Final Report to Defra

Investigation of the taxonomy and biology of the *Cryptosporidium* rabbit genotype.

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EXECUTIVE SUMMARY

Most cases of the human diarrhoeal disease "cryptosporidiosis" are caused by either *Cryptosporidium parvum* or *Cryptosporidium hominis*. In summer 2008, the *Cryptosporidium* sp. rabbit genotype was identified as the causative agent of an outbreak of diarrhoeal disease (cryptosporidiosis) among the human population. This was linked to the drinking water supply and the source of contamination was a wild rabbit that had entered a treated water tank. Prior to this outbreak, there were no published reports of human infection with this genotype and only one infection was known to the UK Cryptosporidium Reference Unit. The outbreak demonstrates that this genotype is a human pathogen of public health importance. However, little is known about it. This study aims to establish the taxonomic status of the rabbit genotype and improve our understanding of the biological features of, and risks from, this newly-identified human pathogen for better prevention and control via the waterborne route. The objectives are:

1. Establish current knowledge by undertaking a literature review.
2. Describe and measure the morphological features of the rabbit genotype in comparison with *C. hominis.*
3. Undertake genetic characterisation of the rabbit genotype at multiple loci.
4. Investigate the comparative experimental host range of the rabbit genotype and *C. hominis.*
5. Establish and submit for publication the taxonomic status of the rabbit genotype on the basis of the data obtained in objectives 1 to 4.
6. Describe the human epidemiology and pathogenicity of the rabbit genotype in a waterborne outbreak.
7. Estimate the prevalence of the rabbit genotype in human cryptosporidiosis by enhanced typing studies.
8. Compare the human epidemiology and pathogenicity of the rabbit genotype with *C. parvum* and *C. hominis* using *a priori* data.
9. Characterise the human infection risk from drinking water by producing a model using drinking water monitoring and water consumption data.

We reviewed the available knowledge about the occurrence and natural host range of the rabbit genotype, and the prevalence of *Cryptosporidium* spp. in rabbits, using systematic review principles. This showed that the rabbit genotype has only been reported in rabbits and humans so far, and that the prevalence of *Cryptosporidium* spp. in wild rabbit populations ranges from 0 to 5%. This is based on just two large studies of >100 animals. Most studies were too small to estimate prevalence and none of the studies provided age or sex data required for meaningful population analysis. Illness appears to be reported more frequently in neonatal or unweaned rabbits than older rabbits. Only four previous studies, in three continents, identified the infecting species/genotypes, and in all four studies the rabbit genotype was the only one found. However, there is experimental evidence that other human-pathogenic cryptosporidia, *C. parvum* and *C. meleagridis,* could also be carried by rabbits. No previous studies of the host range and biological features of the rabbit genotype were identified, although there is some prior data regarding genetic characteristics. This showed that part of only one gene, coding for small subunit ribosomal RNA, had been analysed in all four studies. Characteristic differences between the rabbit genotype and its closest genetic relation, *C. hominis*, were consistent and, over the part of this gene studied, were 0.51%.

Our morphological studies demonstrated that rabbit genotype oocysts are similar in size, shape and appearance to other human-pathogenic cryptosporidia. Rabbit genotype oocysts can be detected using the most commonly used, approved water testing methods in the UK. It is therefore likely that both raw water and treated water testing would detect but not differentiate the rabbit genotype.

Investigation of the rabbit genotype genome, using isolates obtained from the Northamptonshire outbreak and subsequently identified human sporadic cases, involved DNA sequence analysis of parts of six genes commonly used for the differentiation or characterisation of *Cryptosporidium*. The rabbit genotype is identical to *C. hominis* at the COWP and Lib13 genes and therefore cannot be differentiated from it using assays based on these targets. However, differences between it and *C. hominis* were consistent at the following genes, by the amounts shown in brackets: SSU rRNA (0.51%); HSP70 (0.25%); actin (0.12%), and at multiple loci spanning up to 4469 base pairs (0.27%). These differences are substantially less than the differences between *C. hominis* and *C. parvum*, although it must be acknowledged that only a very small proportion of the genome has been studied. Sequencing the GP60 gene is commonly used to identify subtype families within *Cryptosporidium* spp. The rabbit genotype appears to have its own subtype families, which we have published as subtype families Va and Vb.

Although genetic differences are small, biological differences in terms of host infectivity, are large and sufficient for consideration whether the rabbit genotype would naturally be found in the same hosts simultaneously with *C. hominis*. We therefore propose that this variation be acknowledged with the rabbit genotype named *C. hominis cuniculus*, a subspecies of *C. hominis*. Differences between who, when, where and how people become infected were also explored. It appears that a greater proportion of *C. h. cuniculus* infections occur in adults compared with *C. h. hominis* or *C. parvum.* There is a distinct seasonality, also reflected in *C. h. cuniculus* subtypes, and these epidemiological trends may be more linked to exposure opportunities than parasite infectivity or host susceptibility factors. Risk to public health from *C. h. cuniculus* in drinking water appears to be similar to *C. parvum* and *C. h. hominis*.

Key findings of the research are:

- Pet and wild rabbits are a potential source of human cryptosporidiosis and as such, good hygiene practices are recommended following handling rabbits or exposure to their faeces or potentially contaminated surfaces.
- Water supplies should be protected against access by wildlife, including rabbits.
- To estimate more fully the risks from water contamination by wild mammals, population-based prevalence studies of zoonotic pathogens are required.
- Although the rabbit genotype is genetically very closely related to *C. hominis*, this is based on analysis of only a small proportion of the genome. Biological differences in host infectivity are distinct.
- There is sufficient evidence for the rabbit genotype as a subspecies of *C. hominis*, possibly host-adapted to rabbits. We propose *C. hominis cuniculus*. Evidence for separate species status may become available through further studies.
- There is insufficient clarify in the taxonomic position of *Cryptosporidium* “genotypes” and better algorithms for establishing taxonomic status need to be created.
- Routine clinical diagnostic tests, water sampling and testing by approved methods will detect but not differentiate *C. h. cuniculus* from other *Cryptosporidium* spp.
- There is no significant difference in apparent human infectivity or virulence between *C. h. cuniculus* associated with the Pitsford outbreak and the *C. parvum* associated with the Clitheroe outbreak and is substantially less than the variation in infectivity shown between different *C. parvum* strains.
- Differences in epidemiology, compared with *C. h. hominis* and *C. parvum*, may be linked to exposure opportunities.
- In the absence of human volunteer feeding studies, the currently used dose response model for *Cryptosporidium* would appear to be applicable to Quantitative Microbial Risk Assessment analyses of *C. hominis cuniculus.*

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SCIENTIFIC REPORT

Introduction

The protozoan parasite *Cryptosporidium* is a major cause of gastrointestinal disease (cryptosporidiosis) worldwide. In the UK, it is the third most commonly reported non-viral cause of gastroenteritis, with between 3000 to 6000 reported cases *per annum* (Health Protection Agency and Health Protection Scotland data). The vast majority of cases in the UK are caused by *C. parvum* and *C. hominis*; rare infections with other species occur including *C. meleagridis*, *C. felis*, *C. canis*, cervine genotype, *C. hominis* monkey genotype, skunk, horse and rabbit genotypes (Robinson *et al*., 2008a; Chalmers and Pollock, 2010; Chalmers *et al*., 2009a). Risk factors for cryptosporidiosis include contact with farmed animals (*C. parvum*), especially young ruminants, toileting young children, changing nappies or caring for someone with diarrhoea (*C. hominis*) and foreign travel (Hunter *et al*., 2004). Outbreaks have been linked to drinking water supplies (mains and private), recreational waters, foods, institutions and farm visits. In summer 2008, the *Cryptosporidium* sp. rabbit genotype was identified as the causative agent of an outbreak of cryptosporidiosis among the human population (Chalmers *et al*., 2009b). This was linked to the drinking water supply and the source of contamination was a wild rabbit that had entered a treated water tank at the Pitsford Water Treatment Works which serves a population of 258,000. Prior to the outbreak, there were no published reports of human infection with this genotype and only one infection was known to the UK Cryptosporidium Reference Unit. The outbreak demonstrates that this genotype is a human pathogen of public health importance. However, little is known about it. This study aims to establish the taxonomic status of the rabbit genotype and improve our understanding of the biological features and risks for better prevention and control via the waterborne route.

The principal question addressed in this project is whether the rabbit genotype is a separate species from, or a variant of *C. hominis*. After undertaking a literature review (Objective 1), we followed the guidelines for investigating the taxonomy of *Cryptosporidium* genotypes, described by Xiao and colleagues (2004) to examine the taxonomic status of the rabbit genotype:

1. Describing and measuring the morphological features of the oocyst (Objective 2).
2. Genetic characterisation at multiple loci, to include the small subunit ribosomal RNA (SSU rRNA) and other functional genes (Objective 3).
3. Establishing natural and experimental host range (Objective 4).
4. Comply with International Committee for Zoonotic Nomenclature species-naming rules (Objective 5).

In addition to the comparative biology, the epidemiology and human health effects of infection were also investigated by detailed analysis of the outbreak cases (Objective 6), and compared with sporadic cases identified by enhanced testing and with *C. parvum* and *C. hominis* cases (Objectives 7 and 8). To evaluate the need for prevention and control via the waterborne route, public health risks from drinking water were investigated using Quantitative Risk Assessment (Objective 9).

Objective 1. Establish current knowledge by undertaking a literature review.

Introduction

Cryptosporidium spp. have been found in the faeces of over 150 mammalian host species, but the risks to public health from wildlife are poorly understood. To establish the current knowledge base about *Cryptosporidium* spp. infecting Lagomorphs, and rabbits in particular, and the potential risks to public health that rabbits may pose in the transmission of zoonotic cryptosporidiosis, we undertook a literature and data review, which has now been published in the journal Zoonoses and Public Health (Robinson and Chalmers, 2010).

Methods

Systematic principles were used in developing the search strategy (Knipschild, 1994). Electronic searches of seven online databases (PubMed, PubMed Central, Web of Knowledge, GenBank, Open System for Information on Grey Literature in Europe [openSIGLE], National Research Register of the National Institute for Health Research UK, Intute Website), not limited by either document type or language, covered the full time-span for each database from its inception up to 7th October 2008. Full details of search terms and processing retrieved references are in Appendix 1.

To estimate published prevalence in wild rabbits, Wilson's 95% confidence intervals for proportions were calculated (Confidence Interval Analysis v2.1.2, Southampton, UK).

To provide contemporary identification of *Cryptosporidium* isolates from rabbits, DNA sequences published on GenBank were compared at the partial small subunit ribosomal RNA (SSU rRNA) gene, for which data are available for the majority of isolates, sequence alignments were generated in ClustalX 2.0 and manually edited in BioEdit 7.0.9. Phylogenetic analysis was carried out by a neighbour-joining in TREECON. Evolutionary distances were calculated by the Kimura two-parameter model with *Eimeria tenella* as an outgroup.

Results

A total of 629 references were identified in the literature databases. Once duplicates had been removed 570 papers remained, of which 47 were identified as potentially relevant. In addition to these, Intute also identified 20 websites that required screening from which four further relevant references were found. Manual searching identified a further 11 references and consultation with experts a further 18. Of the 80 potentially relevant references, 74 were finally included in the study. Those investigating wild rabbits are analysed in detail here; for the full results of this literature review see Robinson and Chalmers (2010).

Cryptosporidium *in Lagomorphs*

Of 27 reports investigating natural infections in European rabbits (*Oryctolagus cuniculus*) in 13 countries, *Cryptosporidium* was detected in 24. Studies of other lagomorphs are rarer and only four were found; *Cryptosporidium* was detected in one cotton tail rabbit (*Sylvilagus floridanus*) but no infections were detected in hares (*Lepus europeaus*), although the numbers tested were small. There were 11 studies of *Cryptosporidium* in wild rabbits, although most reported incidental findings rather than population-based prevalence studies (Appendix 1, Table 1.1). However, prevalence can be estimated for each of two large studies (>100 samples), at 0.9% (95%CI 0.2% to 5.0%) and 0.0% (95%CI 0.0% to 1.6%). In smaller studies, prevalence ranged from 0.0% to 7.1%, with wider confidence intervals up to 32.4%. No epidemiological studies detailing age and sex profiles were found.

No *Cryptosporidium*-specific pathology was reported in wild rabbits due to paucity of data or co-infections with other pathogens. In captive rabbits pathogenicity appears to be related to young age, resulting in high mortality and liquid diarrhoea (data not shown) (Peeters, 1988; Pavlasek *et al.*, 1996; Shiibashi *et al.*, 2006). Older animals seem to lack clinical signs, but all ages may shed low numbers of oocysts (Rehg *et al*., 1979; Inman and Takeuchi, 1979; Peeters, 1988; Pavlasek *et al*., 1996; Cox *et al*., 2005; Shiibashi *et al*., 2006).

