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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

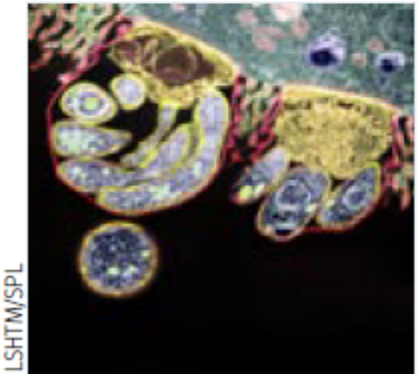
suggested relapse in inflammatory bowel disease e.g. Crohn's

A patient's experience

For the full versions of these articles see bmj.com

BMJ 2009;339:b4168

CLINICAL REVIEW

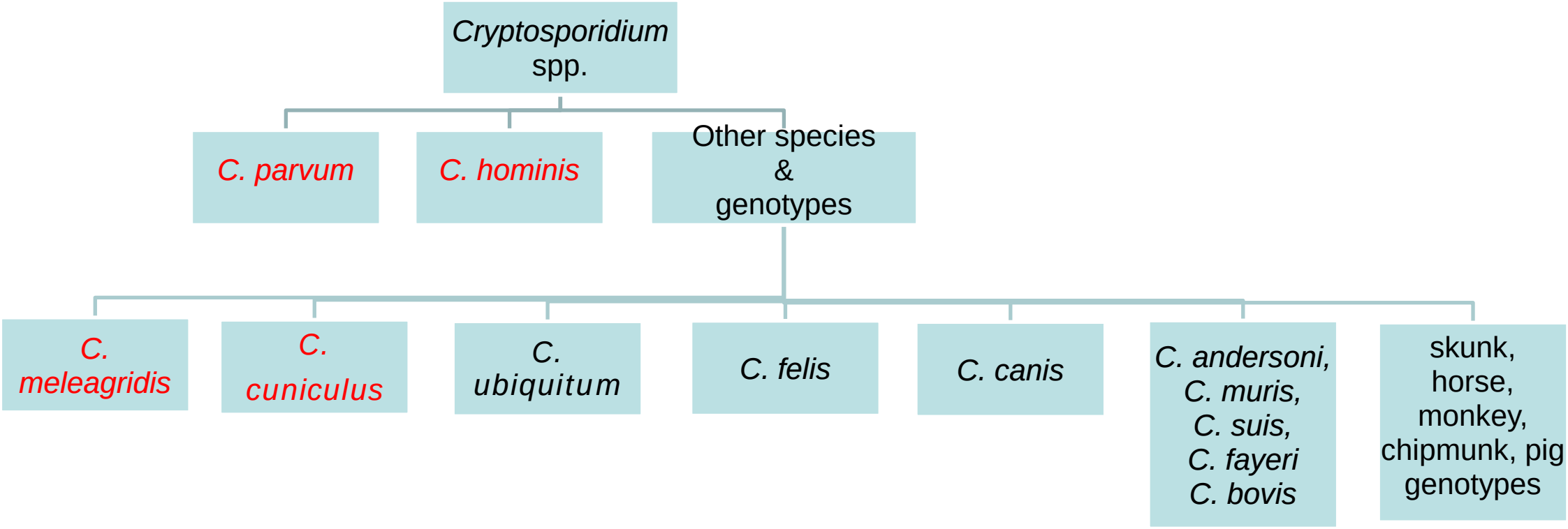


LSHTM/SPL

Cryptosporidiosis

A P Davies,[1] R M Chalmers[2]

Worldwide diversity of *Cryptosporidium* spp. in human infection



Evidence for human pathogenicity of *Cryptosporidium* species.

