Methods for Surveillance of Antimicrobial Resistant Bacteria in Environmental Water and Wastewater

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Presented Graciously by Dr. Emanuele Sozzi

Antibiotic Resistance is an Increasing Threat to Health

Few if Any New Antimicrobials

Many Resistance Mechanisms


**AMR Hotspots as Targets for Interventions to Reduce Risks**

**Waterborne Disease Interactions in the Water Environment: A One Health Issue**

- There are currently no globally coordinated efforts for AMR surveillance, response, and prevention. (We’re getting there...)

- Little is actually known about the magnitude, global landscape, and trends of ARB due to lack of harmonized, coordinated data and method for data collection.

- A simple but robust monitoring method is needed for the direct detection and quantification of target or indicator ARB in exposure relevant hotspots.

- System should be accessible for both high and lower income countries and applicable to clinical, agriculture, community, and environmental settings.

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Project Objectives:

• Address the need for a simple, culture-based microbial method to detect and quantify target ARBs of concern in environmental samples.

• Implement indicator system proposed by World Health Organization as a proof of concept.

• Enumerate target AMR in environment, including *E. coli* and other coliforms with reduced susceptibility to Extended-spectrum-β-lactams

• Performance evaluation of AMR culture media (CHROMagar ESBL bacteriologic culture medium)
• Preparation of Bio Rad Rapid E. coli 2 agar medium, CHROMagar™ ESBL, Tryptic Soy Agar (Difco™), Phosphate-buffered Saline, 1X Trypticase Soy Broth, and 40% Glycerol aqueous Solution

• Sample Collection, Transport, and Storage
• Direct, One-Step Membrane Filter Method for analysis of samples on Bio Rad Rapid E. coli 2 and CHROMagar™ ESBL using procedures form EPA Method 1604

Day 1
• Preparation of Bio Rad Rapid E. coli 2 agar medium, CHROMagar™ ESBL, Tryptic Soy Agar (Difco™), Phosphate-buffered Saline, 1X Trypticase Soy Broth, and 40% Glycerol aqueous Solution
• Sample Collection, Transport, and Storage
• Direct, One-Step Membrane Filter Method for analysis of samples on Bio Rad Rapid E. coli 2 and CHROMagar™ ESBL using procedures form EPA Method 1604

Day 2
• Sample Collection, Transport, and Storage
• Direct, One-Step Membrane Filter Method for analysis of samples on Bio Rad Rapid E. coli 2 and CHROMagar™ ESBL using procedures form EPA Method 1604

Day 3
• 20 mL filter volume (Filtered 3 dilutions in triplicate)
• 0.45μm pore size, 47 mm diameter, gridded membrane filter (Millipore HA filter)
• 60 x 15 mm plates (Bio-Rad, ESBL and KPC)
• Incubation for 24 hours at 37°C.

Day 4
• One colony from each TSA plate was then inoculated into 1X tryptic soy broth into a separate, 8mL sterile vial and incubated for 24 hrs at 37°C.

Day 5
• Revival, purification, re-isolation
• MALDI-TOF MS
• VITEK 2 Analysis

Day 6
• Enrichment vials with discernable regrowth were stored in 1mL aliquots (0.5mL TSB culture and 0.5mL 20% glycerol stock solution) in 1.5mL sterile freezer tubes and stored at -80°F for future characterization.

Day 7
• Streak on 100mmx15mm ESBL plates of the same medium and incubate for about 24 hours at 37°C.

