced time to result for all kind of samples

Opota et al., CMI, 2015
Point of care (point of impact) for ID positive blood cultures

- MR(SA)
- VAN A / VAN B
- C. diff
- TB/RIF

You only see what you are looking for
Microbiome (16 S) targeted NGS

- Sequencing of all bacteria in a sample by barcoded sequencing of the V3/V4 or V5-V7 regions of the 16S-rDNA gene
Diagnosis of Bacterial Bloodstream infections using 16S metagenomics

- 75 children (median age 15 months) with severe febrile illness in Burkina Faso
- Standard blood culture and malaria testing
- 16S (V3–V4 regions) metagenomics retrospectively
- Miseq 600 cycles (56 hrs)
- ID using full length 16S SILVA reference database
- 4 negative control samples (contaminants)
- Increase in number of positive samples
- Detection of more than one bacterial species in almost half of the confirmed bBSI patients, a finding which was completely missed by blood culture
- 51 samples:
  - reads only identified to genus level and not clinically relevant → negative
  - reads not reproducible identification to species level → negative
- Species identified in negative control
- Read depths of pathogens varied between replicates
  - limited blood volumes tested
  - possible amplification bias by PCR during the library preparation
  - the inherent variation between deep sequencing
Microbiome in blood samples from healthy donors

16S copies per ml whole blood

B. Phyla

C. 16S copies in Whole Blood (log scale)

Paisse et al. TRANSFUSION 2016;56;1138–1147
Summary 16 S Metagenomics

• a diversified microbiome exists in healthy blood
• appropriate control samples crucial to detect background signals
• no AMR genes
• no viruses/fungi/parasites
• not always identification to the species level
Improved identification/resolution by 16 – 23 S NGS

- DNA extraction
- 16S-23S rRNA PCR (~ 4.5 kB)
- Library preparation
- Next Generation Sequencing (MiSeq, V3, 600)
- de novo reads assembly (Qiagen (CLC) Genomic Workbench)
- basic local alignment search (BLAST, le BiBi)

Project leader: Mirjam Kooistra
Sabat et al, submitted to SREP
16S-23S on spiked samples

% identified

Species | Genus

Acinetobacter | Corynebacterium | Enterobacteriaceae | Enterococcus | Staphylococcus | Streptococcus | Other | All

16S rRNA sequencing

16S-23S rRNA sequencing

Ion 16S Metagenomics kit (Thermo Fisher)

Vitek MS (BioMérieux)