Species	Outbreaks of disease	Human experimental infectivity	Epidemiologic evidence	
C. parvum	√	√	√	Multiple studies
C. hominis	√	√	√	Multiple studies
C. cuniculus	√	X	√	Dose response in waterborne outbreak
C. meleagridis	X	√	√	In a birth cohort in Lima, Peru, these species were associated with diarrhoea.
C. felis	X	X	√	
C. canis	X	X	√	
C. ubiquitum	X	X	X	

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

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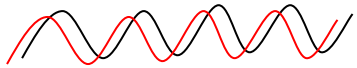
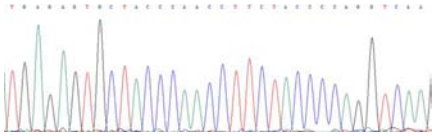
Workflow 2000-2010

1. Separate oocysts from faecal debris
2. Disrupt oocysts

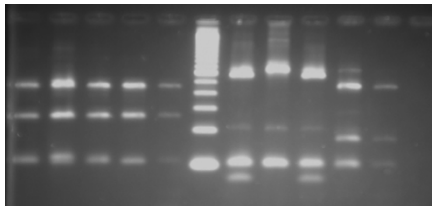
3. Extract DNA
4. Amplify DNA by PCR



5. Identify species by:
   - Benchmark method DNA sequence analysis ssu rRNA gene



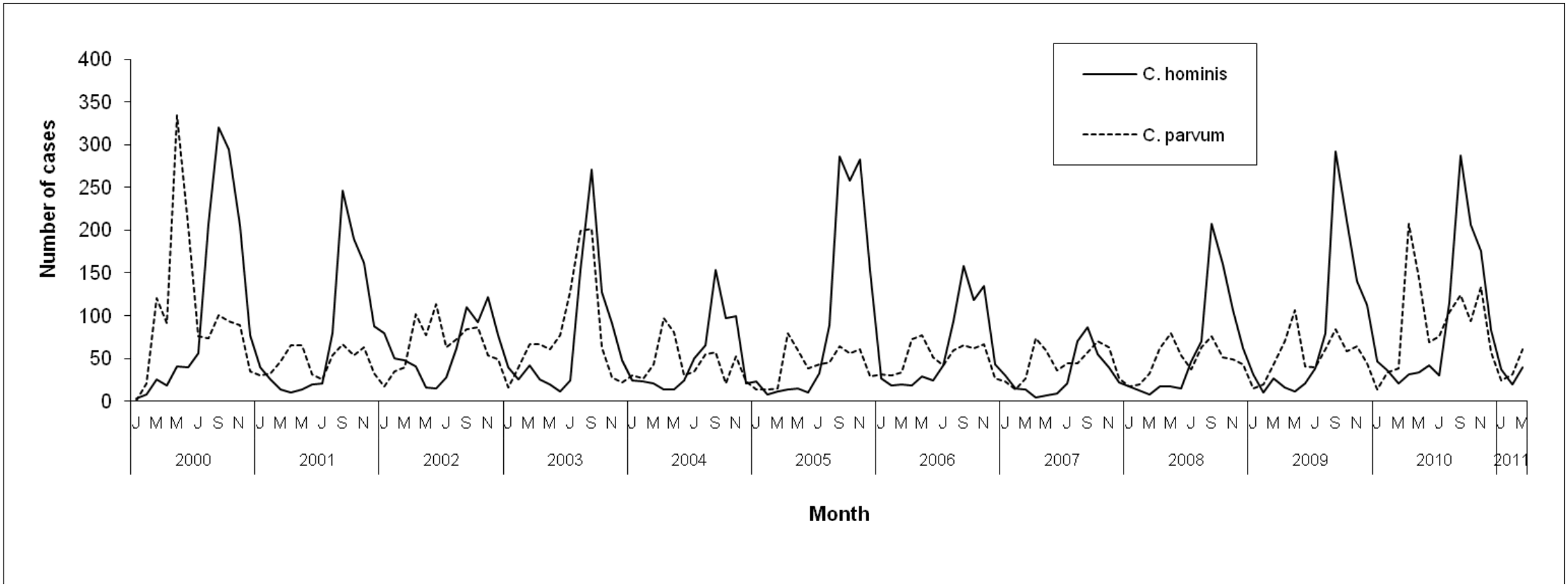
   - Tools to look for markers of sequence variation e.g. Restriction fragment length polymorphisms (RFLP)



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

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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

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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	Real-time PCR first year of use 2 321 samples
• 97% typed • 3% untyped • ~10% samples require repeat tests to achieve this	• 99.5% typed • 0.5% untyped • No repeat testing
• 44% *C. parvum* • 51% *C. hominis* • 0.4% both	• 49% *C. parvum* • 47% *C. hominis* • 0.6% both
• 1.1% Other	• 3% Other

Improved performance and efficiency, reduced turnaround time and costs.

