Environmental Microbiology

Microbial Source Tracking and Quantitative Detection of Potential Pathogens in Roof Harvested Rainwater

Warish Ahmed and Ted Gardner
March 2008
Brief background of recently completed project

1. The “Smoking Gun” study

Microbial source tracking (MST) methods to estimate of site impacts of on-site wastewater treatment systems in Pine Rivers Shire

Clients:
- Healthy Waterways
- SEQ Catchment
- Local councils

Collaborators:
- University of the Sunshine Coast (USC)
- Pine Rivers Shire
2. PCR detection of pathogens in rainwater

Real-time PCR detection of pathogens in roof harvested rainwater samples collected from Southeast Queensland

Clients:
- Local Councils
- State Government
- Qld Water Commission

Collaborator:
- Qld University of Technology
Overview of “Smoking Gun” Scoping study

Aim:
Identify human faecal pollution in stormwaters from non-sewered catchments in Pine Rivers Shire via septic systems.
What is Microbial Source Tracking (MST) ???

- Methods to identify the sources of faecal pollution in waters
- Fingerprints of indicator bacteria found in sources are compared to the fingerprints found in water samples
- Experimental technique gaining popularity
Identify the dominant sources of faecal pollution

Build a faeces database
Biochemical fingerprinting procedure

Bacterial isolates are inoculated into the PhPlates containing the growth medium and 11 discriminatory substrates.

Plates are incubated and read at different time intervals; data transferred to a computer and processed.
Catchment sampling

- Number of samples collected 21
- 19 base flow and 2 rising stage
- Up to 7 samples were collected from each site
- Rainfall 18-30 mm

Four Mile Creek
River Oaks Drive
Bergin Creek
### Quantification of faecal pollution

#### Bergin Creek

<table>
<thead>
<tr>
<th>Sources</th>
<th>E. coli</th>
<th>Ent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>8%</td>
<td>9%</td>
</tr>
<tr>
<td>Animal</td>
<td>53%</td>
<td>57%</td>
</tr>
<tr>
<td>Unknown ??</td>
<td>39%</td>
<td>34%</td>
</tr>
</tbody>
</table>

#### Four Mile Creek

<table>
<thead>
<tr>
<th>Sources</th>
<th>E. coli</th>
<th>Ent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>Animal</td>
<td>55%</td>
<td>66%</td>
</tr>
<tr>
<td>Unknown ??</td>
<td>41%</td>
<td>30%</td>
</tr>
</tbody>
</table>

#### River Oaks Drive

<table>
<thead>
<tr>
<th>Sources</th>
<th>E. coli</th>
<th>Ent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>10%</td>
<td>9%</td>
</tr>
<tr>
<td>Animal</td>
<td>48%</td>
<td>65%</td>
</tr>
<tr>
<td>Unknown ???</td>
<td>42%</td>
<td>26%</td>
</tr>
</tbody>
</table>
Harsh realities

Large database is required for success
At what cost?

Only Fraction of indicator can be quantified
What about unknowns?
More harsh realities

**Host-specificity**

*E. coli and enterococci lack host-specificity***

**Database catchment specific**

*Need a new database for each catchment***
Alternative approaches for MST

- PCR based detection of human-specific molecular “marker”

  A molecular “marker” can be defined as a specific gene or sequence of a gene that is associated with faecal indicator of a particular host.