Experimental infection in rabbits has been established with *Cryptosporidium* including human pathogenic species: *C. parvum* IOWA isolate and *C. meleagridis* in neonate rabbits (Mosier *et al*., 1997; Darabus and Olariu, 2003). *C. parvum* infection resulted in pasty, unformed faeces, villous blunting and inflammation of the lamina propria (Mosier *et al.*, 1997). Additionally, *C. muris* infects neonate and weanling rabbits (Iseki *et al.*, 1989; Aydin and Ozkul, 1996).

Identity of Cryptosporidium *species in rabbits and rabbit genotype in other samples*

Due to a lack of comprehensive molecular characterisation, the identity of cryptosporidia causing most natural infections in rabbits is uncertain. Twelve *Cryptosporidium* DNA sequences from rabbits, or of rabbit genotype in humans, were identified (Table 1). Four sequences from four rabbit studies in three continents were from the partial SSU rRNA gene; all were rabbit genotype. All but one are identical, differing from *C. hominis* by the same four polymorphisms. Two additional polymorphisms occur close to the reverse primer site in one isolate (Appendix 1, Figure 1.1). The close relationship between rabbit genotype, *C. hominis* and *C. hominis* monkey genotype at this locus is shown in Figure 1. The five available rabbit genotype sequences at the HSP70 locus are also identical, but only show a single bp difference from *C. hominis*. Only single DNA sequences are published at the actin and *Cryptosporidium* oocysts wall protein (COWP) genes. The actin gene also only differs from *C. hominis* by a single bp, while the COWP gene is identical to *C. hominis*.

Table 1. *Cryptosporidium* DNA sequences and identity from rabbits, and rabbit genotype isolates from humans and water samples identified on the GenBank database.

Reference	**Country** (population type)	***Cryptosporidium* genes investigated**	**DNA sequence GenBank accession numbers**	**% similarity to *C. hominis* GenBank accession # AJ849462 (SSU rRNA) XM_661095 (actin) XM_661662 (HSP70) XM_661099 (COWP)**	***Cryptosporidium* sp. identity, inferred from SSU rRNA sequence alignment**
Isolates from rabbits					
Xiao *et al*., 2002	China (captive)	SSU rRNA actin	AY120901 AY120924	780bp/784bp: 99.5% 989bp/990bp: 99.9%	rabbit genotype
Ryan *et al*., 2003	Czech Republic (captive)	SSU rRNA HSP70	AY273771 AY273775	481bp/485bp: 99.2% 396bp/397bp: 99.7%	rabbit genotype
Learmonth *et al*., 2004	New Zealand (wild)	SSU rRNA	AY458612	749bp/754bp: 99.3%	rabbit genotype
Anon 2008a	UK (wild)	SSU rRNA HSP70	FJ262725 FJ262728	783bp/787bp: 99.5% 278bp/279bp: 99.6%	rabbit genotype
Yu *et al.*, unpublished	China (captive)	23 kDa sporozoite surface antigen mRNA	EU498678	Insufficient data for confirmation as rabbit genotype	
Yu *et al.*, unpublished	China (captive)	15 kDa sporozoite surface antigen (CP15) mRNA	EF453376	Insufficient data for confirmation as rabbit genotype	
Isolate from a human sporadic case					
Robinson *et al*., 2008a	UK	SSU rRNA HSP70 COWP	EU437413 EU437412 EU437411	783bp/787bp: 99.5% 278bp/279bp: 99.6% 506bp/506bp: 100%	rabbit genotype

SSU rRNA = small subunit ribosomal RNA; HSP70 = 70 kDa heat shock protein; GP60 = 60kDa glycoprotein; COWP = *Cryptosporidium* oocyst wall protein.

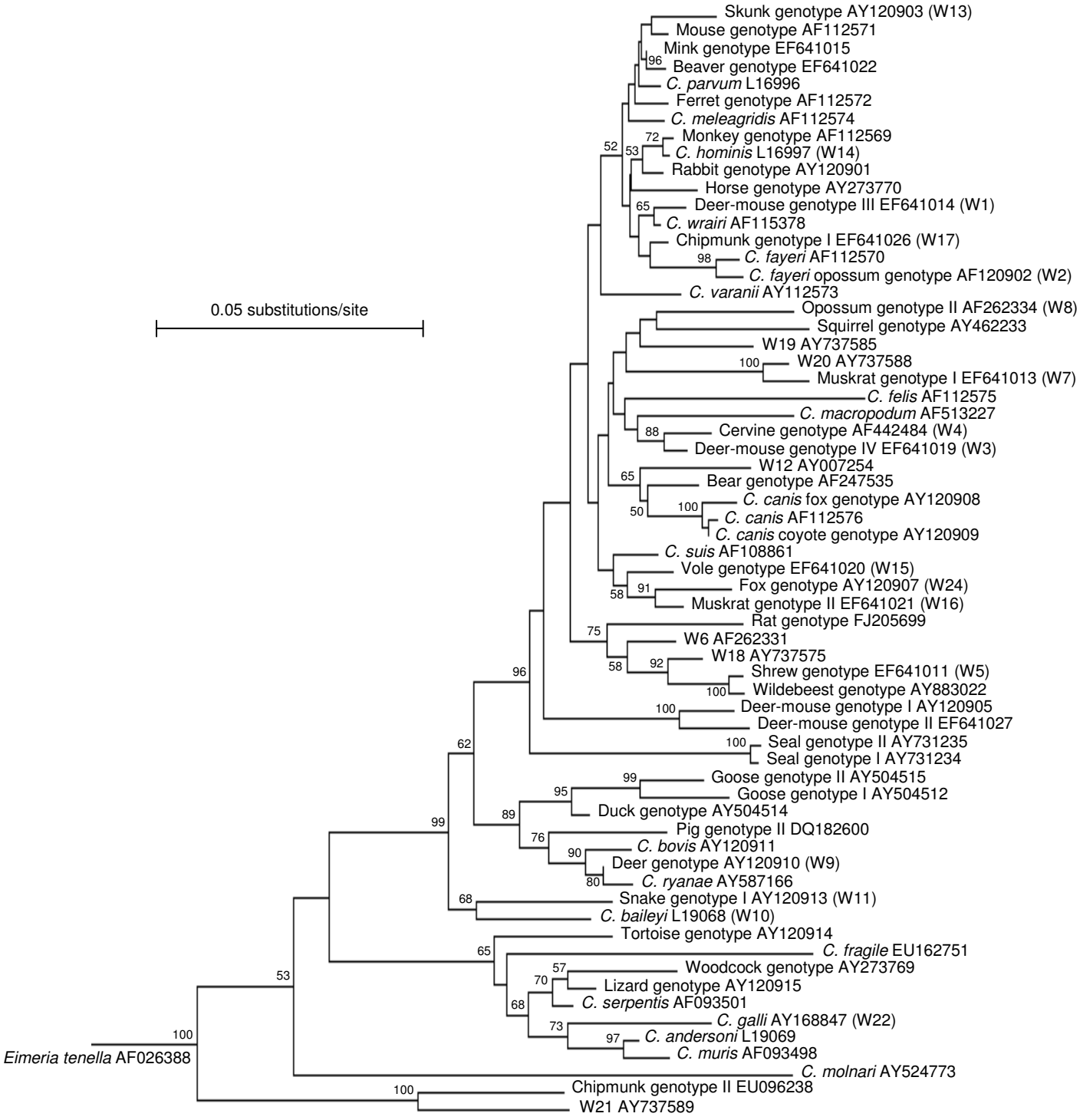

Figure 1. Phylogenetic relationships between *Cryptosporidium* sp. rabbit genotype and known *Cryptosporidium* species/genotypes as inferred by a neighbour-joining analysis of the SSU rRNA gene. Bootstrapping values over 50% from 1,000 pseudoreplicates are shown at branches.

Evidence for rabbits as a potential source of human infection with Cryptosporidium

Prior to the outbreak of waterborne cryptosporidiosis in the UK in July 2008 (Anon., 2008a; Chalmers *et al*., 2009b), there were no published reports of human infection with the rabbit genotype, although an adult female who had been to Spain had been recorded by the CRU, a finding published in November 2008 (Robinson *et al*., 2008a). No associations between rabbits and human cryptosporidiosis had been identified in population-based analytical epidemiologic studies where this was investigated (Bern *et al.,* 2002; Hunter *et al*., 2004; Smith *et al*., 2009). However, individual risks may be present. A human case of cryptosporidiosis identified as *C. hominis* was reported to have no other identifiable risk factors than contact with rabbits (McLauchlin *et al*., 2000). While *Cryptosporidium* oocysts have been detected in pet rabbits, the species were not identified although it is clear from experimental studies that human pathogenic *Cryptosporidium* species are infectious for rabbits.

Discussion

Current knowledge and understanding of *Cryptosporidium* in rabbits is limited. Estimated prevalence in wild rabbits is up to 5.0%, although the data are from just two large scale studies. The rabbit genotype is most commonly identified although the number of studies is small and few rabbits were studied. The true prevalence of *Cryptosporidium* in wild rabbits is likely to be affected by several factors including population density and structure, the age of the host, and exposure, while the estimated prevalence will depend on the study design, sampling frame and method, nature of the samples collected, sensitivity and specificity of the detection test. None of the studies found provided any epidemiological data. The apparent low number of oocysts shed by many rabbits is noteworthy when designing a sampling study as many of the traditional methods of detection have low analytical sensitivity and have the potential to generate false negatives (Pereira *et al*., 2002). This can be overcome by using IMS to concentrate oocysts prior to detection by either IF microscopy or molecular methods (Webster *et al*., 1996; Deng *et al*., 1997; Pereira *et al*., 1999; Deng *et al*., 2000; Atwill *et al*., 2003; Davies *et al*., 2003; Cox *et al*., 2005; Robinson *et al*., 2008b).

The rabbit genotype has a close genetic relationship with *C. hominis* and the monkey genotype inferred from phylogenetic analyses at the SSU rRNA, HSP70, actin and COWP loci (Xiao *et al*., 2002; Ryan *et al*., 2003; Learmonth *et al*., 2004; Robinson *et al*., 2008a) but is readily differentiated at the SSU rRNA gene where all rabbit genotype isolates except one were identical. This is consistent across geographical and host (rabbit and human) boundaries.

It appears that rabbits are susceptible to infection with *Cryptosporidium* rabbit genotype, *C. parvum* and *C. meleagridis*. All these species and genotypes are human pathogens and the role of rabbits as a potential source of zoonotic *Cryptosporidium* must be considered. Rabbits and humans are the only known natural hosts of the rabbit genotype (Xiao *et al*., 2002; Ryan *et al*., 2003; Learmonth *et al*., 2004; Robinson *et al*., 2008a; Anon., 2008a; Chalmers *et al*., 2009b). Although exposure through drinking water has caused a human outbreak of illness, direct contact with rabbits or their faeces has not been identified as a risk factor for human cryptosporidiosis. However, a documented Vero cytotoxigenic *Escherichia coli* O157 outbreak among people who had contact with rabbit faeces while visiting an animal park (Bailey *et al*., 2002) demonstrates the potential of rabbits as a direct zoonotic risk to public health.

The risks to rabbits from *Cryptosporidium* appear to be age-related with high rabbit mortality, diarrhoeic faeces and higher oocysts counts observed in neonatal rabbits and in rabbits 30 to 40 days of age. This has also been observed with *Cryptosporidium* sp. in other host species, such as with *C. parvum* in cattle (Fayer *et al*., 2007).

The literature returned demonstrates that *Cryptosporidium* spp. occur in rabbit populations. Although only the rabbit genotype has been positively identified in wild rabbits, other species including *C. parvum* have established experimental infections and this could occur naturally. Further studies, incorporating epidemiological data and molecular characterisation, are required to fully understand the prevalence, range of *Cryptosporidium* species and genotypes that can infect rabbits and the potential risk to public health. In addition, the potential host range and pathogenicity of the rabbit genotype compared with other closely related species need to be examined. Both pet and wild rabbits are a potential source of human cryptosporidiosis and as such, good hygiene practices are recommended following animal handling or exposure to faeces or potentially contaminated surfaces (Anon., 2008b). Water supplies should be protected against access by wildlife, including rabbits.