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Unusual *Cryptosporidium* spp. in clinical samples, E&W, 2000-2010

Species	Number (in 18 488 samples)	
C. meleagridis	149	
C. felis	53	
C. ubiquitum	30	
C. canis	3	
Horse genotype	2	
Skunk genotype	2	
Novel genotypes	10	
C. cuniculus (rabbit gt)	48	(2007 and 2008 only)

Elwin et al., 2011; Chalmers et al., EID 2010; CRU unpublisjed data)

Epidemiol. Infect., Page 1 of 11. © Cambridge University Press 2011
doi:10.1017/S0950268811000860

The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000–2008

K. ELWIN, S. J. HADFIELD, G. ROBINSON AND R. M. CHALMERS*

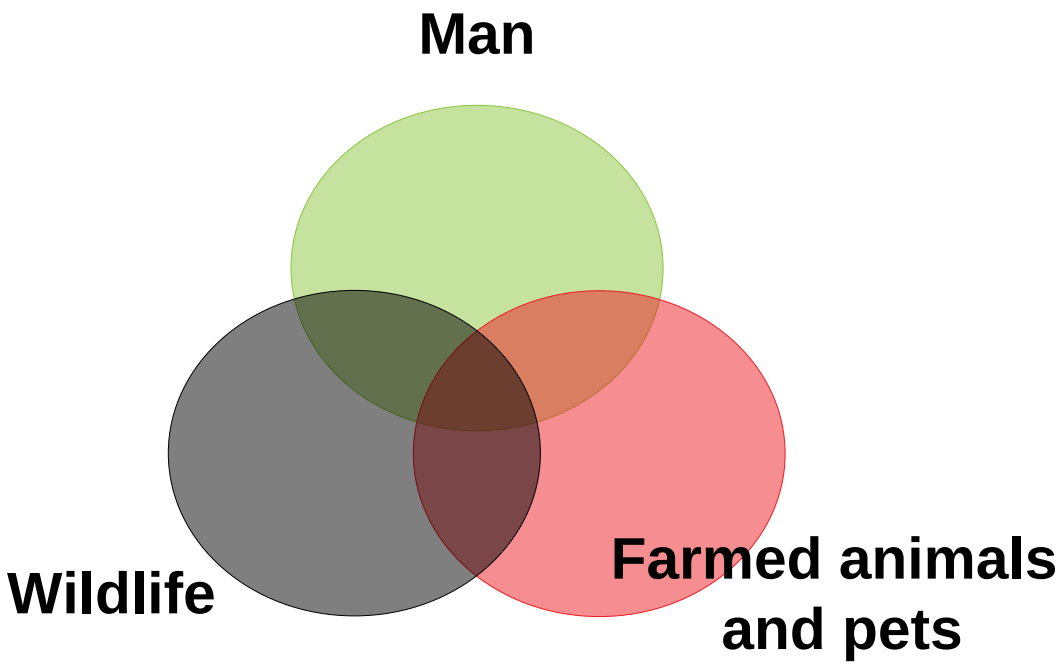
UK Cryptosporidium Reference Unit, Public Health Wales Microbiology, Singleton Hospital, Swansea, UK