Microflex MS (Bruker)
### Identification of pathogens

**TABLE 3.** Bacterial identification results from 23 positive blood culture bottles based on culture and NGS of 16S-23S rRNA region.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Patient</th>
<th>Bottle</th>
<th>Culture (Maldi-TOF MS)</th>
<th>NGS of 16S-23S rRNA region (% of total reads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC01</td>
<td>Patient A</td>
<td>anaerobic</td>
<td><em>Escherichia coli</em></td>
<td><em>Escherichia coli</em> (100%)</td>
</tr>
<tr>
<td>BC02</td>
<td>Patient B</td>
<td>aerobic</td>
<td><em>Streptococcus dysgalactiae</em></td>
<td><em>Streptococcus dysgalactiae</em> (100%)</td>
</tr>
<tr>
<td>BC03</td>
<td>Patient C</td>
<td>anaerobic</td>
<td><em>Klebsiella oxytoca</em></td>
<td><em>Klebsiella oxytoca</em> (100%)</td>
</tr>
<tr>
<td>BC05</td>
<td>Patient D</td>
<td>aerobic</td>
<td><em>Staphylococcus hemolyticus</em></td>
<td><em>Staphylococcus hemolyticus</em> (100%)</td>
</tr>
<tr>
<td>BC06</td>
<td>Patient E</td>
<td>anaerobic</td>
<td><em>Staphylococcus hominis</em></td>
<td><em>Staphylococcus hominis</em> (100%)</td>
</tr>
<tr>
<td>BC07</td>
<td>Patient F</td>
<td>aerobic</td>
<td><em>Staphylococcus capitis</em></td>
<td><em>Staphylococcus capitis</em> (100%)</td>
</tr>
<tr>
<td>BC08</td>
<td>Patient G</td>
<td>anaerobic</td>
<td><em>Streptococcus pneumoniae</em></td>
<td><em>Streptococcus pneumoniae</em> (100%)</td>
</tr>
<tr>
<td>BC09</td>
<td>Patient H</td>
<td>aerobic</td>
<td><em>Staphylococcus epidermidis</em></td>
<td><em>Staphylococcus epidermidis</em> (100%)</td>
</tr>
<tr>
<td>BC10</td>
<td>Patient I</td>
<td>anaerobic</td>
<td><em>Staphylococcus hominis</em></td>
<td><em>Staphylococcus hominis</em> (100%)</td>
</tr>
<tr>
<td>BC11</td>
<td>Patient J</td>
<td>anaerobic</td>
<td><em>Bacteroides sp.</em></td>
<td><em>Bacteroides fragilis</em> (100%)</td>
</tr>
<tr>
<td>BC12</td>
<td>Patient K</td>
<td>aerobic</td>
<td><em>Staphylococcus hominis</em></td>
<td><em>Staphylococcus hominis</em> (100%)</td>
</tr>
<tr>
<td>BC13</td>
<td>Patient L</td>
<td>anaerobic</td>
<td><em>Bacteroides ovatus</em></td>
<td><em>Bacteroides ovatus</em> (100%)</td>
</tr>
<tr>
<td>BC14</td>
<td>Patient M</td>
<td>anaerobic</td>
<td><em>Staphylococcus aureus</em></td>
<td><em>Staphylococcus aureus</em> (100%)</td>
</tr>
<tr>
<td>BC15</td>
<td>Patient N</td>
<td>aerobic</td>
<td><em>Escherichia coli</em></td>
<td><em>Escherichia coli</em> (100%)</td>
</tr>
<tr>
<td>BC16</td>
<td>Patient O</td>
<td>anaerobic</td>
<td><em>Staphylococcus aureus</em></td>
<td><em>Staphylococcus aureus</em> (100%)</td>
</tr>
<tr>
<td>BC17</td>
<td>Patient P</td>
<td>anaerobic</td>
<td><em>Streptococcus pneumoniae</em></td>
<td><em>Streptococcus pneumoniae</em> (100%)</td>
</tr>
<tr>
<td>BC18</td>
<td>Patient Q</td>
<td>aerobic</td>
<td><em>Escherichia coli, Streptococcus infantis</em></td>
<td><em>Escherichia coli</em> (69.3%), <em>Streptococcus lutensis</em> (30.7%)</td>
</tr>
<tr>
<td>BC19</td>
<td>Patient R</td>
<td>anaerobic</td>
<td><em>Escherichia coli</em></td>
<td><em>Escherichia coli</em> (100%)</td>
</tr>
<tr>
<td>BC20</td>
<td>Patient S</td>
<td>anaerobic</td>
<td><em>Escherichia coli</em></td>
<td><em>Escherichia coli</em> (100%)</td>
</tr>
<tr>
<td>BC21</td>
<td>Patient T</td>
<td>aerobic</td>
<td><em>Escherichia coli</em></td>
<td><em>Escherichia coli</em> (100%)</td>
</tr>
<tr>
<td>BC22</td>
<td>Patient U</td>
<td>anaerobic</td>
<td><em>Bacteroides vulgatus</em></td>
<td><em>Bacteroides vulgatus</em> (100%)</td>
</tr>
<tr>
<td>BC23</td>
<td>Patient V</td>
<td>aerobic</td>
<td><em>Staphylococcus hominis</em></td>
<td><em>Staphylococcus hominis</em> (100%)</td>
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<tr>
<td>BC24</td>
<td>Patient W</td>
<td>aerobic</td>
<td><em>Staphylococcus epidermidis</em></td>