- For the first time in Australia, we introduced 3 human-specific PCR markers:

  - *Bacteroides* HF183
  - *Bacteroides* HF134
  - *E. Faecium* esp
**Advantages**

- No database is required
- Rapid
- Human-specific
- More sensitive and accurate measures of faecal pollution
- Comparatively cheaper

**Limitations**

- Markers not available for wild animals
- Host-specificity needs to be tested before field application
- The concentration of some of the markers could be low
- Quantitative methods are not available for all markers
What is Polymerase Chain Reaction (PCR)

- The PCR is a technique for copying a piece of DNA a billion-fold
- PCR requires
  1) an enzyme called *Taq*
  2) Short pieces of DNA called primer
  3) DNA template to copy

**Double-stranded DNA separation or denaturation at 95°C**

**Primer annealing to template DNA at 59°C**

**Primer elongation at 72°C**
Conventional PCR

Master Mix (Taq, MgCl2, DNTPs)
Forward primer
Reverse primer
DNA template

Thermal Cycler (30 cycles takes about 2-3 hrs)

Gel electrophoresis

Gel documentation
How good the PCR markers are???

- 52 samples from septic and STP
- 155 samples from 12 animal species

Each faecal sample was weighted

DNA was extracted Using DNA stool kit

Samples were tested with PCR using specific primers
### Host-specificity results

<table>
<thead>
<tr>
<th>Sources</th>
<th>HF183</th>
<th>HF134</th>
<th>esp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septic system</td>
<td>12/12</td>
<td>12/12</td>
<td>7/12</td>
</tr>
<tr>
<td>Primary influent</td>
<td>15/15</td>
<td>15/15</td>
<td>15/15</td>
</tr>
<tr>
<td>Secondary effluent</td>
<td>15/15</td>
<td>15/15</td>
<td>14/15</td>
</tr>
<tr>
<td>Treated effluent</td>
<td>10/10</td>
<td>9/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Ducks, chickens</td>
<td>0/30</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td>Kangaroos, deer</td>
<td>0/25</td>
<td>0/25</td>
<td>0/25</td>
</tr>
<tr>
<td>Cattle, horses, goats</td>
<td>0/44</td>
<td>0/44</td>
<td>0/44</td>
</tr>
<tr>
<td>Dogs</td>
<td>0/20</td>
<td>7/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Pigs</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Pelican, wild birds</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Goats, sheep</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>100%</td>
<td>95.5%</td>
<td>100%</td>
</tr>
</tbody>
</table>
## Detection of faecal pollution in Pine Rivers Catchment

<table>
<thead>
<tr>
<th>Catchments</th>
<th>HF183</th>
<th>HF134</th>
<th>esp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bergin Creek S1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bergin Creek S2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bergin Creek S3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bergin Creek S4</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Four Mile Creek S1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Four Mile Creek S2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Four Mile Creek S3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>River Oaks Drive S1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>River Oaks Drive S2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>River Oaks Drive S3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Conclusions.......”the smoking gun study”

- Both biochemical fingerprinting method and PCR markers indicated human faecal pollution in storm water samples collected from 3 catchments in Pine rivers Shire.

- According to biochemical fingerprinting method, the percentage of human derived faecal pollution was lower than animal faecal pollution.

- Host-specific molecular markers performed well in identifying human sourced faecal pollution.
QC/QA and Peer review

- For PCR analysis, peer reviewed methods were used as there is no standard method available for PCR.

- PCR detection of markers were set up in consultation with the researchers who originally developed these methods.

- Each manuscript was sent to independent reviewers in the field of MST before submission in a journal.

- Each manuscript has gone through at least 7 independent reviews before being accepted for publication.
Publications from “the Smoking Gun” scoping study

**International peer reviewed journals**

2. Ahmed et al. (2008a) – Letters in Applied Microbiology
4. Ahmed et al. (2008c) – Journal of Applied Microbiology

**National journal**

1. Ahmed et al. (2008d) – AWA Water (Review article)
Exploring quantitative PCR

- Quantitative PCR also called real-time PCR

- Detection and quantification of fluorescence reporter which increases in direct proportion to the amount of PCR product in a reaction

- Does not measure the end product like conventional PCR, instead its measure product in real time
Real-time PCR Cycler

Liquid Handler

Real-time PCR machine
Real-time PCR Quantification process

- gDNA Quantified using Spec (ng/µl)
- Gene copy numbers are calculated using formula
- Gene copies are serially diluted ranged from $10^7$ to $10^0$/µl of DNA

