Molecular diagnosis of Rotavirus and 31 other enteropathogens in Dhaka, Bangladesh
• A “Complete” Stool Workup using Conventional Methods
  – is never complete, is costly, qualitative only not quantitative, and difficult for the lab

• Our goal with Molecular Diagnostics
  • Comprehensive number of enteropathogens
  • Sensitive as possible (minimize “no pathogen found”)
  • Practical for field studies (easy, reproducible, accurate)
  • Quantitative/semi-quantitative
    – In developing country settings, mixed infections the norm, many infections asymptomatic. Value to quantitation has been observed for Norovirus, EPEC, Cryptosporidium, etc. (Haque et al, CID 2009; Phillips et al, BMC ID 2009; Barletta et al, CID 2011).
UVA Assay Development

\[\text{Linearity} \quad \text{Limit of Detection} \quad \text{Repeatability} \quad \text{Reproducibility} \quad \text{Accuracy} \quad \text{Interfering Substances} \quad \text{Stability} \quad \text{Carryover}\]

\[\text{UVA Assay Validation} \]

Site Verification and Use
(AKU Pakistan, KCRI Tanzania, ICDDR,B Bangladesh, AFRIMS Thailand, MRC Gambia)

\[\text{Broader use}\]
<table>
<thead>
<tr>
<th>Multiplex PCR &gt;Luminex</th>
<th>Multiplex qPCR</th>
<th>Taqman Array Card</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral panel</strong></td>
<td><strong>Panel 1</strong></td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Adeno, Astro, Rota, Noro GII/GI, Sapo, internal control</td>
<td>Astro, Rota, Noro GII, Sapo, internal control</td>
<td>Astrovirus</td>
</tr>
<tr>
<td><strong>E coli panel</strong></td>
<td><strong>Panel 2</strong></td>
<td>Rotavirus</td>
</tr>
<tr>
<td>EAEC (aaiC+aatA), EPEC (bfpa+eae), ETEC (LT + ST), internal control</td>
<td>aaiC,aatA, bfpA, eae, internal control</td>
<td>Norovirus GII</td>
</tr>
<tr>
<td><strong>Other bacteria panel</strong></td>
<td><strong>Panel 3</strong></td>
<td>Sapovirus</td>
</tr>
<tr>
<td>Aeromonas, Campy, Salmonella, Shigella/EIEC/EIEC, Vibrio cholera, internal control</td>
<td>ST, LT, adeno, ipaH, internal control</td>
<td></td>
</tr>
<tr>
<td><strong>Protozoa panel</strong></td>
<td><strong>Panel 4</strong></td>
<td>RNA control</td>
</tr>
<tr>
<td>E.his, Giard, Crypto, internal control</td>
<td>Aeromonas, Campy, Salm, Vib, internal control</td>
<td></td>
</tr>
<tr>
<td><strong>Panel 5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ehis, Giard, Crypto, internal control</td>
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</table>
Summary thus far

• Linearity has been assessed across all enteropathogen templates, generally a wider linear range with real-time vs PCR-luminex, with consistent, similar performance across sites

• Repeatability and Reproducibility better with real-time vs PCR-luminex (but partly artifactual)

• Accuracy more-or-less similar
Accuracy on analytical specimens
- all sites averaged

Accuracy %

Adenovirus-LX
Astrovirus-LX
Norovirus Gil-LX
Rotavirus-LX
Sapovirus-LX
Astrovirus-RT
Norovirus Gil-RT
Rotavirus-RT
Sapovirus-RT
Campylobacter-LX
Salmonella-LX
IpaH-LX
Vibrio-LX
Campylobacter-RT
Salmonella-RT
Vibrio-RT
<table>
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<tr>
<th>Pros</th>
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</table>
|      | • Calls are easy (e.g., cMFI > 3) | • Most places have qPCR  
• Tight precision | • Easiest for the field  
• Great to screen for lots of targets |
| Cons | • Lots of steps | • Calls require post-PCR curve analysis (thresholds/baseline) | • Expensive repeats – still need individual assays |
| Platform cost (+7-15%/yr) | $25-65K | $38-110K | $110K |
| Reagent costs (15 targets) | $30 | $49 | $50 |
What these molecular diagnostics reveal when taken to childhood diarrhea in developing countries