<td><em>Staphylococcus epidermidis</em> (100%)</td>
</tr>
<tr>
<td>Sample</td>
<td>Patient</td>
<td>Material</td>
<td>Culture</td>
<td>NGS of 16S-23S rRNA region (% of total reads)</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>----------</td>
<td>---------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>KM1</td>
<td>Patient A</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>Propionibacterium acnes (9.1%), Haemophilus parasuis (2.3%), eukaryotic DNA (88.6%)</td>
</tr>
<tr>
<td>KM2</td>
<td>Patient A</td>
<td>punctate (fluid)</td>
<td>Negative</td>
<td>eukaryotic DNA (100%)</td>
</tr>
<tr>
<td>KM3</td>
<td>Patient A</td>
<td>punctate (fluid)</td>
<td>Negative</td>
<td>Salmansleria salmonae (0.3%), eukaryotic DNA (89.7%)</td>
</tr>
<tr>
<td>KM4</td>
<td>Patient A</td>
<td>punctate (fluid)</td>
<td>Negative</td>
<td>Gemella sanguinis (1.3%), Haemophilus parasuis (1.0%), eukaryotic DNA (97.7%)</td>
</tr>
<tr>
<td>KM5</td>
<td>Patient A</td>
<td>punctate (fluid)</td>
<td>Negative</td>
<td>Herminimonas sp. (10.5%), Propionibacterium acnes (9.7%), Moraxella catarrhalis (7.5%), eukaryotic DNA (72.3%)</td>
</tr>
<tr>
<td>KM6</td>
<td>Patient B</td>
<td>pus</td>
<td>Negative</td>
<td>Streptococcus intermedius (100%)</td>
</tr>
<tr>
<td>KM7</td>
<td>Patient C</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>eukaryotic DNA (100%)</td>
</tr>
<tr>
<td>KM8</td>
<td>Patient C</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>No identification</td>
</tr>
<tr>
<td>KM9</td>
<td>Patient D</td>
<td>joint puncture (fluid)</td>
<td>Negative</td>
<td>Enhydrobacter aerogenes (49.8%), Acinetobacter sp. (18.1%), Moraxella olosaensis (14.0%), Staphylococcus sp. (5.8%), Pseudomonas aeruginosa (3.1%), Staphylococcus epidermidis (0.6%), Psychrobacter sp. (0.5%), Propionibacterium acnes (1.3%), AkabERP (0.6%), Acinetobacter sp. (0.4%), Chryseobacterium sp. (0.3%)</td>
</tr>
<tr>
<td>KM10</td>
<td>Patient D</td>
<td>joint puncture (fluid)</td>
<td>Negative</td>
<td>No identification</td>
</tr>
<tr>
<td>KM11</td>
<td>Patient D</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>Propionibacterium acnes (9.8%), Bacillus nealsomii (6.7%), Pseudomonas fluorescens (0.6%), eukaryotic DNA (82.9%)</td>
</tr>
<tr>
<td>KM12</td>
<td>Patient D</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>eukaryotic DNA (100%)</td>
</tr>
<tr>
<td>KM13</td>
<td>Patient D</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>Unalbacter oligocarbonophilum (3.5%), Propionibacterium acnes (0.7%), eukaryotic DNA (95.9%)</td>
</tr>
<tr>
<td>KM14</td>
<td>Patient D</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>Propionibacterium acnes (1.4%), eukaryotic DNA (98.6%)</td>
</tr>
<tr>
<td>KM15</td>
<td>Patient D</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>Veillamella parvula (0.9%), eukaryotic DNA (99.1%)</td>
</tr>
<tr>
<td>KM16</td>
<td>Patient D</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>eukaryotic DNA (100%)</td>
</tr>
<tr>
<td>KM17</td>
<td>Patient E</td>
<td>blood</td>
<td>n.d.</td>
<td>Bacillus cereus (0.5%), eukaryotic DNA (99.5%)</td>
</tr>
<tr>
<td>KM18</td>
<td>Obstruction material A</td>
<td>formaline captured, biopsy (tissue)</td>
<td>n.d.</td>
<td>Propionibacterium acnes (64.4%), Staphylococcus epidermidis (25.4%), Paracoccus sanguinis (10.1%)</td>
</tr>
<tr>
<td>KM19</td>
<td>Obstruction material B</td>
<td>formaline captured, lung biopsy (tissue)</td>
<td>n.d.</td>
<td>Staphylococcus epidermidis (56.0%), Propionibacterium acnes (34.6%), Pseudomonas fluorescens (9.4%)</td>
</tr>
<tr>
<td>KM20</td>
<td>Patient F</td>
<td>joint puncture (fluid)</td>
<td>Negative</td>
<td>eukaryotic DNA (100%)</td>
</tr>
<tr>
<td>KM21</td>
<td>Patient F</td>
<td>biopsy (tissue)</td>
<td>Negative</td>
<td>Acinetobacter sp. (18.6%), Paucibacter sp. (12.8%), Herminimonas arsenicoxydans (5.2%), eukaryotic DNA (63.4%)</td>
</tr>
</tbody>
</table>