Y (Ct) = -3.5 X (concentration of genomic copies) + 36.78

$R^2 = 0.99$
The concentration of HF183 and the *esp* marker in sewage

<table>
<thead>
<tr>
<th>Raw sewage</th>
<th>HF183 gene copies/100 mL</th>
<th><em>esp</em> gene copies/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>STP 1-Sample 1</td>
<td>9.3 X 10⁹</td>
<td>3.8 X 10⁴</td>
</tr>
<tr>
<td>STP 1-Sample 2</td>
<td>3.9 X 10⁹</td>
<td>1.5 X 10⁴</td>
</tr>
<tr>
<td>STP 1-Sample 3</td>
<td>4.6 X 10⁹</td>
<td>1.3 X 10⁴</td>
</tr>
<tr>
<td>STP 1-Sample 4</td>
<td>7.3 X 10⁹</td>
<td>2.3 X 10⁴</td>
</tr>
<tr>
<td>STP 2-Sample 1</td>
<td>9.1 X 10⁸</td>
<td>1.1 X 10⁴</td>
</tr>
<tr>
<td>STP 2-Sample 2</td>
<td>2.1 X 10⁹</td>
<td>2.0 X 10⁴</td>
</tr>
<tr>
<td>STP 2-Sample 3</td>
<td>1.3 X 10⁹</td>
<td>9.8 X 10³</td>
</tr>
<tr>
<td>STP 2-Sample 4</td>
<td>9.8 X 10⁸</td>
<td>1.0 X 10⁴</td>
</tr>
</tbody>
</table>
Application of the *esp* marker in Ningi Creek catchment

**Mixed landuse catchment**

**Entire catchment serviced by septic tanks**

A recent study used ARA database and identified human sourced faecal pollution (Carroll *et al.* 2007)

16 grab samples were collected on 2 occasions after storm events (76 mm)
**MST Results**

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>esp gene copies/100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Event 1 NC1</td>
<td>1.1 X 10^2</td>
</tr>
<tr>
<td>Event 1 NC3</td>
<td>1.6 X 10^2</td>
</tr>
<tr>
<td>Event 1 NC4</td>
<td>5.3 X 10^2</td>
</tr>
<tr>
<td>Event 1 NC6</td>
<td>5.2 X 10^2</td>
</tr>
<tr>
<td>Event 2 NC4</td>
<td>4.3 X 10^2</td>
</tr>
<tr>
<td>Event 2 NC5</td>
<td>3.1 X 10^2</td>
</tr>
</tbody>
</table>

1.6 X 10^4 marker = 100 mL raw sewage
2.9 X 10^2 marker = 100 mL creek water

Therefore, 100 mL of creek water samples contained **1.8 ml** of raw sewage

- **Campylobacter spp.** 180 cfu
- **Salmonella spp.** 9 cfu
- **Rotavirus** 720 pfu
- **Giardia lamblia** 180 cysts
- **Cryptosporidium parvum** 0.36 oocysts
- **Adenoviruses** 1100 genomic copies
- **Noroviruses** 90 genomic copies

PROOF IN CONCEPT as the number would vary STP to STP
Journal Publications

International peer reviewed journals

1. Ahmed et al. (2008e) – Environmental Microbiology (under review)
2. Ahmed et al. (2008f) – Water Science and Technology (under review)

International conference

1. Health Related Water Microbiology (HRWM) – Tokyo 2007 (poster presentation)
Limitations faecal indicators

May originate from non-faecal sources

Ability to replicate in environmental waters

Cannot be used to differentiate the sources of faecal pollution

Weak association with the presence of pathogens
How about direct monitoring of pathogens ???

Direct monitoring of pathogens is an attractive option!!!