Objective 2. Describe and measure the morphological features of the *Cryptosporidium* rabbit genotype in comparison with *C. hominis.*

Introduction

There have been no previous studies of the biological features of the *Cryptosporidium* rabbit genotype, and description of the morphological features, at the very least of the oocyst stage, is an essential element of taxonomic description. Here we describe the detailed microscopical examination of rabbit genotype oocysts in field, clinical and experimental samples compared with *C. hominis* and other cryptosporidia.

Methods

Sources of Cryptosporidium *oocysts for examination*

The field isolate was from the bowel contents of a juvenile rabbit (CRU reference number W17211) retrieved from a contact tank at Pitsford water treatment works (Northamptonshire, UK), considered the source of contamination in the outbreak. In addition to *Cryptosporidium*, *Eimeria* spp. oocysts were also seen in the sample. The human clinical isolate examined (W17330) was from a patient considered to be part of the outbreak, and provided the inoculum in the animal transmission study from which oocysts were also examined (Objective 4).

Morphological examination of oocysts

Oocysts were separated from faecal debris by saturated salt flotation (Ryley *et al.*, 1976), and washed in reverse osmosis (RO) water to remove salt residue and return an osmotic balance to the final suspension (100µl). Equal volumes (15µl) of oocyst suspension and an anti-*Cryptosporidium* FITC conjugated antibody (CryptoCel, Cellabs) were mixed, incubated at room temperature for 15 minutes and a 10µl sealed wet preparation made on a microscope slide. Epifluorescence microscopy was used to detect and measure oocysts (n=50) at x1000 with Differential Interference Contrast (DIC) microscopy using the calibrated measuring capability of a DS-5M camera with DS-L1 control unit (Nikon UK Ltd.). Sizes were compared with previously published measurements of *C. hominis* and *C. parvum*.

To gather data on the recovery of the rabbit genotype oocysts by IMS (Isolate™, TCS Biosciences), 2g faeces was tested according to Robinson *et al*. (2008b).

To provide data on the staining characteristics of the rabbit genotype by DWI approved methods, 15µl of each oocyst suspension and the IMS material was applied to single-well slides, air-dried, fixed with methanol and stained with the CryptoCel FITC antibody (Cellabs) and 4′,6′-diamidino-2-phenylindole (DAPI, Sigma) (Smith *et al.*, 2002).

To gather data on the staining characteristics of the rabbit genotype oocysts when prepared using the methods employed in UK primary diagnostic laboratories, two faecal smears were also prepared from the human clinical sample and stained using auramine phenol

and modified Ziehl-Neelsen (Anon, 2007). The FITC, DAPI and auramine phenol stained samples were examined by epifluorescence at x400 and x1000 magnification. The modified Ziehl-Neelsen stained smear was examined at x400 and x1000 magnification with bright field microscopy.

Results

Oocyst morphology

Rabbit genotype oocysts were subspherical to ellipsoidal with a thick, clear and smooth oocyst wall with a suture at one pole. Sporulation occurred endogenously and sporulated oocysts were detected in freshly passed faeces. Four naked vermiform sporozoites were present and generally positioned parallel to each other at the perimeter of the oocyst. A central vacuole contained the oocyst residuum; fine granules roughly 0.6µm in diameter and a larger spherical globule 1.4µm in diameter.

Rabbit genotype oocysts from the wild rabbit measured, in suspension, a mean of 5.98 x 5.38µm with a length to width ratio of 1.11. The oocysts from the human clinical sample were the same size and shape. These measurements are slightly larger than *C. parvum* and *C. hominis* although the shape of the oocysts is similar (Appendix 2, Table 2.1).

Oocysts were recovered from bowel contents by IMS and fixed oocysts stained for IF as per the DWI monitoring protocol demonstrated bright, uniform FITC staining and up to four individual sporozoite nuclei were visible, fluorescing brightly following DAPI staining (Figures 2). Oocysts in the faecal smears stained by auramine phenol and modified Ziehl-Neelsen showed typical *Cryptosporidium* staining characteristics (Figure 2). Oocysts dried onto slides for staining were smaller than those measured in suspension, with a mean of 5.08 x 4.28µm and a length to width ratio of 1.19.

Rabbit genotype oocysts recovered from the faeces of rabbits, immunosuppressed gerbils and immunosuppressed adult mice which shed oocysts in the transmission studies had mean length and width dimensions of 5.21 x 4.74µm, 5.06 x 4.59µm and 5.06 x 4,46µm with a length to width ratio of 1.11, 1.10 and 1.14 respectively. These are similar to those of *C. parvum* and *C. hominis* and provide further evidence that oocyst morphology cannot be used to differentiate between these species and the rabbit genotype.

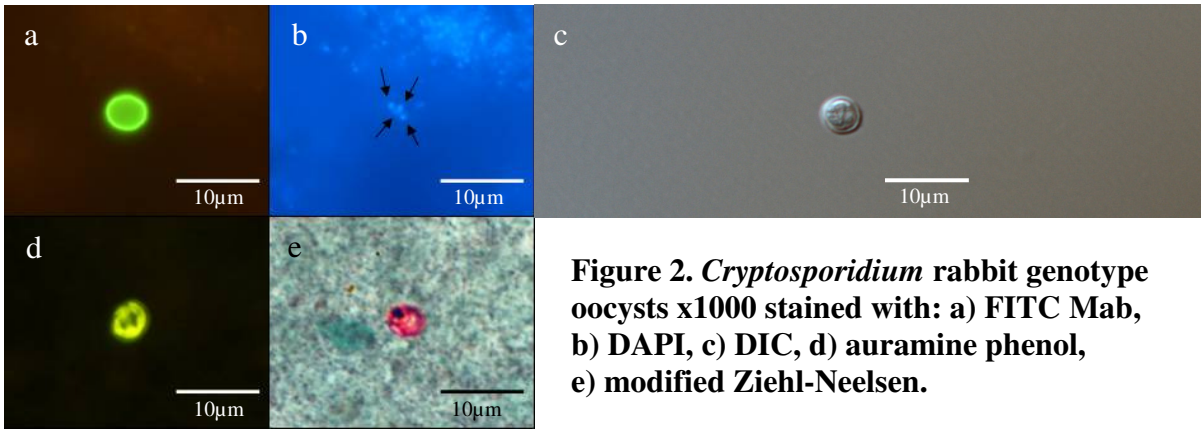

Figure 2. *Cryptosporidium* rabbit genotype oocysts x1000 stained with: a) FITC Mab, b) DAPI, c) DIC, d) auramine phenol, e) modified Ziehl-Neelsen.

Discussion

The morphology of rabbit genotype oocysts is typical of the genus, particularly the intestinal species which include *C. parvum* and *C. hominis*. While the size ranges and shape index overlap, oocysts from the rabbit genotype when measured in suspension are slightly larger than *C. hominis*. The size and shape of oocysts can vary depending on preparation and fixation (Fayer *et al.*, 1997), which is apparent in this study where unfixed oocysts were

slightly larger. Rabbit genotype oocysts recovered from experimentally infected animals, were slightly smaller than those from the wild rabbit and human stool (Objective 4). The reasons for this are unclear.

For diagnostic and detection purposes the size range of rabbit genotype oocysts falls within the 4 to 6 µm range that is stated in the protocols for water monitoring (Anon, 2005; Anon, 2009) and clinical diagnosis (Anon, 2007) but cannot be differentiated from other *Cryptosporidium* species by morphology alone. The rabbit genotype is clearly amenable to recovery by immunomagnetic separation using Isolate™ (TCS Biosciences), and Dynabeads® anti-*Cryptosporidium* (Invitrogen) used by Anglian Water for routine monitoring (Nick Humphries, Anglian Water, personal communication), and detection with IFM (Crypto-Cel, Cell Labs) demonstrating that this genotype would be detected in samples tested by approved methods. This is despite the fact that the antibodies in the IMS and IFM kits were originally raised against *C. parvum*.

This work has demonstrated that the oocyst morphology of the rabbit genotype is not sufficiently different from other closely related species, such as *C. hominis* and *C. parvum*, to enable differentiation, which can only be achieved using molecular methods. The staining and oocyst recovery properties of the rabbit genotype are the same as these other species, ensuring that they can be detected using the methods employed by clinical diagnostic and water testing laboratories.

Objective 3. Undertake genetic characterisation of the *Cryptosporidium* rabbit genotype at multiple loci.

Introduction

The literature review (Objective 1) revealed that only limited genetic data were available for the rabbit genotype. As an essential requirement of the taxonomic description, we investigated several isolates at multiple loci, in comparison with known human pathogenic *Cryptosporidium* species. Measurement of genetic variation between *Cryptosporidium* isolates provides a quantitative assessment of similarity, or difference (presented as % difference over a defined sequence length of base pairs, bp). This is usually undertaken by analysis of at least the SSU rRNA gene, and preferably other genes too. However, it is acknowledged that intraspecific variation also occurs, and there are no absolute % difference cut-off values for delineating *Cryptosporidium* species with functional phlyogenetic similarities. A cautious approach would be to define a new group (species) as one which differs from its nearest neighbour by the same amount or more than is observed between other defined species, also taking into account other biological features.

We investigated five genes (SSU rRNA, HSP70, COWP, actin, and LIB13) which are commonly used for the differentiation or characterisation of *Cryptosporidium* isolates. A sixth gene, glycoprotein (GP) 60, was investigated as it provides valuable epidemiological linkage information between isolates (see Objective 7), but being under selective pressure it is highly polymorphic even within species and cannot enlighten the taxonomic relationship.

Furthermore, to provide additional information on the relationship between *C. hominis, C. parvum* and the rabbit genotype, hierarchical cluster analysis over many thousand bp was undertaken as part of an EU funded project (Healthy Water).

Methods

Cryptosporidium *isolates, oocyst preparation, DNA extraction and characterisation at six genetic loci*

Human clinical isolates (23 outbreak and a subset of 15 sporadic) and one field isolate obtained from the intestinal contents of the rabbit, which was the suspected source of the outbreak, were processed to extract DNA by saturated NaCl flotation, heat treatment,

proteinase K digestion in lysis buffer and a spin-column filtration technique (QIAamp DNA mini kit, Qiagen) as described in Elwin *et al*., 2001. DNA extracts were stored at -20◦C prior to use. PCR products were obtained as follows:

1. SSU rRNA gene: ~830bp fragment using a nested protocol (Jiang *et al*., 2005).
2. HSP70 gene: 448bp fragment using external primers F4 and R4 (Morgan *et al*., 2001), carried out on the field isolate and a representative sample of 25 clinical isolates.
3. Actin gene: ~830bp fragment using a nested protocol was carried out on the field isolate and a representative sample of 25 clinical isolates. (Ng *et al*., 2006).
4. COWP gene: 550bp fragment (Spano *et al*., 1997) generated from the field isolate and two clinical isolates. Since the rabbit genotype and *C. hominis* are homologous no further isolates were investigated at this locus.
5. LIB13 coding gene with unknown function: ~160bp fragments generated in a species-specific real time PCR (Tanriverdi *et al*., 2003) from the field isolate and the two clinical isolates also characterised at the COWP locus. The sequence targeted in the LIB13 assay revealed homology between *C. hominis* and the rabbit genotype and hence no further isolates were tested.
6. GP60 gene: ~850bp fragment using a nested PCR protocol (Alves *et al*., 2003). Within each allele family there exist multiple subtypes, varying from each other according to the number of trinucleotide repeats (TCA, TCG or TCT) coding the amino acid serine; this forms the basis of the full subtype nomenclature (Sulaiman *et al*., 2005).

Confirmation of amplification and sequence analysis

With the exception of the LIB13 real time assay, PCR amplicons from each of the conventional PCR assays were separated by 2% (w/v) agarose gel electrophoresis to confirm the presence or absence of the expected product. Melt curve analysis was carried out in the real time assay to confirm amplification of the correct target. PCR products were purified prior to sequencing using the Qiaquick clean up kit (Qiagen Ltd). Bidirectional sequencing was carried out by Geneservice, Cambridge, UK using the BigDye Terminator Cycle Sequencing kit on an ABI3730 automated sequencer (Applied Biosystems UK). Consensus sequences were created for each isolate at each locus tested (ChromasPro 1.4a, Technelysium Ltd, Australia), and compared with published sequences in the GenBank database using the National Institutes of Health National Center for Biotechnology Information basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST). As a QC exercise 3% (4/133) of assays were repeated.

To permit a more extensive comparison between isolates, over 4469 bp, a further 11 *Cryptosporidium* genes, identified using reciprocal basic local alignment search tool) (BLAST) (Altschul *et al*., 1990) of the published *C. hominis* and *C. parvum* genomes was performed as part of an EU funded project "Healthy Water". Ten genes were selected from a possible 304 candidates, with preference being given to annotated genes, and were investigated along with analysis of the COWP gene on 4 different rabbit genotype isolates, and compared with *C. hominis*, *C. parvum* and *C. meleagridis* by hierarchical cluster analysis (Norusis, 2005).