1Species present in negative control(s) and regarded as contamination introduced during sample preparation.
2Genus absent in negative controls but previously reported as contamination of DNA extraction kits, PCR and other laboratory reagents.

Sabat et al, submitted to SREP
Detection of antimicrobial resistance

- Check-Direct BD Max CPE/ESBL Screen
  - direct clinical material (eSwab)
  - also blood cultures (not yet CE-IVD)

  **carbapenemases** - KPC, OXA-48, VIM and NDM - including the emerging OXA-181 variant

  **ESBL target genes** CTX-M-1 group, CTX-M-2 group, CTX-M-9 group, SHV-ESBL

A. Williams et al., BCAS poster 2014
High-throughput deep-sequencing

- Sequence all the DNA and/or RNA
- Profiling of microbial communities
- Universal pathogen detection
- Host response to infectious diseases
Metagenomic analysis of bloodstream infections

- 27 blood samples from 9 patients with acute leukemia and suspected BSI at different time points of their antimicrobial treatment
- DNA extraction (Molzym)
- DNA processed to NebNext microbiome enrichment
- Amplification and Nextera XT library preparation
- 2*100 base pair PE sequencing on a HiSeq 2500
1,005,260,502 reads (33.5 million reads per sample) sequenced per sample on average
• Tetracycline resistance genes in 2 samples (F, PF)
• Macrolide Lincosamide Streptogramin resistance genes in 4 samples (1 F, 3 PF)
Conclusions of this paper

• Routinely used blood culture only detects a proportion of pathogens
• Viral and fungal pathogens present in immunocompromised patients
• Bacterial infections diagnosed with blood culture and treated with antibiotics
• Fungal and viral infections often undetected in BSI, although associated with higher mortality rates in specific patient groups
Detection and characterization of Dengue viruses

- Library preparation: 1.5 days
- Sequencing: 1 day
- Data Analysis: 10 minutes – 1 hour

TruSeq RNA sample preparation V2 (Illumina)

MiSeq Instrument Kit V2 300 cycles Paired-end (Illumina)

• Library validation
• Cluster generation

Qubit Agilent technologies

Natacha Couto, Erwin Raangs, Erley Lizarazo
Bioinformatics analysis

1. Ultra fast web-tool: Taxonomer

Raw reads

2. CLC Genomics Workbench v9.5.4.

Raw reads

QC

Quality Trimming

Map against human genome (hg19)

Mapped reads

Unmapped reads

Map against reference

De novo assembly

Blast

Robert Schlaberg
Taxonomer results

<table>
<thead>
<tr>
<th></th>
<th>human</th>
<th>viral</th>
<th>bacterial</th>
<th>ambiguous</th>
<th>unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample1</td>
<td>1799273 (91.9%)</td>
<td>55286 (2.8%)</td>
<td>32116 (1.6%)</td>
<td>40762 (2.1%)</td>
<td>31664 (1.6%)</td>
</tr>
<tr>
<td>Sample2</td>
<td>1061246 (83.0%)</td>
<td>11865 (0.9%)</td>
<td>29354 (2.3%)</td>
<td>37695 (3.0%)</td>
<td>137676 (10.8%)</td>
</tr>
<tr>
<td>Sample3</td>
<td>1059851 (75.6%)</td>
<td>144024 (10.3%)</td>
<td>31810 (2.3%)</td>
<td>51882 (3.7%)</td>
<td>113368 (8.1%)</td>
</tr>
<tr>
<td>Sample4</td>
<td>1448970 (79.6%)</td>
<td>83284 (4.6%)</td>
<td>46983 (2.6%)</td>
<td>76781 (4.2%)</td>
<td>163584 (9.0%)</td>
</tr>
<tr>
<td>Sample5</td>
<td>1046940 (74.5%)</td>
<td>28552 (2.0%)</td>
<td>42867 (3.1%)</td>
<td>79974 (5.7%)</td>
<td>206749 (14.7%)</td>
</tr>
</tbody>
</table>

Dengue 2 (DENV2) was identified in all samples

Quick analysis: 2-5 minutes
Full analysis: 2 hours
## CLC workbench results

### Mapping against human genome

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number of reads</th>
<th>Mapped reads against hg19</th>
<th>Unmapped reads against hg19</th>
<th>Dengue-2 mapped reads</th>
<th>Average coverage</th>
<th>Longest contig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3,918,662</td>
<td>3671259 (93.7 %)</td>
<td>247403 (6.3 %)</td>
<td>110770</td>
<td>1480.6</td>
<td>10694</td>
</tr>
<tr>
<td>2</td>
<td>2,556,120</td>
<td>2186315 (85.5 %)</td>
<td>369805 (14.5 %)</td>
<td>13517</td>
<td>176.5</td>
<td>10712</td>
</tr>
<tr>
<td>3</td>
<td>2,802,530</td>
<td>2220772 (79.2 %)</td>
<td>581758 (20.8 %)</td>
<td>288694</td>
<td>3747.2</td>
<td>10763</td>
</tr>
<tr>
<td>4</td>
<td>3,640,058</td>
<td>3038591 (83.5 %)</td>
<td>601467 (16.5 %)</td>
<td>165422</td>
<td>2146.9</td>
<td>10711</td>
</tr>
<tr>
<td>5</td>
<td>2,810,772</td>
<td>2251377 (80.1 %)</td>
<td>559395 (19.9 %)</td>
<td>53517</td>
<td>704.6</td>
<td>10619</td>
</tr>
</tbody>
</table>

hg19: human genome 19

- Near full-length genome sequences in all samples
- Proportion of DENV2 mapped reads varied from 0.5% to 10.3%
Phylogenetic analysis