• Birth cohort study in Dhaka, Bangladesh (PI Haque/Petri, CID 2011)

• N=147 children, ages 0-12 mo
• Collected surveillance stools monthly (n=1385) and with every diarrheal episode (n=420)
• All stools, RNA/DNA extraction, PCR for 32 targets = 57,760 results
Our Multiplex PCR-Luminex laboratory developed panels for enteropathogens

### Viral

- Adenovirus
- Astrovirus
- Norovirus GI
- Norovirus GII
- Rotavirus
- EAEC
- EHEC
- EPEC
- EPEC/EHEC
- ETEC
- EIEC
- Shigella
- Salmonella (pan)
- Campylobacter (Cj, Cc)
- Aeromonas (pathogenic)
- Vibrio (cholera & parahaemolyticus)
- Yersinia (pan)
- Shigella (pan)
- Cryptosporidium spp.
- Giardia lamblia
- Entamoeba histolytica
- Cyclospora cayetanensis*
- Cystoisospora belli

### E. coli

- Hexon
- Capsid
- ORF1-ORF2
- ORF1-ORF2
- NSP3
- aaiC
- aatA
- Stx1
- Stx2
- bfpA
- Eae
- LT
- ST
- iaa/ipaH
- invA
- cadF
- Aerolysin
- toxR
- lysP
- ipaH
- COWP
- 18S rRNA
- 18S rRNA
- ssrRNA
- 5.8s & ITS2

### Other bacteria

- Enterocytozoon bieneusi
- Encephalitozoon intestinalis
- Ascaris lumbricoides
- Necator americanus
- Ancylostoma duodenale
- Strongyloides stercoralis
- Trichuris trichiura

### Protozoa 1

- Taniuchi Diag Micr ID 2012

### Protozoa 2

- Taniuchi AJTMH 2011

### Helminth

- Taniuchi AJTMH 2011
Severity

The majority of diarrhea episodes were mild in this community study. Only 39 (9.3%) were moderate or severe (Ruuska score > 6).

<table>
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<tr>
<th>% of probable/dominant detections</th>
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<tbody>
<tr>
<td>1. EAEC (10.9%)</td>
<td>1. Rotavirus (13.2%)</td>
</tr>
<tr>
<td>2. Campy (10.5%)</td>
<td>2. E. histolytica (10.1%)</td>
</tr>
<tr>
<td>3. EPEC (9.7%)</td>
<td>3. EAEC (9.4%)</td>
</tr>
<tr>
<td>4. Rotavirus (7.6%)</td>
<td>4. Campylobacter (8.4%)</td>
</tr>
<tr>
<td>5. E. histolytica (6.6%)</td>
<td>5. Cryptosporidium (7.9%)</td>
</tr>
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Severity

- The majority of diarrhea episodes were mild in this community study.
- Only 39 (9.3%) were moderate or severe (Ruuska score > 6).
Remember it’s not just about 1 pathogen

- There were, on average,
- $2.0 \pm 1.7$ dominant
- $1.4 \pm 0.8$ probable
- $2.4 \pm 1.9$ less likely targets identified with each diarrheal specimen
- Including rotaviral probable/dominant diarrhea, where average $3.8 \pm 1.8$ other pathogens were found
Conclusions

• In this community based study of enteropathogens...
• Diarrhea is associated with multiple dominant/probable enteropathogens, and is not caused by 1. Multiple pathogens appear to be acting as a gang.
• Detection of several of the bacterial genes in stool must be carefully interpreted (many detections are “less likely” contributory)
• Rotavirus is clearly one of or the most important pathogen, and detection generally is significant (i.e., “probable” or “dominant”, rarely “less likely”), but it occurs with other probable/dominant pathogens as well
• This has implications for how we understand the etiology of diarrhea, and how much diarrheal burden can be reduced with a single pathogen vaccine – depends on how dominant rotavirus is in the context.
• Some rotavirus+ stools after vaccination will have many other pathogens, and these episodes may not reflect vaccine failure.
For instance, in the PROVIDE study

98 Rotavirus+ diarrheal specimens after RV vaccination/placebo
The Project team

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