Day 8
• Streak on 100mmx15mm TSA plates, incubate for 24 hours at 37°C

CFUs were counted for presumptive E. coli and other coliforms via colony color interpretation based on manufacturers’ descriptions

CFU – LDL = 1, UDL = 250, TNTC >250

60x15mm filter plates of all 4 media after incubation
Determine presence, concentration, and relative proportion of presumptive ESBL producing *E. coli* and other coliforms at sampled hotspots

**Timeline and Summary of Events**

- 13 sampling events
- 67 Assays
- Calculation of Concentrations for all sites, assays, and media) using colony counts of presumptive *E. coli* and other coliforms
- Calculations of the relative proportion of presumptive ESBL and KPC producing target Organisms

**Hospital Sewage**

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>Other coliforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average concentration</td>
<td>CFU / 100 mL 95% CL (+/-)</td>
<td>CFU / 100 mL 95% CL (+/-)</td>
</tr>
<tr>
<td>Ave BR</td>
<td>4.30E+06 1.02E+03</td>
<td>7.74E+07 3.86E+03</td>
</tr>
<tr>
<td>Ave ESBL</td>
<td>5.00E+05 3.10E+02</td>
<td>1.62E+06 5.09E+02</td>
</tr>
</tbody>
</table>

**Results**

<table>
<thead>
<tr>
<th></th>
<th>% ESBL E. coli</th>
<th>% ESBL Other coliforms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hospital Sewage</strong></td>
<td>27%</td>
<td>44%</td>
</tr>
</tbody>
</table>

Concentrations of Target Organisms on Bio-Rad and ESBL media (N = 24)
Results

Raw Sewage

<table>
<thead>
<tr>
<th>Average concentration</th>
<th>E. coli</th>
<th>Other coliforms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU / 100 mL</td>
<td>95% CL (+/-)</td>
</tr>
<tr>
<td>Ave BR</td>
<td>2.58E+06</td>
<td>1.11E+03</td>
</tr>
<tr>
<td>Ave ESBL</td>
<td>2.70E+05</td>
<td>3.22E+02</td>
</tr>
</tbody>
</table>

Secondary Effluent

<table>
<thead>
<tr>
<th>Average concentration</th>
<th>E. coli</th>
<th>Other coliforms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU / 100 mL</td>
<td>95% CL (+/-)</td>
</tr>
<tr>
<td>Ave BR</td>
<td>3.93E+04</td>
<td>1.23E+02</td>
</tr>
<tr>
<td>Ave ESBL</td>
<td>2.89E+02</td>
<td>1.05E+01</td>
</tr>
</tbody>
</table>
Performance Evaluation of CHROMagar ESBL media

Timeline and Summary of Events

1. Select Isolate revival, purification, and re-isolation
2. Identity confirmation via MALDI-TOF MS analysis
3. Antimicrobial susceptibility testing via VITEK 2

Results

Hospital Sewage – ESBL MALDI-TOF Confirmation (N = 104)

- Presumptive *E. coli* - 14% Correctly Confirmed
- Presumptive Other coliforms - 96% Correctly Confirmed
- Total Isolates, post-MALDI - 85% Correctly Confirmed

<table>
<thead>
<tr>
<th>Category</th>
<th>Presumptive</th>
<th>Post-MALDI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>14%</td>
<td>85%</td>
</tr>
<tr>
<td>Other coliforms</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td>Other Gram-Negative</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Gram-Positive</td>
<td>10%</td>
<td></td>
</tr>
</tbody>
</table>
Results

Raw Sewage – ESBL MALDI-TOF Confirmation (N = 61)

- Presumptive *E. coli*: 70% Correctly Confirmed
- Presumptive Other coliforms: 73% Correctly Confirmed
- Total Isolates, post-MALDI

Secondary Effluent – ESBL MALDI-TOF Confirmation (N = 60)

- Presumptive *E. coli*: 85% Correctly Confirmed
- Presumptive Other coliforms: 79% Correctly Confirmed
- Total Isolates, post-MALDI
Results

Hospital Sewage – ESBL VITEK 2 Confirmation (N = 45)

- E. coli
  - Cef: N = 2
  - Imp: N = 3
- Other coliforms
  - Cef: N = 36
  - Imp: N = 20
- Other Gram-Negative bacteria
  - Cef: N = 2
  - Imp: N = 1

Raw Sewage – ESBL VITEK 2 Confirmation (N = 57)

- E. coli
  - Cef: N = 14
  - Imp: N = 3
- Other coliforms
  - Cef: N = 32
  - Imp: N = 4
- Other Gram-Negative bacteria
  - Cef: N = 33
  - Imp: N = 5

Legend: Red = Resistant, Yellow = Intermediate, Green = Susceptible
Results

Secondary Effluent – ESBL VITEK 2 Confirmation (N = 44)

Discussion:

- Presumptive ESBL and KPC producing bacteria were found and confirmed at all sites.