Results

Characterisation at six genetic loci:

1. SSU rRNA gene: all 39 isolates tested produced amplicons with sequences homologous with each other and a published sequence from a rabbit AY120901 (Xiao *et al*., 2002). The two additional polymorphisms seen in isolate AY458612 from New Zealand, identified in the literature review (Objective 1) were not seen in this collection

2. HSP70: 24/25 isolates produced amplicons and when sequenced 23 were homologous with each other and a published sequence from a rabbit AY273775 (Ryan *et al*., 2003). One clinical isolate showed 356/357bp (99.7%) similarity with these isolates.
3. Actin: All 25 isolates tested produced amplicons with sequences homologous with each other and a published sequence from a rabbit AY120924 (Xiao *et al*., 2002).

4 & 5. COWP & LIB13: The field isolate and the two clinical isolates tested at these two loci all produced amplicons which showed homology with each other and with published *C. hominis* sequences.

6. GP60: all 39 UK isolates tested produced amplicons, revealing two different subtype families, Va and Vb, with four and seven subtypes respectively.

   QC exercise – data from the retested isolates gave the same result as the original analysis.

These analyses confirm that the rabbit genotype and *C. hominis* are identical at the COWP and LIB13 genes and therefore cannot be differentiated from it using assays based on these targets. We also confirm the UK rabbit genotype differs from *C. hominis* at the following genes, and assays based on these genes potentially differentiate the rabbit genotype:

SSU rRNA: 4 bp differences in 787 bp (0.51%)
HSP70: 1 bp difference in 403 bp (0.25%)
Actin: 1 bp difference in 833 bp (0.12%)

These differences are substantially less than between *C. hominis* and *C. parvum*, which differ by 1.02, 1.51, 1.51% at these loci respectively. No evidence for mating between the rabbit genotype and *C. hominis* was observed in any of the isolates analysed.

The rabbit genotype appears to have its own GP60 subtype families, which we have proposed as Va and Vb (Chalmers *et al*., 2009b). As part of separately funded work, we have also developed a novel real time PCR-based assay to rapidly, specifically and sensitively detect and differentiate these two subtype families (Hadfield *et al*., in preparation).

Four of the additional 10 loci failed to amplify for *C. meleagridis* so differences between this species and the others are based only on 2853 bp comparisons. Of note is that the rabbit genotype isolates differ from *C. hominis* but only in 0.27% of base pairs. This is close to the difference between *C. parvum* and anthropogenic *C. parvum* isolates (0.13%) (Figure 3). This is substantially less than the differences between *C. hominis* and *C. parvum* (1.72%) and those between *C. hominis* or *C. parvum* and *C. meleagridis* (5.5% and 5.05%, respectively).

DNA sequences from the original rabbit field isolate were deposited on GenBank under accession numbers FJ262725, FJ262728, FJ262731, GU327781 to GU327783.

Discussion

Genetic characterisation of a larger number of rabbit genotype isolates in comparison with *C. hominis* has been undertaken using two approaches; through evaluation of individual (SSU rRNA, HSP70, Actin, COWP, LIB13 and GP60) and combined, multiple (4469bp) loci. The results support previously limited descriptions of similarity amongst rabbit genotype isolates and small variation from *C. hominis*, identified during the literature review (Objective 1). Nevertheless, the genetic differences are shown to be consistent and conserved, both in samples from the UK and worldwide. The polymorphisms in the SSU rRNA, HSP70 and Actin genes in the rabbit genotype are never seen in *C. hominis*. However, only about 0.07% of the entire genome has been studied and further investigation of the whole genome would be helpful in establishing the true genetic differences between the rabbit genotype and other cryptosporidia. Such data have only so far been published for single *C. hominis*, *C. parvum* and *C. muris* isolates (Xu *et al*., 2004; Abrahamsen *et al*., 2004; Silva, unpublished), but as next generation sequencing technologies become more readily available and affordable such

analyses on greater numbers of isolates will greatly improve our understanding of genetic relatedness within the *Cryptosporidium* genus.

The very small differences detected so far between the rabbit genotype and *C. hominis* are much less than those between other accepted *Cryptosporidium* species. However, species definition cannot be achieved solely through the measurement of genetic variability and other biological observations must be included in making conclusions.

The GP60 gene is a commonly used target for subtyping *Cryptosporidium* spp. and substantial variation in the rabbit genotype has led to the proposal of two distinct subtype families Va and Vb, each containing different subtypes (Chalmers *et al*., 2009b).

Based on the genetic analyses alone, there is no good evidence that the rabbit genotype differs sufficiently from *C. hominis* for it to be designated a separate species, and would probably be deemed to be a subspecies of *C. hominis*, but with its own GP60 subtypes.

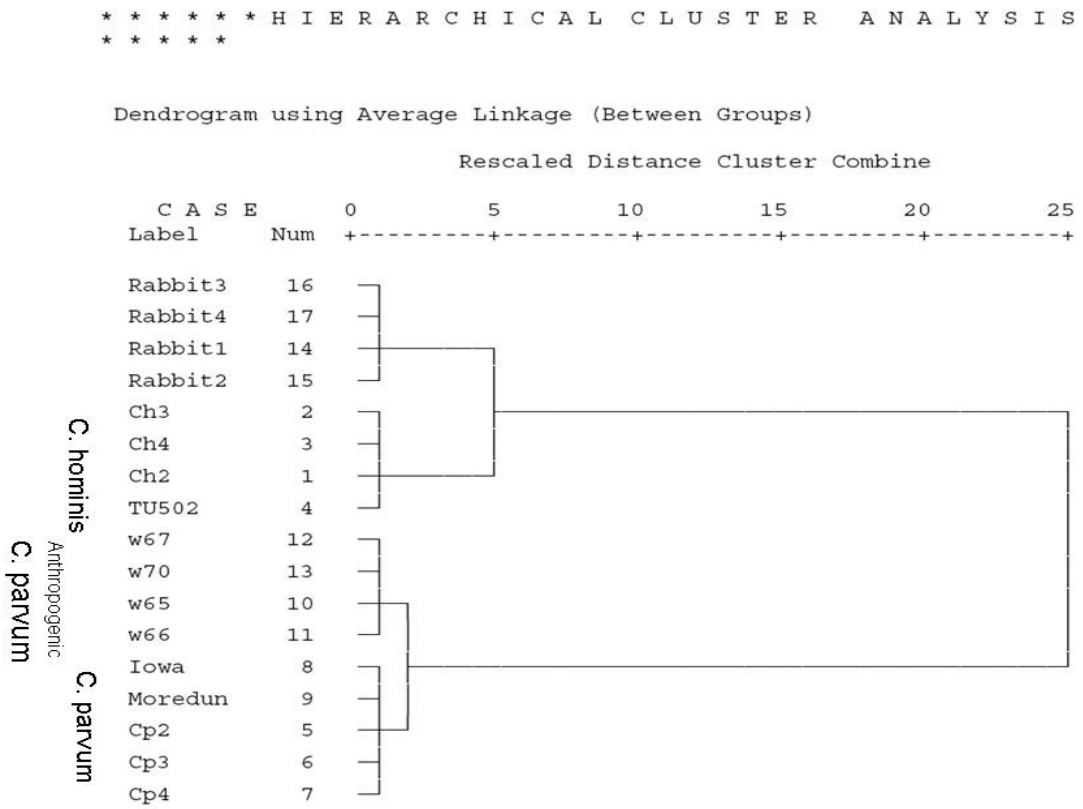

Figure 3. Relationships between *C. parvum*, *C. hominis* and rabbit genotype.

Objective 4. Investigate the comparative experimental host range of the rabbit genotype and *C. hominis.*

Introduction

As the rabbit genotype and *C. hominis* are genetically very similar, the infectivity of each isolate in experimental transmission studies using both immunocompetent and immunocompromised animals was observed. In addition to infection in rabbits, the usual host for the rabbit genotype, and neonatal mice, which is a widely used infectivity model for *C. parvum*, we also used immunosuppressed mice and Mongolian gerbils. Immunosuppressed mice have been utilised for *Cryptosporidium* studies for over 20 years, while the use of Mongolian gerbils for *C. hominis* studies has been reported to have some success (Baishanbo *et al*., 2005; Moredun Institute unpublished data), thus providing a small mammal "model" for infection with this species. In our experiments, for each animal type there was also a non-

infected control group of animals. The experimental design was scrutinised and approved by the Moredun Institutes' in-house ethics committee, and a statistician, to ensure efficient and ethical use of animals.

Methods

Inoculum preparation and administration

The rabbit genotype inoculum was prepared from faeces from one of the outbreak cases and *C. hominis* from a sporadic case from outside the outbreak affected area who had not travelled abroad and had no other routinely-looked for pathogens detected in their stool sample. It was not possible to use the rabbit faeces as they were insufficient in volume/numbers of oocysts and were co-infected with *Eimeria*. Prior to inoculation, the oocysts were separated from faecal matter by sucrose flotation and stored in antibiotic solution (10 units Penicillin, 10µg Streptomycin in 1ml RO water), surface-sterilised by immersion in 70% ethanol for 30 minutes, and resuspended in sterile phosphate buffered saline. A portion of each inoculum was also tested for viability using a 2 step i*n-vitro* excystation method (Lally *et al.* 1992). The inoculum, administered by oral gavage, was 10^5 semi-purified oocysts per animal, this being the largest practicable dose given the quantities of oocysts available, and a dose known to be sufficient to enable ready detection of *C. parvum* infection in neonatal mice. For the rabbit genotype, excystation rates at inoculation were 93.9% and the sporozoite:shell ratio was 2.25. For *C. hominis* excystation rates at inoculation were 98.5% and the sporozoite:shell ratio was 3.15 Age matched control animals were untreated.

Host animals, outcome measurements and data analysis

New Zealand white rabbits were sourced from Harlan UK, and the Mongolian gerbils from B&K Universal Ltd. Mice (Porton strain) were bred in-house, a closed colony maintained at Moredun for over 40 years. Animals were mainly group housed; in pairs or fours (initial rabbit experiment), and in trio's (gerbils and adult mice). The second group of rabbits (see Results, below) were housed individually. Neonatal mice were housed as litters. All animals were fed proprietary diets and water *ad libitum*, the rabbits also receiving autoclaved hay. Out-sourced animals were allowed one week to settle and acclimatise to their new surroundings. The welfare of the animals was monitored throughout. Details of immunosuppression, animal husbandry, sampling and sample preparation are in Appendix 3.

The first outcome measurement was oocyst counts per cage sample by AP microscopy (40 fields of view; x400 magnification or scanning for 5 minutes if no oocysts seen), recorded for up to 44 days post infection (PI). The exact count was not recorded except where < ten. The data were coded as: 0 = oocysts not seen; 1 = <10; 2 = ≥10; 3 = oocysts seen in the majority of fields; 4 = large numbers of oocysts seen in every field.

Cryptosporidium oocysts at peak shedding times were also typed at the SSU rRNA and GP60 loci (Objective 3) to ensure they matched those used to initiate the infections.

The second outcome measurement was subjective histopathology. Pairs of animals from each group were sacrificed during oocyst shedding, and the gastrointestinal tract removed. Sections of stomach, duodenum, ileum, caecum and colon were fixed in formol saline, processed through paraffin wax blocks, cut into 5 µm sections and stained with haematoxylin and eosin and examined for evidence of *Cryptosporidium* endogenous stages.

For all the cages, the counts on successive days were highly correlated, a high count of oocysts on a particular day generally being followed by a relatively high count the following day. Because of the small number of cages used for each host species-isolate combination, the ordinal nature of the data and the lack of independence between successive oocyst counts it was not possible to fit any sophisticated statistical models to the data. Two approaches were

used for the analyses. First, the study period was arbitrarily split into four 10 day periods and the number of cages in each isolate group with a high maximum count (code 3 or 4) determined. The differences in proportions between the two isolates were assessed using a Fisher's exact test. Secondly, the median times taken for cages to reach code 3 were compared for the two isolates using a Mann-Whitney U-test.

Cell culture infectivity

Rabbit genotype oocysts were also used *in-vitro* (Rochelle *et al.,* 2002). Briefly, surface sterilised oocysts were excysted as described above, then inoculated onto confluent layers of HCT-8 cells (a human ileo-caecal carcinoma cell line) grown on multiwell slides. Seventy two hours post inoculation, the cell sheets were fixed with methanol, then stained with 'Sporoglo' (Waterborne Inc, New Orleans) a monoclonal antibody stain which binds to endogenous stages but not to oocysts. Clusters of developing endogenous stages were observed using an Olympus BX 50 fluorescence microscope, and measured using a commercial image analysis system (Image Pro Express, Media Cybernetics).