American/Asian Genotype
Sub-cluster B
Shotgun metagenomics

- Improved turn around time to detect and characterize dengue virus
- Universal method for all kind of viruses
- Allows detection of genetic variants within the sample
- Superior resolution of DENV dynamics given by phylogenetic analysis of complete genome

What to improve?

- The proportion of human DNA should be as low as possible (human DNA depletion)
- Increase the coverage per sample
iDTECT™ Dx Blood test

- CE-IVD metagenomic test
- identify bacteria and viruses in blood samples

Source: Pathoquest website
- species qualified with identification confidence index
  - genome coverage
  - alignment distribution metric p-value
  - number of segments for segmented viruses

- thresholds defined prior to the study based on in silico analyses of simulated samples
Blood spiked with 5 viruses and 4 bacteria (Gram +/-, DNA, RNA, ss, ds)

<table>
<thead>
<tr>
<th>Method</th>
<th>Installation</th>
<th>Usability</th>
<th>Method</th>
<th>Nucl / Prot</th>
<th>Databases</th>
<th>Maintained</th>
<th>Speed (5Mlo)</th>
<th>Reported</th>
<th>Bacteria</th>
<th>Missed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surpi</td>
<td>Align</td>
<td>N</td>
<td>RefSeq/NT</td>
<td>Q2 2015</td>
<td>25 min</td>
<td>150</td>
<td>361</td>
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<tr>
<td>Kraken</td>
<td>K-mer LCA</td>
<td>N</td>
<td>RefSeq</td>
<td>Q1 2016</td>
<td>1 min</td>
<td>231</td>
<td>874</td>
<td>1 virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ReadScan</td>
<td>Align</td>
<td>N</td>
<td>NC</td>
<td>Q3 2012</td>
<td>21 h</td>
<td>65</td>
<td>395</td>
<td>1 bact.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taxonomer</td>
<td>K-mer LCA</td>
<td>N+P</td>
<td>Greengenes Uniprot</td>
<td>Q2 2016</td>
<td>30 min</td>
<td>203</td>
<td>1949</td>
<td></td>
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<tr>
<td>CosmosID</td>
<td>Cloud</td>
<td>?</td>
<td>Proprietary</td>
<td>Q2 2017</td>
<td>10 min</td>
<td>20</td>
<td>18</td>
<td>1 virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iDTECT</td>
<td>Align</td>
<td>N</td>
<td>iDTECT</td>
<td>Q1 2017</td>
<td>15 min</td>
<td>5</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Identification Confidence Index
- True positives / False positives
  - Low: 0/5
  - Medium: 1/3
  - High: 3/2

High FP:
- *P. acnes*
- *S. mitis*
Untargeted next-generation sequencing-based first-line diagnosis

- multicentre, blinded, prospective, proof-of-concept study
- no fungi and parasites in this version
- 101 immunocompromised adults followed for 30 days
- more clinically relevant viruses/bacteria with uNGS 36/101 (36%) vs SP 11/101 (11%) [after 30 days 19/101 (19%)]
- high negative predictive value compared with conventional methods (64/65, 95% CI)

Parize et al. CMI 2017
http://dx.doi.org/10.1016/j.cmi.2017.02.006
Two discordant cases:

- **CMV**(10⁵ copies/ml)
  - uNGS → plasma; CMV → whole blood
  - qPCR on plasma also negative

- **E. coli** bacteraemia
  - considered as contaminants
  - some reagents used for uNGS contain residual amounts of **E. coli** DNA
Summary

• Metagenomics will significantly reduce the turnaround time of the microbial diagnosis of BSI
• Benefit for patient care
• Possibility to differentiate bacteria to the species level
• Detection of AMR genes → important stewardship implications
Limitations of metagenomics (on blood)

- Genotype not always phenotype → RNA sequencing?
- Presence of a high quantity of non-microbial nucleic acids
- Presence of contaminant DNA → reagents, sample taking
- Presence of microbial DNA in healthy individuals
- Persistence of DNA from dead microbes
- Documentation required for impact on costs (length of stay, hospitalization, additional diagnostic tests)
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