- The highest concentrations and relative proportions of presumptive ESBL and KPC production in *E. coli* and other coliforms were detected in hospital sewage.

- Lower, but still detectable concentrations and proportions of presumptive ESBL and KPC producing bacteria found in raw sewage and secondary effluent.

- CHROMagar ESBL medium performed the best in secondary effluent samples, indicating the potential influence of selection pressure during treatment.
Conclusions:

- Elevated concentrations of highly AMR bacteria in hospital and municipal sewage indicates the widespread presence in the population and their possible spread to other from exposure via environmental, food and person-to-person transmission routes.

- Global spread of ARB merits evaluation across other geographic regions in US and abroad using similar methods to identify ARB threats and detect outbreaks.

- These media and methods have promise as a candidate indicator system to detect and quantify ARB of health concern in environmental media as a monitoring system to support environmental surveillance as an element of a global action plan to combat AMR.

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### Materials and Methods:

<table>
<thead>
<tr>
<th>Bacteriologic Culture Media</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Rad Rapid' <em>E. coli</em> 2</td>
<td>Chromogenic environmental medium validated for the detection and enumeration of <em>E. coli</em> and other coliforms bacteria in food and waste waters.</td>
</tr>
<tr>
<td>CHROMagar™ ESBL</td>
<td>Chromogenic medium for the detection of Gram-negative bacteria producing ESBL / resistant to extended beta lactams in stools and urine.</td>
</tr>
<tr>
<td>CHROMagar™ KPC</td>
<td>Chromogenic medium for the detection of Gram-negative bacteria with reduced susceptibility to most carbapenem agents in stools and urine.</td>
</tr>
</tbody>
</table>

**Target Organisms**

*E. coli* and Other Non-*E. coli* coliforms (*Klebsiella, Enterobacter, Citrobacter*, and *Serratia*)
### Materials and Methods:

<table>
<thead>
<tr>
<th>Enumeration</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrations and Proportions of target organisms</td>
<td>Colony forming units (CFUs) for presumptive <em>E. coli</em> and other coliforms were totaled for each plate and recorded as discrete counts according to colony color guides provided by the manufacturer. Proportions were calculated by dividing target organism CFU/100 mL, plated on ESBL or KPC by the CFU/100 mL in parallel assay, plated on Bio-Rad Rapid<em>E. coli</em> 2, for the same sample.</td>
</tr>
<tr>
<td>Bacteria Speciation via MALDI-TOF MS</td>
<td>Matrix-assisted laser desorption, time of flight mass spectrometry – soft ionization process that analyzes biomolecules and large organic molecule and compares them to a digital library of well characterized organisms.</td>
</tr>
<tr>
<td>Susceptibility analysis via VITEK 2</td>
<td>For isolate originally detected on CHROMagar ESBL, reduced susceptibility to Extended-β-lactams (Cefpodoxime) and carbapenems (Imipenem) was evaluated via Vitek2 (Objective 3).</td>
</tr>
</tbody>
</table>

### Recommendations:

- Initial and iterative performance evaluation
- Bacteria colony color and morphology referencing catalog
- Cost and Availability of Indicator System (specifically the for lower-income labs)
- Animal-free products
- Spread plate vs. membrane plate
- Partners, personnel, and planning
- Incorporate the One Health Philosophy by expanding AMR environmental surveillance using approaches and tools consistent with current medical and clinical methods
Limitations:

- VERY limited funding
- Retro-active confirmation of isolates during preliminary stage
- Mixed isolate cultures, repeated revivals, and freeze / thaw
- Sample comparison are hindered by disparate sample sizes and limited overlap in temporality
- Reliance on Beta-D-Galactosidase (GAL) and Beta-D-Glucuronidase (GLUC) to differentiate and positively ID coliform and *E. coli*, respectively.