The importance of unusual *Cryptosporidium* species and genotypes in human cryptosporidiosis

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What is cryptosporidiosis?

“An illness caused by Cryptosporidium and characterized by diarrhoea, abdominal cramps, loss of appetite, low-grade fever, nausea, and vomiting”.

2002 – FDA approved nitazoxanide in children
2005 – FDA approved nitazoxanide in adults
No licensed treatment in UK

The disease can be prolonged, invasive and life-threatening in severely immunocompromised persons.
Cryptosporidium and the immunocompromised patient

1996 – HAART introduced: controls problems of cryptosporidiosis in AIDS patients in developed world

Cryptosporidiosis is now increasingly recognised in other T-cell immunodeficiencies (esp. haematological and T-cell primary)

Has a devastating effect where treatment is not available (lack of HAART, fake drugs)

Undefined treatment modalities (nitazoxanide trials still underway)
Long term sequelae

Infection developing countries:
children exhibit poor growth, depressed cognitive function

Generally:
possible links to reactive arthritis and irritable bowel syndrome
suggested relapse in inflammatory bowel disease e.g. Crohn’s
A patient’s experience

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Cryptosporidiosis

A P Davies,1 R M Chalmers2
Worldwide diversity of *Cryptosporidium* spp. in human infection

- *C. parvum*
- *C. hominis*
- Other species & genotypes
  - *C. meleagridis*
  - *C. cuniculus*
  - *C. ubiquitum*
  - *C. felis*
  - *C. canis*
  - *C. andersoni, C. muris, C. suis, C. fayeri, C. bovis*
  - skunk, horse, monkey, chipmunk, pig genotypes
Evidence for human pathogenicity of *Cryptosporidium* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Outbreaks of disease</th>
<th>Human experimental infectivity</th>
<th>Epidemiologic evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. parvum</em></td>
<td>√</td>
<td>√</td>
<td>√ Multiple studies</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>√</td>
<td>√</td>
<td>√ Multiple studies</td>
</tr>
<tr>
<td><em>C. cuniculus</em></td>
<td>√</td>
<td>X</td>
<td>√ Dose response in waterborne outbreak</td>
</tr>
<tr>
<td><em>C. meleagrisi</em></td>
<td>X</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td><em>C. felis</em></td>
<td>X</td>
<td>X</td>
<td>√</td>
</tr>
<tr>
<td><em>C. canis</em></td>
<td>X</td>
<td>X</td>
<td>√</td>
</tr>
<tr>
<td><em>C. ubiquitum</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

In a birth cohort in Lima, Peru, these species were associated with diarrhoea.
Clinical typing assay requirement

- Ideally, be able to detect all *Cryptosporidium* spp. or at least detect and differentiate all species that infect humans

- Must be suitable for the population served and the resources available
UK strategy for understanding Cryptosporidium epidemiology, sources and risks

Create a national collection of clinical isolates
- Started in January 2000
- Diagnostic labs asked to send in Cryptosporidium positive stools
- In UK, stools are unpreserved

Use conventional PCR-RFLP to generate baseline data
- Efficient DNA extraction from semi-purified oocysts
- Supported by sequencing the SSU rRNA gene

Develop rapid tests based on gathered information
- 10 years of data
- Seasonal, geographic, temporal trends and changes understood
Methods for typing from clinical samples

Challenge 1
• Getting the sporozoite DNA out of the oocysts

Challenge 2
• Amplifying the DNA from faeces which contains inhibitors
The CRU approach for typing clinical samples

1. Semi-purify the oocysts

2. Use heat and lysis buffer to open the oocysts

3. Use spin-columns to extract the DNA: highly stable, good quality

Chalmers et al., Eurosurveillance 2009 14(2) 15 January
Workflow 2000-2010

1. Separate oocysts from faecal debris
2. Disrupt oocysts
3. Extract DNA
4. Amplify DNA by PCR
5. Identify species by:
   • Benchmark method DNA sequence analysis ssu rRNA gene
   • Tools to look for markers of sequence variation e.g. Restriction fragment length polymorphisms (RFLP)
2000-2010 trends; 14469 samples

- 97% samples typable:
  - 44% C. parvum
  - 51% C. hominis
  - 0.4% both
  - 1.1% other species/genotypes

Chalmers et al., 2009, 2010; Elwin et al., 2011
Baseline data used for method improvement in 2010

Same semi-purification and DNA extraction process

Real-time PCR
- Automated set-up, reduced handling and contamination risk
- No downstream processing
- Improved PCR performance monitoring
- Semi-quantitative
- Same-day result

Specific targets
- *C. parvum*
- *C. hominis*
- *Cryptosporidium* spp.
- Internal (amplification/inhibition) control

Hadfield *et al.*, Journal of Clinical Microbiology 2011; 49(3): 918-924
More streamlined workflow

1. Salt float
2. Oocyst disruption
3. DNA extraction
4. Conventional PCR, real-time PCR = simultaneous amplification and detection
5. Restriction digest
6. Gel-electrophoresis
7. Gel-inspection, recording and reporting
### Comparative performance (CRU unpublished data)