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*

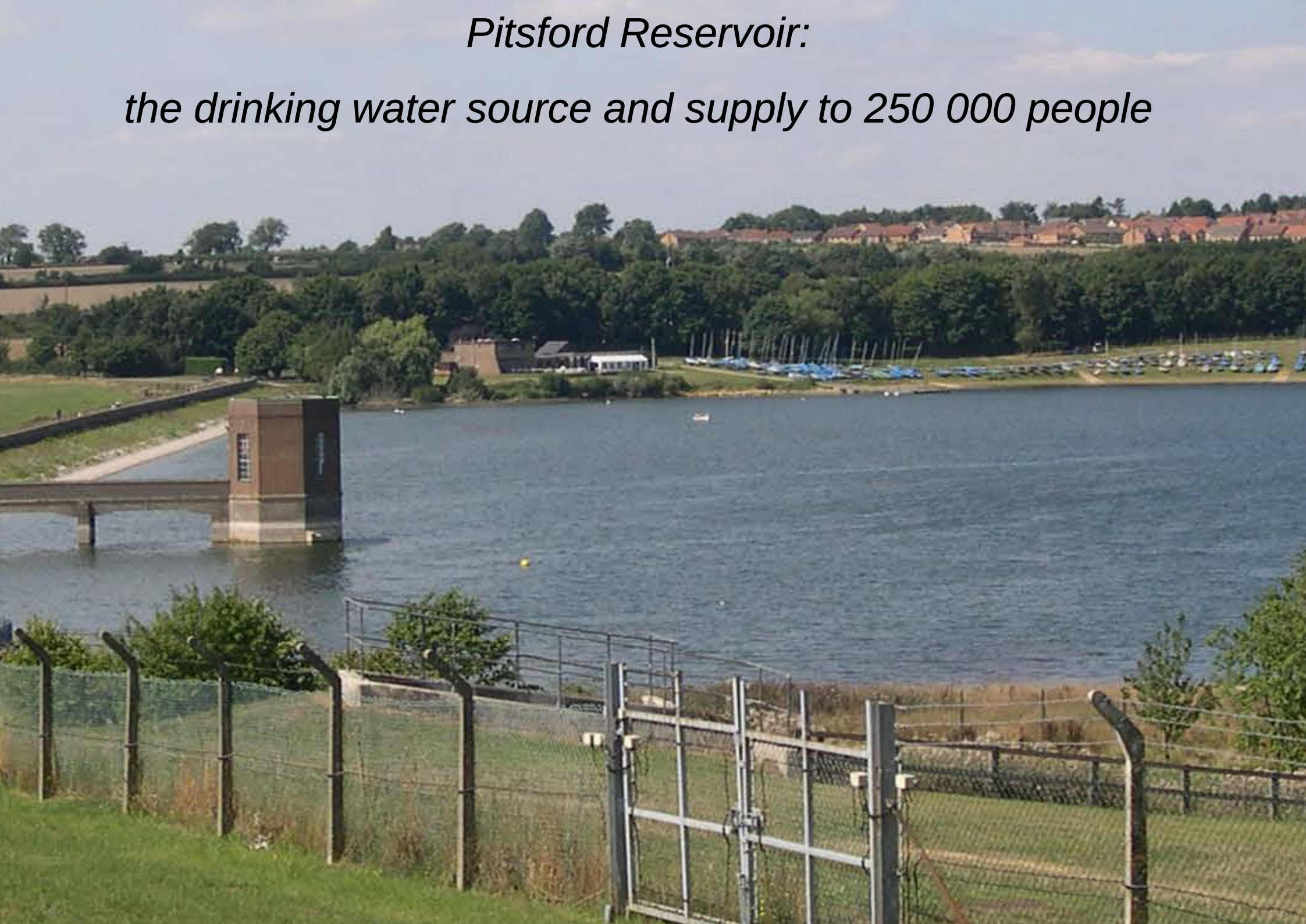
Typing and incident / outbreak management

- Identify clusters of cases
- Help identify source of infection or contamination
- Avoid inappropriate control measures
- With higher-resolution typing, link cases and suspected sources