Results

Infection measured by oocyst shedding (see Figure 4 for a visual summary)

Neonatal Porton mice (n=10 per litter):

no evidence of infection was found with rabbit genotype or *C. hominis.*

Juvenile (weanling) rabbits (n=4 per isolate; 2 controls):

animals infected with the rabbit genotype began shedding oocysts 4 to 7 days PI, and continued to shed at detectable levels until day 14 PI. Numbers of oocysts shed were at best moderate, peaking between days 6 to 10 PI.

The first group of rabbits infected with *C. hominis* provided an anomalous result as the oocysts recovered were the rabbit genotype field isolate. Despite rigorous investigation, it was not possible to determine whether the contamination occurred as a result of cross infection during the course of the first study, or if the field isolate of *C. hominis* used was a mixed infection containing a very small number of rabbit genotype oocysts or if the rabbits had an undetected infection prior to inoculation.

The infectivity study was repeated with a further field sourced *C. hominis* isolate. Excystation rate was 90.9, and the sporozoite/shell ratio was 2.04. Only one animal infected with *C. hominis* shed oocysts, on one day only (d17 PI). Of 4 replicate smears, two were also IFM positive, containing three oocysts each, while two were negative. Subsequent attempts to type the oocysts were unsuccessful, in spite of the use of IMS, indicating the very limited numbers of oocysts present in the faeces.

The differences in shedding scores of the rabbit isolate and the second *C. hominis* isolate in young rabbits were statistically significantly different (Fisher's Exact test $p = 0.029$). However, because of the sample size, the Mann-Whitney test did not give a significant difference for the total shedding period; this test does individual comparisons on each day for the 2 groups and then needs to use a False Discovery Rate (FDR) adjustment because it relies on many individual comparisons. However, if the period was shortened to day 14 (so that each group still has 4 animals) then there are significant shedding differences between the 2 groups on day 6 and days 8 to 12 ($P=0.073$ against a significance threshold of 0.10 when using FDR).

Immunocompromised Mongolian gerbils (n=12 per isolate; 4 controls):

After 7 days immunosuppression, the gerbils were looking 'scruffy', with ruffled coats, but were otherwise in good health. One animal fitted during dosing, and died the following day. The remaining animals continued in good health.

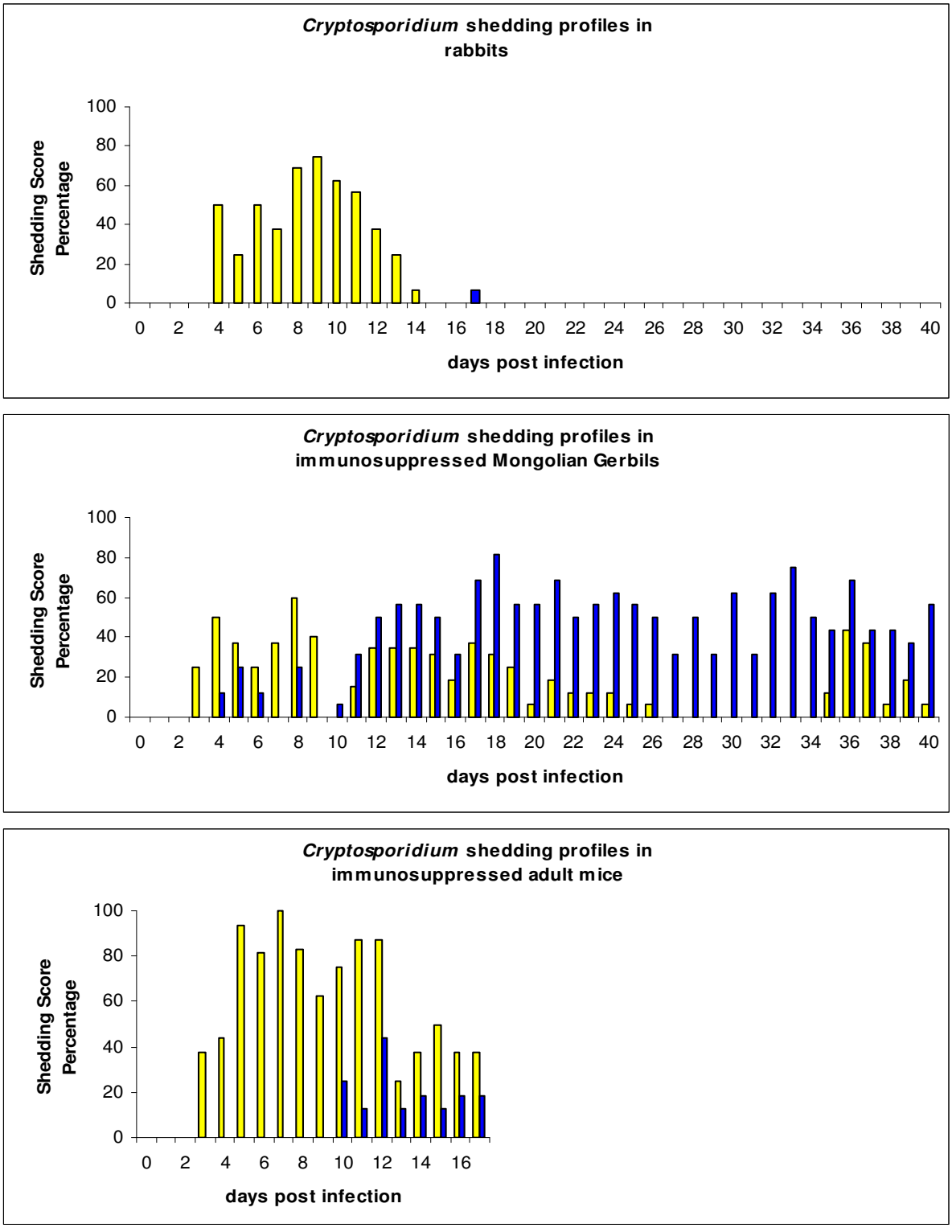

Figure 4. ***Cryptosporidium hominis*** **(blue bars) and rabbit genotype (yellow bars) shedding profiles in experimentally infected animals.**

Gerbils inoculated with the rabbit isolate began shedding oocysts on days 3 or 4 PI, and shedding fluctuated thereafter, peaking between days 8 to 17 PI, though different cages of animals varied widely in the shedding profile obtained. Some continued to shed up to day 28 PI, when shedding in all cages appeared to cease, but some animals started to shed again by day 35 PI, up to day 40 when the monitoring finished.

Gerbils infected with *C. hominis* commenced shedding on days 4-5, but did not shed significant numbers of oocysts until days 11-12, and then continued to shed throughout the remainder of the 40 day observation period without pause.

The proportion of rabbit genotype infected cages having a high count in the first ten days PI was significantly greater than *C. hominis* cages (Fisher's exact test $p = 0.029$). The difference between the median times to high shedding (rabbit genotype 7.5 days compared to *C. hominis* 13.5 days) was statistically significant (Mann-Whitney U-test $p = 0.028$).

Immunosuppressed adult Porton mice (n=12 per isolate; 4 controls):

Problems with the adult mice occurred with illness during immunosuppression, and two died. The animals' health declined rapidly, and the experiment was terminated due to a suspected bacteraemia, exacerbated by the immunosuppression. After consultation with the ethics committee, the experiment was repeated, halving the dose of dexamethazone (it is possible the mice were overdosing themselves, drinking much more water than expected) and adding aureomycin (an antibiotic) to the drinking water. To more closely monitor animal welfare, the daily observation rate was increased, and the monitoring period lengthened. After 7 days immunosuppression all the mice looked well, so we commenced with two groups of 12 infected animals and 4 non infected controls.

The mice infected with rabbit genotype started shedding oocysts on days 3 to 5 PI, and continued to shed oocysts until the end of the 18 day monitoring period.

Those infected with *C. hominis* commenced shedding oocysts on day 10 PI, and continued to shed until the experiment was terminated on day 18 PI, though the numbers of oocysts shed were much smaller than those produced by the animals infected with the rabbit genotype. Problems of ill health persisted in this second group, and we started culling affected animals from day 9 PI onwards. Having achieved a demonstrably different result between the isolates by day 18 PI, we terminated the experiment and culled the remaining mice on welfare grounds.

Infection measured by clinical signs

We found no clinical signs attributable to *Cryptosporidium* infection in any animals for the duration of the experiments.

Infection measured by pathological examination

Neonatal Porton mice:

No pathological examinations were attempted, as there was no evidence from the intestinal or faecal screening that either isolate had established an infection.

Juvenile (weanling) rabbits:

Rabbit genotype. The animal culled during shedding was found to have occasional structures consistent with cryptosporidial infection in the apical brush borders of the small intestinal villi (duodenum and ileum), and infiltrations of eosinophils in the base of the lamina propria and occasionally in the tips of the villi.

None of the *C. hominis* infected animals were culled during shedding as the duration and magnitude of infection were so short.

The age matched control rabbit showed no evidence of infection or pathological lesions.

Immunosuppressed Mongolian gerbils:

Rabbit genotype. Both animals examined showed multiple protozoal parasites on the brush border of the epithelium of the small intestineal villi, with some disruption of the villous structure.

C. hominis. Both animals examined showed multiple protozoal parasites on the brush border of the gastric and small intestinal epithelial villi, while one also demonstrated parasites on the large intestinal villi. There was some disruption of villous structure in both these animals.

The age matched control animal had a few eosinophils in the lamina propria of the small intestine, but no evidence of infection.

Immunosuppressed adult Porton mice:

Rabbit genotype. Both animals showed multiple protozoal parasites in the brush border of the epithelium of the small intestine.

C. hominis. There was no evidence of infection or lesions in the gastrointestinal tract of either of these animals.

The age matched control animal showed no evidence of infection or lesions in the gastrointestinal tract.

Cell Culture with rabbit genotype

72hrs post-inoculation the HCT-8 cell sheets on multiwell slides were fixed in methanol and stained with a commercial monoclonal antibody stain which binds to endogenouos stages but not oocysts. Between 3 and 11 clusters of developing endogenous stages were observed per well, each cluster containing 4 to >20 organisms at varying stages of development. This work establishes that HCT-8 cells can be used as a cell culture model for the rabbit genotype.

Discussion

In contrast with *C. parvum*, for which neonatal mice are an established experimental model, neither the rabbit genotype nor *C. hominis* was infective for neonatal mice. In the other small mammal experimental hosts, the rabbit genotype showed significant differences from *C. hominis*. Although both isolates established infection in immunosuppressed Mongolian gerbils and adult mice, the shedding oocyst profiles were significantly different. In gerbils, more rabbit genotype cages had a high count in first 10 days and shorter median time to high shedding than *C. hominis* (P=0.029). In adult mice all 4 rabbit genotype cages had high levels of shedding in the first ten days compared to none of the *C. hominis* cages (P = 0.029, Fisher's exact test). The observed shedding patterns of *C. hominis* in immunosuppressed adult mice is novel as rodents were previously thought to be refractive to infection with this species, based on neonatal studies (Morgan-Ryan *et al*., 2002; Tzipori and Widmer, 2007).

The shedding profiles of the rabbit isolate and the second *C. hominis* isolate in young rabbits were also clearly different with only marginal evidence of infection in a single rabbit infected with *C. hominis* while the rabbit genotype was shed in high numbers from day 6 PI. The use of two statistical approaches strengthened the argument that there are significant shedding differences; the first test looked at the likelihood of reaching a maximum score for the whole study period and the second test looked at median shedding score differences on individual days/periods. Thus, while the two isolates are genetically very closely related, they are biologically significantly different.

Health complications of immunosuppression are not uncommon and the ruffled coats causing a scruffy appearance in the immunosupproessed Mongolian gerbils was noted previously in the use of this model for the duration of the immunosuppression (Moredun Institute unpublished data), although no mention was made previously by Baishanbo *et al*., (2005).

We observed no clinical signs attributable to *Cryptosporidium* infection in any animals for the duration of the experiments. This contrasts with infection in humans where illness has been reported (Objective 6), but is consistent with other *Cryptosporidium* experimental infections in rabbits where weanlings display few clinical signs compared with neonates. Clinical signs in neonatal rabbits depend upon the infecting *Cryptosporidium* species and vary from mild to fatal pathology (Mosier *et al.*, 1997; Peeters *et al.*, 1986). Our results for the rabbit genotype and *C. hominis* are consistent with other infectivity studies of *C. hominis* in immunosuppressed Mongolian gerbils, which also resulted in asymptomatic infection with no associated mortality (Baishanbo *et al.*, 2005). When compared to *C. parvum* infections no differences in oocyst shedding, presence of parasites or ileal histology were noted (Baishanbo *et al.*, 2005).