#### Conventional PCR
- 14,469 samples
- 97% typed
- 3% untyped
- ~10% samples require repeat tests to achieve this
- 44% *C. parvum*
- 51% *C. hominis*
- 0.4% both
- 1.1% Other

#### Real-time PCR
- First year of use
- 2,321 samples
- 99.5% typed
- 0.5% untyped
- No repeat testing
- 49% *C. parvum*
- 47% *C. hominis*
- 0.6% both
- 3% Other

**Improved performance and efficiency, reduced turnaround time and costs.**
### Unusual Cryptosporidium spp. in clinical samples, E&W, 2000-2010

<table>
<thead>
<tr>
<th>Species</th>
<th>Number (in 18 488 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. meleagridis</em></td>
<td>149</td>
</tr>
<tr>
<td><em>C. felis</em></td>
<td>53</td>
</tr>
<tr>
<td><em>C. ubiquitum</em></td>
<td>30</td>
</tr>
<tr>
<td><em>C. canis</em></td>
<td>3</td>
</tr>
<tr>
<td>Horse genotype</td>
<td>2</td>
</tr>
<tr>
<td>Skunk genotype</td>
<td>2</td>
</tr>
<tr>
<td>Novel genotypes</td>
<td>10</td>
</tr>
<tr>
<td><em>C. cuniculus</em> (rabbit gt)</td>
<td>48 (2007 and 2008 only)</td>
</tr>
</tbody>
</table>

Elwin et al., 2011; Chalmers et al., EID 2010; CRU unpublished data
Significant (p<0.05) risk factors among “unusuals” were:

- Travel abroad – *C. meleagridis*
- Being immunocompromised – all, most especially *C. felis*
- Contact with cats – *C. felis*
Typing and incident / outbreak management

• Identify clusters of cases

• Help identify source of infection or contamination

• Avoid inappropriate control measures

• With higher-resolution typing, link cases and suspected sources
Pitsford Reservoir: the drinking water source and supply to 250,000 people
Pitsford WTW process schematic 2008 (Bob Markell, Anglian Water)

Large distribution system
7 to 10 day transit time
Water Quality Incident 25th June 2008

- Cryptosporidium oocysts detected in the treated water continuously sampled between 19-23rd June (0.05/10L)

- Oocysts again detected in 24 hr sample on 24th June (0.8/10 L)

- Previously no detections

- Wed 25th June 2008 at 6.00 am

- Precautionary notice to boil drinking water
Investigating the source of contamination

- All source water samples were negative for *Cryptosporidium*
- Faecal indicators satisfactory
- All water treatment processes working optimally
- Yet oocysts in final water.............and throughout distribution system
- WHY?
Source of contamination

- 26\textsuperscript{th} June: oocysts and a dead rabbit found in a contact tank
- Extensive monitoring and flushing of distribution system (storage tanks and towers)
Genotyping from water samples by benchmark method

Oocyst disruption and DNA extraction

Multiple aliquots

Ssu rRNA nested PCR….

Clean up amplicons, sequencing reaction

Sequence analysis:
- edit
- analyse
- compare
- Issue report
What was known about rabbit genotype?

- Uncertain taxonomic status: closely related to *C. hominis*; indistinguishable by routine typing tools

- GenBank
  4 x 18s sequences*, 2 x HSP70*, 1 x Actin, 1 x COWP*
  *from world’s only previously reported human isolate
  Rest from 3 rabbits China, NZ, Czech Republic

- Distribution and prevalence in rabbits: not known

- Risk to public health: not known

- Requires enhanced clinical testing to differentiate from *C. hominis*

Differentiation of *C. cuniculus* in routine diagnosis

- Identical to *C. hominis* at COWP, Lib13
- HSP70 99.7% similarity
- Actin 99.9% similarity
- SSU rRNA gene 99.5% similarity
- *SspI* RFLP (L18)

Outbreak rabbit genotype cases and isolates

- Age range 10 to 60 years (median 29 years)
- 70% female
- Many reported drinking large volumes water (median 1.8 litres/day; national median is 0.8 L)
- Cryptosporidium isolates from the rabbit, the water and the patients were indistinguishable at multiple loci (18s, Actin, HSP70, GP60)
The estimated mean incubation period is 6.8 days, median 6.2 days and mode 5.5 days.

Taking the 80% credible interval, the range = 2 to 11 days.

Probability / risk of infection – similar to *C. parvum* outbreak

### Continuing clinical method improvement in 2011

<table>
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<th>DNA extraction</th>
<th>Real-time PCR</th>
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<tr>
<td>• Semi-purification and DNA extraction process</td>
<td>• Compared with direct-from stool extraction</td>
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<tr>
<td>Specific targets</td>
<td></td>
</tr>
<tr>
<td>• specific unusual Cryptosporidium spp.: C. cuniculus, C. meleagridis, (C. felis, C. ubiquitum)</td>
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</table>
“Monitoring this complex environmental system is technologically and practically challenging. ..... 

Agencies need detailed understanding of the behaviour of pathogens in the environment so that they can apply the risk assessments intrinsic to these approaches....... 

Detailed molecular epidemiology strongly coupled to environmental monitoring is required to systematically connect pathogen strains with environmental sources and pathways to exposure and disease.”