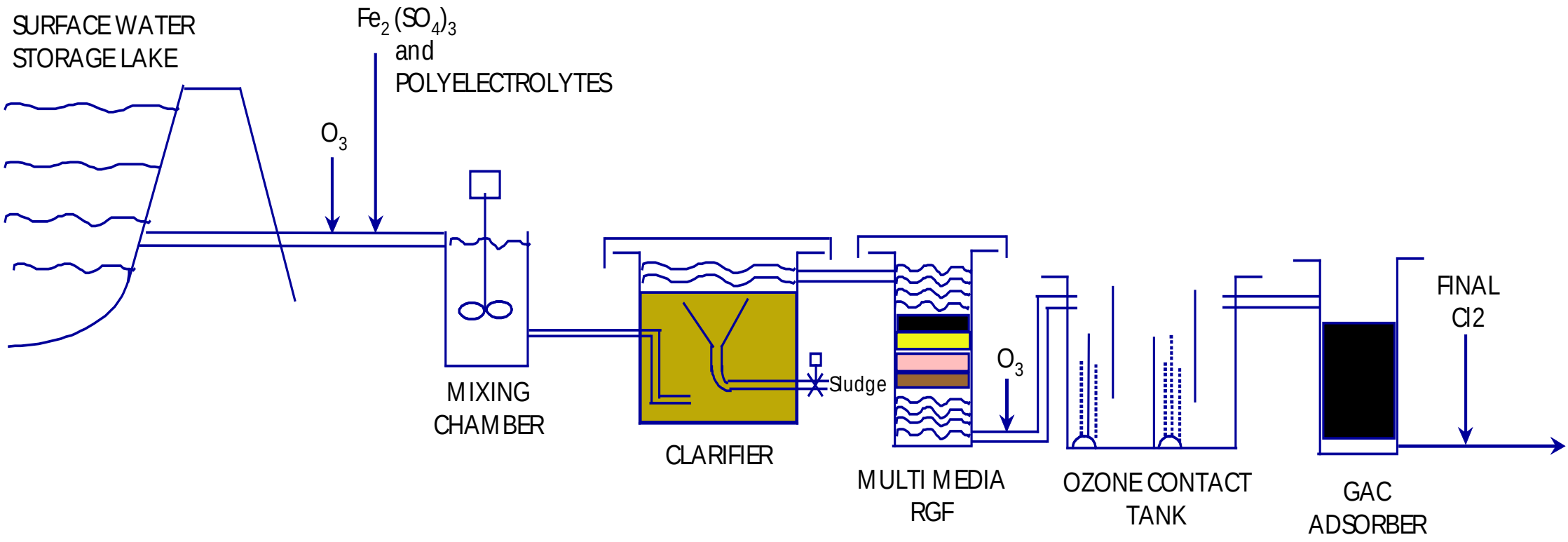
Pitsford Reservoir:

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?