Structures consistent with cryptosporidial infection were observed in the small intestine by histopathological examination of rabbits, gerbils and adult mice infected with rabbit genotype. This is consistent with other intestinal cryptosporidia in their type hosts.

The infection of HCT-8 cells with the rabbit genotype resulted in clusters of endogenous stages in the cell layer. This is similar to that reported for *C. hominis* by Hashim *et al.* (2004). They compared HCT-8 cell infection with *C. hominis* and *C. parvum* and found that *C. hominis* infection was less efficient, and formed discrete clusters in the monolayer compared to the even distribution of *C. parvum* (Hashim *et al.*, 2004).

Objective 5. The taxonomic status of the *Cryptosporidium* rabbit genotype.

The current algorithm for investigating the taxonomy of *Cryptosporidium* genotypes, described by Xiao and colleagues (2004) is based on morphological, limited genetic and host infectivity data. Based on these investigations, morphology was indistinguishable from *C. hominis*, genetic differences at the loci studied were small and although clear biological differences in infectivity were seen there remain uncertainties in the taxonomic status. By including assessment of the low likelihood of *C. hominis* and rabbit genotype being in the same host at the same time, it could be argued that there is a case for separate species status. However, it would also be desirable to investigate whether mating could occur, and whether, when the two populations coincide, the differences between them change (sympatric character displacement studies). This may be difficult *in vitro* or *in vivo* as the infectivity patterns differ and it is possible that co- or staggered inoculation might lead to overgrowth of one to the exclusion of the other. However, it might be achieved by *in silico* modelling.

On the basis of the available evidence we propose that the rabbit genotype has the proper taxonomic designation as a subspecies of *C. hominis, C. hominis cuniculus.* The nominotypical subspecies must now be *C. hominis hominis*. Should further data become available, re-assessment of species status from this level is readily achieved. The current practice of naming new "genotypes" without linking to a species is not satisfactory.

Cryptosporidium hominis cuniculus

Description:	Sporulated oocysts 5.98 x 5.38µm with a length to width ratio of 1.11
Type host:	Rabbit (*Oryctolagus cuniculus*)
Other hosts:	Natural infections humans (*Homo sapiens*)
Type locality:	UK
Other localities:	China, Czech Republic, New Zealand

Location in host: Microvillous border of small intestinal epithelial cells
Prepatent period: 4 to 7 days to oocyst shedding
Patent period: Oocysts shed for 7 days
Sporulation time: Sporulate *in vivo*
Material deposited: Phototype; UK Cryptosporidium Reference Unit, Swansea, Wales, UK. GenBank accession numbers for type strain rabbit isolate W17211 at SSU rRNA, HSP70, GP60, LIB13, COWP and Actin genes, respectively: FJ262725, FJ262728, FJ262732, GU327781 to GU327783.
Etymology: *Cryptosporidium hominis cuniculus*
Transmission studies: Experimental infections established in weanling rabbits, immunosuppressed Mongolian gerbils and immunosuppressed Porton mice but not in neonatal mice.
Pathogenicity studies: No clinical signs in rabbits, immunosuppressed Mongolian gerbils or Porton mice. Occasional structures consistent with cryptosporidial infection in the apical brush borders of the small intestinal villi (duodenum and ileum), and infiltrations of eosinophils in the base of the lamina propria and occasionally in the tips of the villi.
Diagnosis: Oocysts in faeces are morphologically similar to *C. h. hominis* and *C. parvum,* stain with acid fast and fluorescent reagents and cross react with antibodies in commercially available immunomagnetic separation kits (Isolate™, TCS Biosciences and Dynabeads® anti-*Cryptosporidium*, Invitrogen) and immunofluorescent microscopy reagents(CryptoCel, Cellabs); differentiated by analysis of SSU rRNA, HSP70 or Actin genes. GP60 subtype families Va and Vb; subtypes within each.

Objective 6. Describe the human epidemiology and pathogenicity of the *Cryptosporidium* rabbit genotype in a waterborne outbreak.

Introduction

Before the outbreak in July 2008, only a single human infection with the rabbit genotype had been identified in the CRU, and indeed worldwide, a finding published in November 2008 (Robinson *et al*., 2008a). This was a sporadic infection in 2007 in a 48 year old female from the northwest of England who had no reported contact with rabbits (Robinson *et al*., 2008a). Based on this single case it was not possible to predict that the rabbit genotype was a human pathogen, capable of causing an outbreak of human illness. Following detection of *Cryptosporidium* oocysts in samples of treated water on 23rd June 2008 (3 day sample) and 24th June (24 hour sample) to 258000 population, Anglian Water placed a precautionary boil water advisory notice at 6am on 25th June. Enhanced surveillance for cases was established by East Midlands South Health Protection Unit on the same day.

The incubation period is the time between exposure to the infectious agent and the onset of clinical symptoms. This was not known for the rabbit genotype in humans. Whilst most people have a reasonably good idea when illness started, at least to within a day or so, few cases will be able to time their exposure. It is reasonable to assume that mean incubation period is approximately equal to the time between the period of peak oocyst concentration in the drinking water and the median date of onset. However, this does not give a very clear idea of the range of possible incubation periods. A further problem is that the best data for concentration of oocysts in drinking water comes from sampling at the water treatment plant. There is also a delay in the distribution of water from the plant to people's taps and it is not clear when each case would have been exposed to the peak concentration. This section of the

report undertakes MonteCarlo modelling using @Risk (Palisade Co) in order to estimate the likely distribution of incubation periods.

Methods

Data collected during the outbreak investigation were provided by the East Midlands South Health Protection Unit (EMSHPU) and were subjected to further cleaning and re-analysed for this report. First, data were collected on a standard "trawling" questionnaire routinely administered on behalf of EMSHPU by the Local Authority Environmental Health Department (Appendix 4). An extended questionnaire additionally administered to collect more detailed data on water consumption, prior illness and medication (Appendix 5). Due to the small number of cases and incomplete data for some, the focus here is on descriptive epidemiology. For some findings comparison has been made with data collected in a case control study of sporadic cases of cryptosporidiosis (Hunter *et al*., 2004) and the national tap water consumption study (Anon, 2008c).

To investigate incubation periods, the distribution of oocysts in water leaving the plant were estimated from all samples taken and the actual estimates of exposure were performed by MonteCarlo modelling. Samples were from filters run over three days prior to oocyst detection and then run over much short periods subsequently. Most samples were taken from the outlet of the treated water contact tank, but after the incident had been identified, samples were also taken from the district pumps. Oocyst concentrations were calculated for each 1/10th day (2.4h) period. Samples were assumed to have equal flow and a constant concentration of oocysts through their sampling period. Oocyst concentrations were calculated from the total flow sampled in each time period and the number of oocysts assumed to have been detected during that period. The exception was the first sample positive which had been collected over three days. In this case, it was assumed that all of the oocysts had been released into the water over a short time period and that the concentration was as high as in the following sample, but for only the proportion of the time needed to explain the final concentration.

Results (see Appendix 6 for full patient details)

The total number of confirmed rabbit genotype cases in the Northamptonshire outbreak was 23. All lived in the area supplied by Pitsford Water Treatment Works. At least one, and possibly two, of the cases was secondary, acquired from a household member. The earliest onset of illness was 24th June 2008 and the latest was 17th July 2008 (Figure 5).

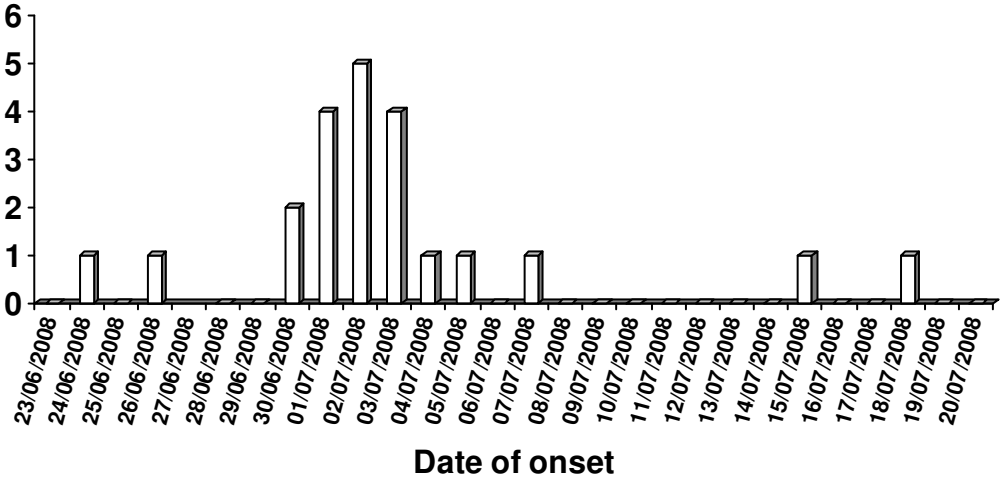

Figure 5. Epidemiological curve of the Northamptonshire out break: confirmed rabbit gentotype cases

There were 7 (30%) male and 16 (70%) female cases. The age range was 10 to 60 years, with a median of 29 years. Symptoms varied across cases. All had diarrhoea, self-described as watery in all cases, five with mucous, and as "severe" in the majority of cases (only one "moderate"). Only 4 described vomiting as a symptom, but 14 were nauseated. Abdominal pain and cramping was a predominant feature across the cohort (77%). Less than 50% experienced fever or flushing with the infection. Duration of diarrhoea ranged from 2 days to 39 days (median 13 days). No cases were hospitalised.

15 of the 22 cases providing additional data reported having a prior medical complaint and / or a bowel problem (5 both). The underlying medical condition (not bowel) ranged from skin to mental health, endocrine and prior (not current) malignancy problems. The cases with a history of a bowel problem had conditions ranging from reflux to prior surgery. 10 cases were taking prior medication (ranging from treatment for diabetes, mental health problems to blood pressure medication and oral contraception). 6 were taking antacid treatment (including acid suppression). Of note, 10 had taken medication to mitigate the effects of the diarrhoea (e.g. anticolic / antidiarrhoeals) and 7 had taken analgesia (paracetamol / paracetamol opiate combined and 2 were on antibiotics (reason unclear).

Water consumption data show that the volume of unfiltered, unboiled tap water consumed by cases ranged from 0 to 3.6 litres per day (lpd) (median 1.8 litres). This is higher than that reported in the most recent National Tap Water Consumption Study undertaken in 2008 (DWI 70/2/217) for summer consumption which is 0.777 lpd.

The *Cryptosporidium* contamination at the water treatment works occurred in the time period between 19th June 2008 and 23rd June 2008. The period to onset of diarrhoea varied widely. At least two cases appeared to commence with symptoms substantially later than the majority. It may be that these were secondary cases. Figure 6 shows the estimated concentration of oocysts in the finished water calculated for each 2.4h period. It can be seen that there is a fairly rapid increase in concentration followed by an exponential decline in concentration over the course of about 72 hours. The mean concentration of oocysts in finished water over the 24 hours of 23rd June was 0.048/L, on the 24th this was 0.078, on the 25th 0.013 and on the 26th 0.001. No oocysts were detected after the 26th June despite some 73 samples being taken up to 12th August that filtered almost 150,000L.