<table>
<thead>
<tr>
<th>Conventional culture methods</th>
<th>PCR-based methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>❑ Injured or stressed cells</td>
<td>❑ Direct monitoring of pathogens</td>
</tr>
<tr>
<td>❑ Viable but not culturable (VBNC)</td>
<td>❑ Detect pathogens that are difficult to grow</td>
</tr>
<tr>
<td>❑ Labour intensive</td>
<td>❑ Rapid</td>
</tr>
<tr>
<td>❑ Lack of sensitivity</td>
<td>❑ Sensitive</td>
</tr>
<tr>
<td></td>
<td>❑ Inability to distinguish between viable and non-viable cells</td>
</tr>
</tbody>
</table>
Pathogens in roof harvested rainwater

**Aims:**

1. Detection of pathogenic microorganisms in roof harvested rainwater using PCR

2. Quantification of *Campylobacter jejuni*, *Salmonella* spp., *Legionella pneumophila*, and *Giardia Lamblia* using real-time PCR

3. Quantitative Microbial Risk Assessment (QMRA) of rainwater
Microbiological quality of rainwater

- Rainwater quality is generally acceptable for drinking and household use

- Studies in New Zealand and in the USA reported the presence of enteric pathogens in rainwater samples

- The quality of rainwater is assessed based on the concentration of E. coli

- Question remains “what is the correlation between E. coli and pathogens in rainwater???
Faecal indicators and pathogens

No. of samples tested = 27

Faecal indicators tested
- E. coli
- Enterococci
- C. perfringens
- Bacteroides spp.

Pathogens tested
- Aeromonas hydrophila
- Campylobacter
- Salmonella
- L. pneumophila
- G. lamblia

Queensland Government the Smart State
Preliminary results

$n=27$

Indicators
- E. coli: 63%
- Enterococci: 78%
- Bacteroides: 48%
- C. coli: 89%

Pathogens
- L. pneumophila: 41%
- A. hydrophila: 26%
- Salmonella spp.: 15%
- C. jejuni: 11%
- G. lambia: 4%

Real-time quantification
Our future........Microbiological water quality toolbox

**Indicators**
- Faecal coliforms
- *E. coli*
- Enterococci
- *C. perfringens*
- *Bacteroides spp.*
- Coliphage
- F+RNA coliphage

**MST**
- Biochemical fingerprinting
- ARA
- MLST typing
- Human-specific *Bacteroides*
- Human-specific *esp*
- Human-specific *E. coli* clone
- Cattle-specific *Bacteroides*
- Human-specific adenovirus
- Human-specific polyomavirus
- Dog-specific *Bacteroides*

**Pathogens**
- *C. jejuni, C. coli*
- *E. coli* O157, VT1, VT2
- VRE (vanA, vanB, vanC)
- *Salmonella spp.*
- *L. pneumophila*
- *A. hydrophila*
- *G. lamblia*
- *C. parvum*
- Adenoviruses
- Polyomaviruses
- Noroviruses
- Rotaviruses
For indicator analysis standard methods were used and for PCR, Peer review methods were used. The sensitivity, specificity, and intra and inter assay variability, and performance are documented for each PCR method.

Proficiency testing ???

**Manuscript**

Ahmed *et al.* 2008g. Applied and Environmental Microbiology – has been peer reviewed by 2 independent reviewers who are not related to our work – awaiting submission.
Quality of researchers

Warish Ahmed

PhD in MST (2005)
13 journal papers
6 papers are being considered for publications
Reviewed 19 Microbial water quality related research papers since 2005

Flavia Huygens

PhD in Molecular Microbiology (1992)
Experienced working with Campylobacter and MRSA
23 journal papers
National and International peer

Dr. H. Katayama (Japan)

Dr. G. Reischer (Austria)

A/Prof. S. Jiang (USA)

Prof. Joan Rose (USA)

Prof. D. Gordon (Australia)

Dr. M. Katouli (Australia)

Dr. G. Hansman (Japan)