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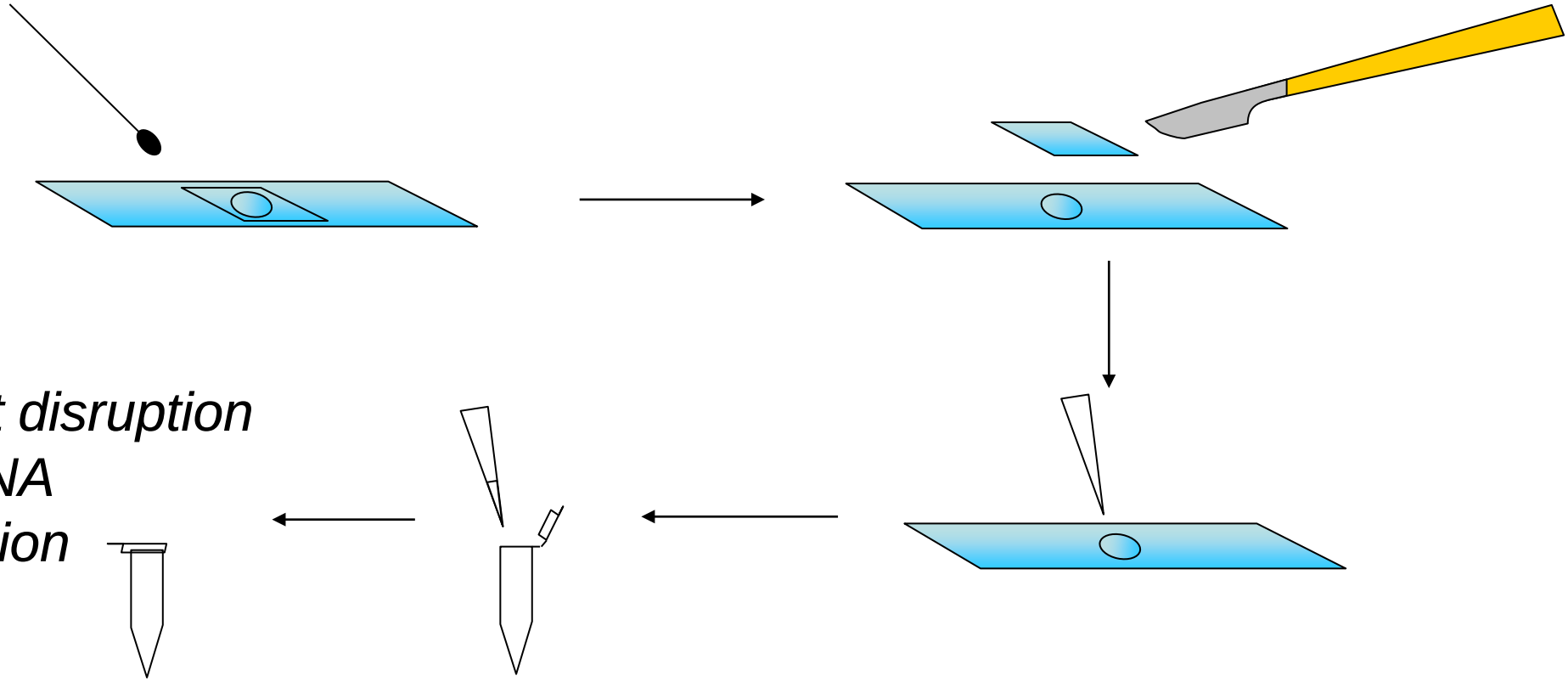
Source of contamination

- 26th June: oocysts and a dead rabbit found in a contact tank
- Extensive monitoring and flushing of distribution system (storage tanks and towers)

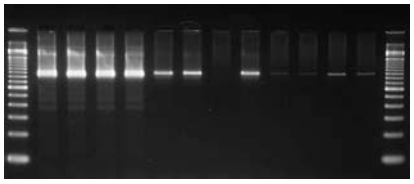
Genotyping from water samples by benchmark method



Oocyst disruption and DNA extraction

Multiple aliquots

Ssu rRNA nested PCR….



Clean up amplicons, sequencing reaction

Based on Xiao *et al*., Appl Environ Microbiol 2001; 67: 1097–1101.