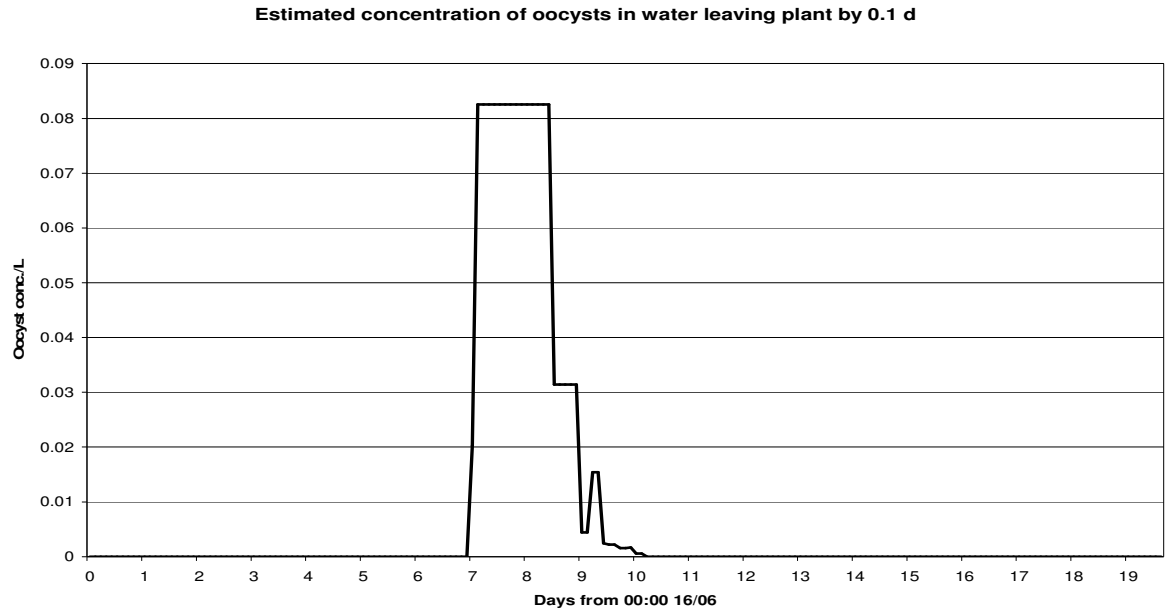

Figure 6. Estimated distribution of oocysts in finished water leaving the treatment works by 1/10th days

The proportion of people estimated to receive their drinking water at various time periods after the water entering the distribution network was obtained from Anglia Water (Table 4). For modelling this distribution the mid point times were taken, i.e. 3, 9, 18, 36, 60 and 84h.

Table 4. Proportion of customers receiving their drinking water within time periods after water had left the treatment works

Time taken for water to travel from Pitsford WTW to the customer	Proportion of customers
Within 6 hours	0.0177
6 to 12 hours	0.0235
12 to 24 hours	0.1335
24 to 48 hours	0.6378
48 to 72 hours	0.1212
> 72 hours	0.0663

The dates of onset were taken from the epidemic curve, though the two cases with onsets > 4 weeks after the event were assumed not to be primary cases.

The actual estimates of exposure were performed by MonteCarlo modelling and are detailed in Appendix 7. The resulting distribution of the incubation periods are shown in Figure 7. It can be seen that that there is a peak around day 6 but that the distribution around this central point is wide and includes negative results which are clearly not possible. These negative incubation periods would arise when an early case is matched with a late exposure date. In part this extended range may also be because the first and last two cases may not have been part of the outbreak or in the case of the longer incubation periods be secondary cases.

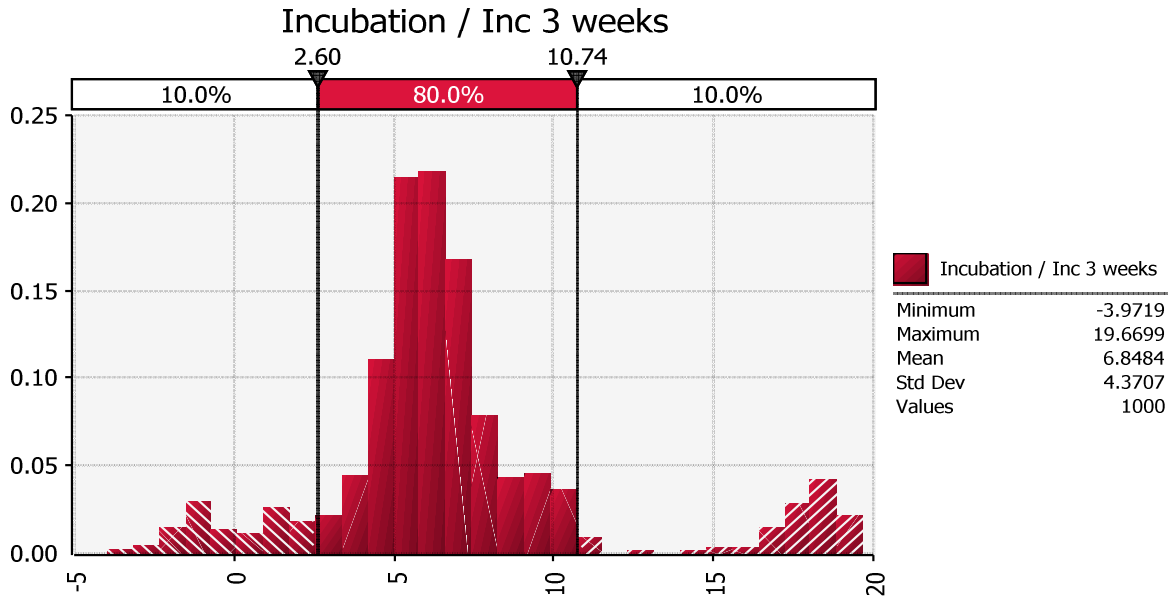

Figure 7. Distribution of possible incubation periods Median 6.2 d, Mode 5.5 d

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 be from about 2 to 11 days. Assuming that the two cases with onset dates of 13th and 14th July really were primary cases and part of the outbreak then the upper limit of the incubation period would be 18 days or more. The case occurring on 24th June was the day after the first contamination and would have an incubation

period of less than 2 days. This is implausibly short and may represent the person misremembering their onset date.

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Discussion

The contamination of the water supply with the rabbit genotype caused known disease in only a small number of individuals overall, in view of the whole population at risk. Symptomatology varied across the cases but was typical of cryptosporidiosis in that, in addition to diarrhoea, the key other symptom was abdominal pain and/or abdominal cramps. However, very few cases (18%) reported vomiting, which contrasts with 65% reported in a large cases series of cryptosporidiosis patients presenting to medical attention (Hunter *et al*., 2004). This difference may be linked to the notable absence of young children in the outbreak cases. The reasons for the lack of childhood cases are not clear, but may relate to fewer exposure opportunities or smaller water volumes consumed by this age group.

The finding of secondary cases, although not surprising, confirms that the rabbit genotype can be transmitted person to person and is important to know for control of the spread of infection. Equally important are the data generated regarding incubation period. The estimated median incubation period (6.2 d) is similar to that reported for three different *C. parvum* isolates in four adult volunteer infectivity studies (5, 6, 6.5, 7), longer than 3 d for a further *C. parvum* isolate and slightly longer than for a single *C. hominis* isolate (4 days) (summarised in Chalmers and Davies, 2009). Thus there is no evidence that the incubation period of the rabbit genotype differs from that of C. *parvum* or substantially from *C. hominis*.

Having a prior medical condition appears to be high in comparison with other cases series. However, not all of these are likely to have a biologically plausible reason for increased vulnerability and so this may reflect a chance finding. In a case control study of sporadic cases (Hunter *et al*., 2004), previous medical illness was reported by 35/231 (15%) cases and 11/403 (3%) cases were taking any medication. In the control group, previous medical illness was reported by 52/228 (23%) and 11/385 (3%) were taking any medication. A notable number of outbreak cases (10) reported a previous history of bowel disorders including reflux through to previous abdominal surgery. This requires further investigation.

Objectives 7 and 8. Estimate the prevalence of the rabbit genotype in human cryptosporidiosis by enhanced typing studies and compare the human epidemiology and pathogenicity of the rabbit genotype with *C. parvum* and *C. hominis* using *a priori* data.

Introduction

The prevalence of the rabbit genotype in human cryptosporidiosis is not known because the routine typing methods for epidemiological purposes used at the UK CRU and elsewhere were not designed to differentiate it from *C. hominis* (Chalmers *et al*., 2009a and b). To estimate prevalence, samples submitted for routine typing in the 12 months since the Northamptonshire outbreak were subjected to enhanced testing for the rabbit genotype. In addition, a look-back exercise was undertaken to differentiate the rabbit genotype among *C. hominis* cases identified from January 2007 to the beginning of the enhanced testing period. Overall, 2 ½ years worth of data were covered by these investigations.

Method

During the outbreak period (July and August 2008) all *Cryptosporidium*-positive stool samples submitted for typing for epidemiological purposes (Chalmers *et al*., 2009a) were tested by nested PCR-RFLP SSU rRNA gene as described in Objective 3. During the look-back exercise (January 2007 to July 2008) and enhanced surveillance periods (September 2008 to July 2009) all samples initially identified with *C. hominis* profiles by routine typing based on PCR-RFLP COWP gene as described in Chalmers *et al*., (2009a) were re-tested by

single SSU rRNA PCR-RFLP employing the rabbit genotype diagnostic restriction enzyme Ssp1. All those with rabbit genotype restriction patterns were confirmed by sequencing SSU rRNA and GP60 genes as described in Objective 3.

The national *Cryptosporidium* typing databases for 2007 and 2008, maintained for epidemiological purposes at the UK CRU for England and Wales (Chalmers *et al*., 2009a) and Scotland (Chalmers and Pollock, 2008; Chalmers and Pollock, 2010) were updated with any new rabbit genotype identifications, merged in Excel using common variables and transferred to EpiInfo (Version 6, Centers for Disease Control and Prevention Atlanta, GA). Data for Ireland and Northern Ireland were not complete over this time period and data from 2009 were incomplete and so not used in this analysis with the loss of three rabbit genotype cases from the dataset; one each from Ireland, Scotland and England. These cases are described in Appendix 4. Sporadic rabbit genotype, *C. parvum* and *C. hominis* cases in the complete two year dataset were compared by age using the Mann-Whitney two sample test, by sex using the Mantel-Haenszel version of the chi-squared test, by month of specimen submission to CRU graphically and by Government Office Region (England and Wales) or health board (Scotland) of diagnostic laboratory. All statistical analyses were undertaken using EpiInfo (Version 6, Centers for Disease Control and Prevention, Atlanta, GA).

Results (see Appendix 8 for sporadic case data)

In the two full years 2007 and 2008, a total of 37/3237 (1.1%) sporadic *Cryptosporidium* cases submitted to the UK CRU for typing for epidemiological purposes from England, Wales and Scotland were identified as rabbit genotype; 23 were in 2007 and 14 in 2008. 25 cases were from England and Wales and 12 were from Scotland. Other cryptosporidia detected were *C. parvum* (1506 cases), *C. hominis* (1383), *C. meleagridis* (26), *C. felis* (8), cervine genotype (8), co-infection *C. hominis* and *C. parvum* (5), novel or unidentified genotypes (5), monkey genotype (1) and 113 that did not amplify with the PCR primers.

The age range of the sporadic rabbit genotype cases was 1 to 74 years (mean 29 years; median 31 years), and was significantly older than *C. hominis* cases (range 0 to 83 years, mean 19 and median 13 years) (p=0.0009) and *C. parvum* cases (range 0 to 86 years, mean 17 and median 29 years) (p=0.00009) (Figure 8). The sex distribution was 14 (37%) female and 22 (58%) male, with one not known, different from the outbreak where there was an excess of female cases and from sporadic *C. parvum* and *C. hominis* cases where the sex distribution is more equal although the differences were not significant (Figure 9).

The monthly distribution of rabbit genotype cases was more similar to *C. hominis* than *C. parvum*, with more cases detected in the late summer and autumn than in the winter and spring (Figure 10). Three rabbit genotype cases were identified in the East Midlands, the outbreak region, but none in Northamptonshire, the outbreak-affected area. Most cases (24%) were in the Eastern Region of England. Two (5.4%) had travelled abroad, one to Spain and the other destination not known.

Environmental exposure data was available for 15 rabbit genotype cases from enhanced surveillance forms, administered by local Environmental Health Departments to unusual cases of cryptosporidiosis (Elwin *et al*., in preparation). Only 1 reported contact with rabbits, a pet rabbit, and two had environmental contact (one played golf and another sat on grass during a walking trip). The enhanced surveillance also identified that two cases had a significant clinical history: one was a paediatric kidney transplant patient on immunosuppressive drugs and another was an elderly lady frequently admitted to hospital with pancreatic problems who also had *Clostridium difficile* infection. None of the sporadic cases reported links with (had visited or received visitors from) the outbreak affected area.