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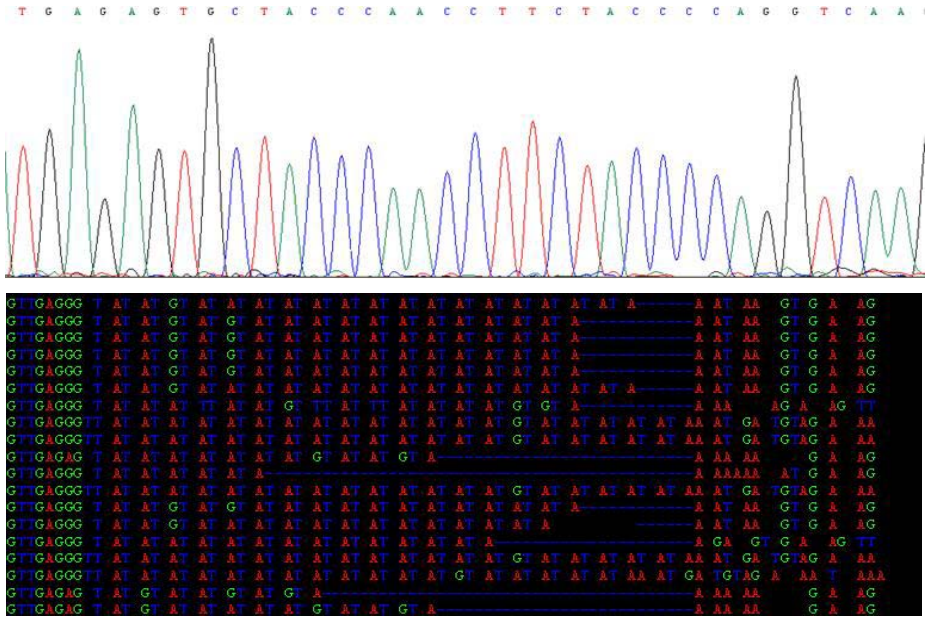
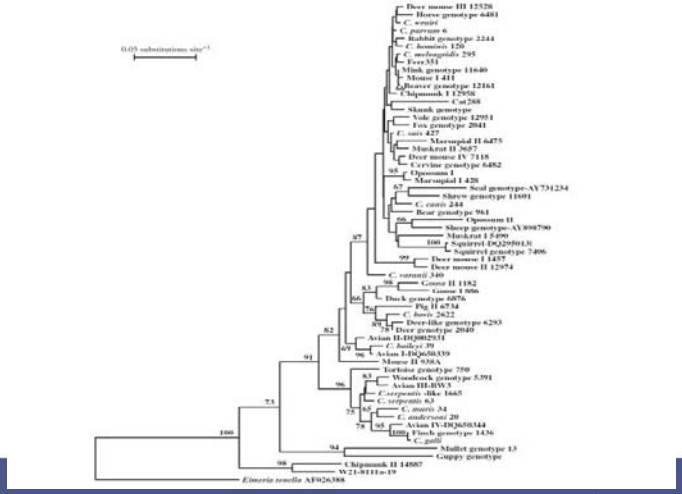
Sequence analysis :

- 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*

Robinson G and Chalmers RM. Zoonoses and Public Health. 2010; 57:e1–e13

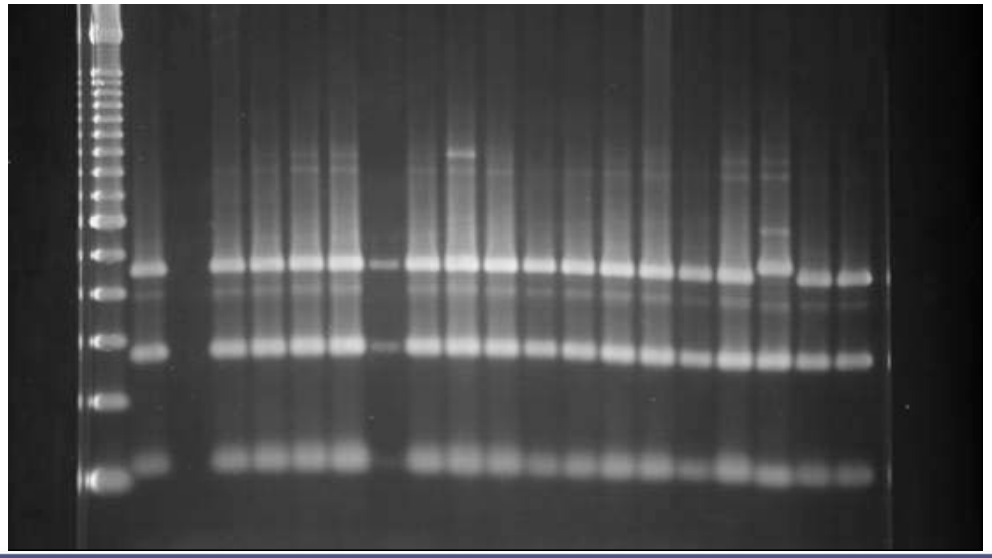
Differentiation of *C. cuniculus* in routine diagnosis

- Identical to *C. hominis* at COWP, Lib13
- HSP70 99.7% similarity
- Actin 99.9% similarity

- SSU rRNA gene

99.5% similarity

*Ssp*I RFLP (L18)



Chalmers *et al*., Emerging Infectious Diseases 2009; 15(5) 829-830.

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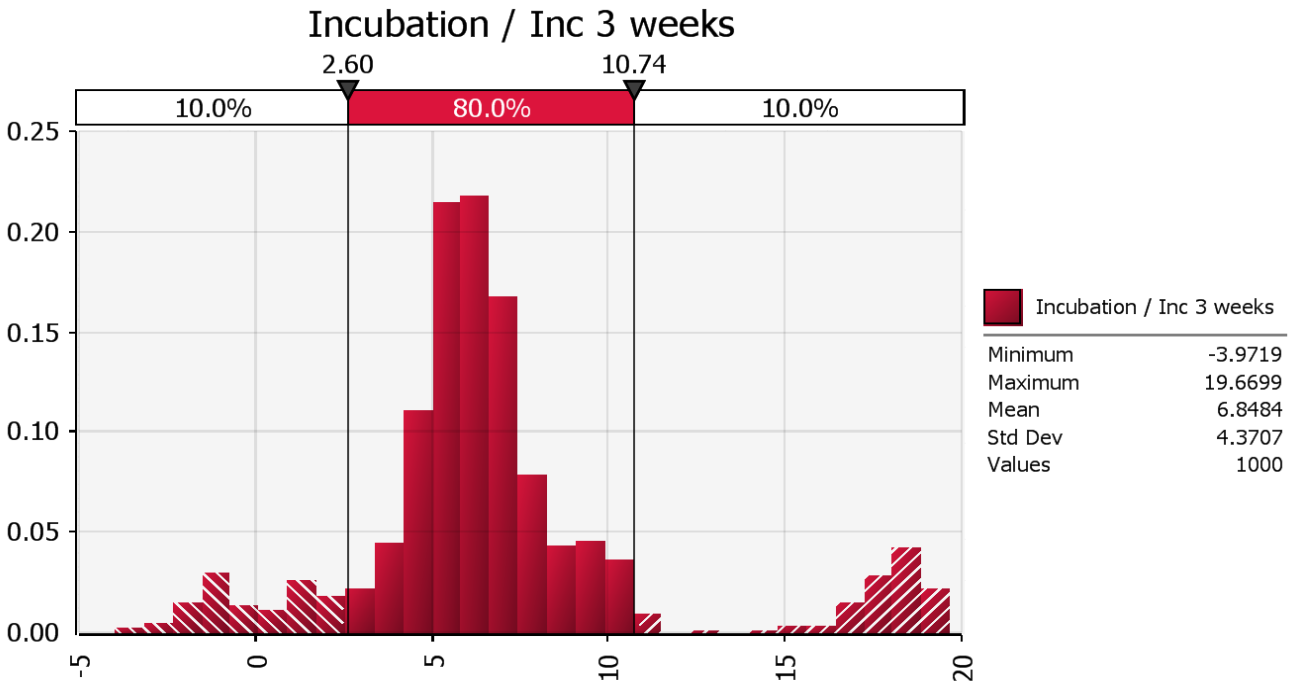
Outbreak rabbit genotype cases and isolates

- 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)

Anon. Health Protection Report 2008;2:29

Distribution of incubation periods based on MonteCarlo modelling of drinking water exposure



The estimated mean incubation period is 6.8 days, median 6.2 d and mode 5.5 d.

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

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

Chalmers *et al*. Final Report to Defra. Drinking Water Inspectorate, 2010.

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Continuing clinical method improvement in 2011

Real-time PCR

DNA extraction	• Semi-purification and DNA extraction process • compared with direct-from stool extraction
Specific targets	• Genus, *C. parvum* and *C. hominis* • specific unusual *Cryptosporidium* spp.: *C. cuniculus, C. meleagridis, (C. felis, C. ubiquitum)*

Bridge *et al.*, Bull. WHO 2010;88:873–875

"*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."