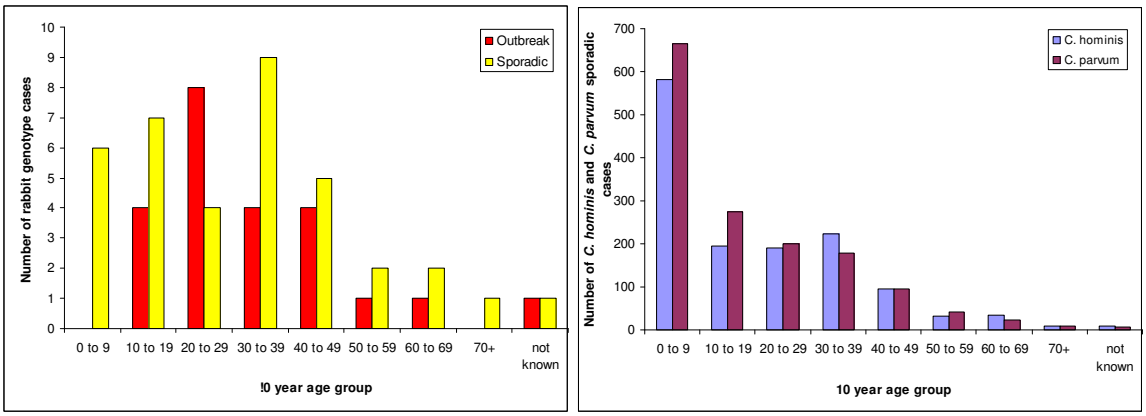

Figure 8. Age distribution of outbreak and sporadic rabbit genotype cases, and sporadic *C. hominis* and *C. parvum* cases in England, Wales and Scotland, 2007 and 2008

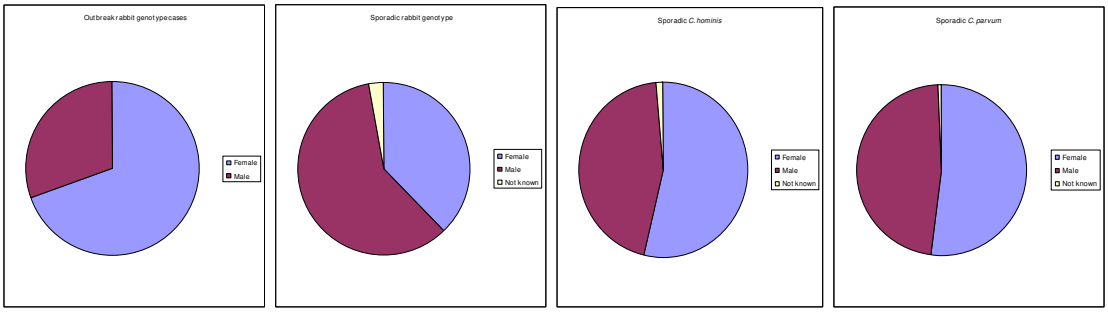

Figure 9. Sex distribution of outbreak and sporadic rabbit genotype cases, and sporadic *C. hominis* and *C. parvum* cases in England, Wales and Scotland, 2007 and 2008

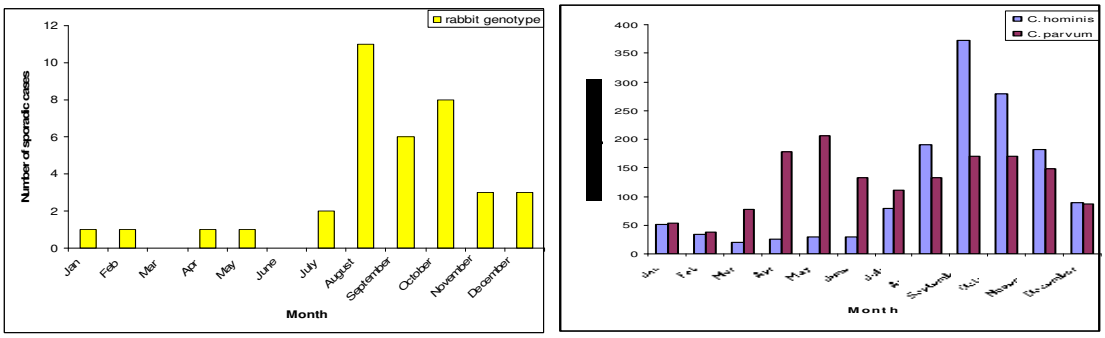

Figure 10. Monthly distribution of sporadic rabbit genotype, *C. hominis* and *C. parvum* cases in England, Wales and Scotland, 2007 and 2008

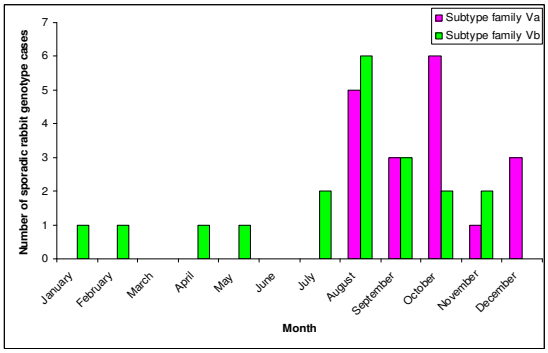

Figure 11. Monthly distribution of rabbit genotype GP60 subtype families from all sporadic cases.

Of all 37 rabbit genotype cases, 18 isolates were GP60 subtype Va and 19 were Vb. This was linked to sex; 10/14 (71.4%) females were Va compared to 31.8% males (χ^2=5.24, df=1, p= 0.022). There was no age or regional distribution in GP60 subtype but Va only occurred between August and December while Vb was found all year round but mostly in August (Figure 11).

Further analysis of the GP60 sequences revealed that within the tandem repeat region coding for the amino acid serine, TCA serine repeats only were detected. Among the Va subtype family isolates, 7 different subtypes with between 9 and 23 serine repeats were detected, significantly fewer than the number of serine repeats, 20 to 37, detected in the 12 different Vb subtypes.

Discussion

A total of 37 sporadic rabbit genotype cases were identified over the study period, positioning it as the third most commonly identified *Cryptosporidium* species or genotype in humans during the study period. The rabbit genotype affects all ages with little age delimination, up to middle age after which numbers decline. However, unlike *C. parvum* and *C. hominis*, there is no link to very young age. Both sexes are affected. Distribution is seasonal, peaking in August through to November and may reflect rabbit breeding seasons although rabbit prevalence studies are lacking. Interestingly, the outbreak occurred in July when few sporadic cases were reported.

Cases are largely indigenous. Pre-disposing factors may be present. There are marked differences in GP60 subtype distribution with Va (the outbreak subtype family) occurring only in the second half of the year while Vb occurs year round but peaks in the second half of the year. Again, it is not known whether this reflects distribution in wild rabbits or not. Va is markedly more common in females than males and may reflect exposure patterns. The outbreak showed that secondary transmission in household with the rabbit genotype occurs but the frequency in sporadic cases is not known.

Objective 9. Characterise the human infection risk from drinking water by producing a model using drinking water monitoring and water consumption data.

Introduction

To investigate the dose response characteristics of the rabbit genotype, we used Quantitative Microbial Risk Assessment (QMRA). This is a process that has developed from the chemical risk assessment paradigm. QMRA has been extensively reviewed in the literature (e.g. Haas *et al.* (1999). The application of QMRA to waterborne pathogens has been more particularly discussed in reports to the European Commission as part of the framework program 5 MicroRisk project (Petterson *et al*. 2006). The QMRA process is a four step process, described in Appendix 9.

In this investigation, the context is given as the risk to human health from the rabbit genotype gaining access to drinking water. That this genotype poses a risk to health is clear from the outbreak at Pitsford and adequately documented elsewhere in this report. A number of QMRAs for *Cryptosporidium*, especially *C. parvum*, in drinking water have been reported (e.g. Masago *et al*. 2002; Smeets *et al*. 2007). The key issue is whether or not these earlier risk assessments are also applicable to the rabbit genotype. In this regard there are two key questions. The first relates to exposure and in particular how many oocysts could be shed by a single rabbit. The number of oocysts present in the bowel contents of the rabbit retrieved from the tank were counted following recovery by IMS and detection of IFM and estimated at $>2x10^5$ opg, although it is not known how long it had shed material into the water. The second, probably more important issue (and certainly more difficult to resolve) is whether the dose response curve differs between the rabbit genotype and *C. parvum*. As regards the

quantitation of oocysts associated with rabbits and the event at Pitsford gives the best estimate of exposure possible. The second issue will be addressed.

Does the dose response characteristics of rabbit genotype differ from that of C. parvum*?*

For *Cryptosporidium*, the dose response curve is the relationship between the number of oocysts consumed and the probability of a subsequent infection. Although dose response curves have been derived from outbreak data for other pathogens (Teunis *et al*. 2004; Teunis *et al*. 2005), there are particular problems for *Cryptosporidium* that would make the direct use of outbreak data problematic. The main problem is the reporting pyramid (Wheeler *et al*. 1999). Most dose response models in risk assessment use, as their end-point, infection which includes symptomatic infection with and without detectable oocyst excretion and also asymptomatic oocyst excretion (Chappell *et al*. 1999). In this context infection can mean the development of symptoms or the asymptomatic shedding of oocysts. Many and possibly the majority of people who become infected will have no symptoms what so ever and will not know they are infected. Even in the event of symptoms developing, the large majority of people are unlikely to seek medical attention. Even if they do seek medical attention a stool sample may not be taken and even if taken may not be positive. Symptomatic illness is well described in cryptosporidiosis in the absence of detectable oocyst shedding (Chappell *et al*. 1999). So in the context of the rabbit genotype differences in prevalence of symptoms may be due to a real difference in infectivity as modelled by the dose response curve or in the proportion of people infected who become sufficiently ill to seek medical attention and then have a diagnostic sample taken.

Differences in apparent health impact as determined by the number of people being identified as having proven illnesses between different species/genotypes may be due to differences in the infectivity/virulence of the strain (Messner *et al*. 2001; Teunis *et al*. 2002a). Indeed, even within strains of the same species the infectivity can vary very widely. For example, the probability of a single oocyst causing an infection can vary by more than 100 fold between different strains of *C. parvum* (Messner et al. 2001).

Also of concern is the impact that variation in host susceptibility may have on the apparent infectivity/virulence. Probably the most important issue is the presence of acquired immunity. In human feeding studies it was shown that there was a sharp decline in susceptibility to infection in volunteers that had pre-existing high levels of anti-cryptosporidium antibody compared to those without such antibody (Chappell *et al*. 1999). It is difficult to extrapolate this finding to population immunity levels. However, along with others we suggested over 10 years ago that the major differences in attack rates between ground water and surface water outbreaks may be due to lower herd immunity in people consuming ground water (Hunter & Quigley 1998; Craun *et al*. 1998). Further evidence for the impact of population immunity on attack rates comes from outbreaks where the cases were not seen amongst residents compared to visitors to the affected area (McAnulty *et al*. 2000) and also from the demonstration that high antibody levels were associated with less reported diarrhoeal disease (Frost *et al*. 2005). In the context of the rabbit genotype it is unlikely that many people will have had much prior exposure. Also it is not clear how much cross immunity there is between infections by different *Cryptosporidium* sp.

In order to gain insight into whether or not the rabbit genotype is more or less infective/virulent than other *Cryptosporidium* sp. we have taken a pragmatic approach that compares the combined effects of both infectivity and virulence. Very good data were available from the Pitsford outbreak on both the confirmed attack rate and also the concentration of oocysts in finished water leaving the treatment works. In a previous *C. parvum* outbreak in Clitheroe in 2000 (Howe *et al*. 2002) there was also good data on confirmed attack rate, and although there were no samples taken at the time of peak exposure

subsequent sampling and hydrological modelling enabled a good estimate to be made of the concentration of oocysts.

The approach we have adopted here is to use the known or modelled oocyst concentration data to predict the proportion of the population likely to have been infected using a standard QMRA methodology for the two outbreaks. We then compared the predicted proportion of people infected to the number of cases of illness actually reported. The ratio between the predicted numbers infected and the actual attack rate is then an estimate of the different infectivity/virulence of the two strains, assuming equal susceptibility and water consumption behaviours in the two populations.

Methods

The daily concentration of oocysts in finished water was modelled for each day that the two populations were likely to be exposed. Actual exposure was then modelled by the product of oocyst concentration and an estimate of the daily unboiled tap water consumption. The water consumption using the estimate of drinking water consumption derived for England, daily consumption of unboiled tap water was modelled as a Poisson distribution with a mean of 2.81 glasses/day (533 ml/day) and each glass being estimated to be 190 ml (Mons *et al*., 2007). The proportion of people infected each day was then calculated from the daily exposure and the dose response curve for Cryptosporidium generally used, namely the Beta-Poisson distribution with α=0.115, and β= 0.176 (Teunis *et al*. 2002b). The overall probability of infection was then derived for the whole period of exposure (Petterson *et al*. 2006).

Figure 12 shows the measured concentration of oocysts in finished water for the two water systems with the exception of day 0 for the Clitheroe outbreak where the day 0 concentration was derived from the hydrological model (Howe *et al*., 2002). It can be seen that exposure in the Clitheroe outbreak was substantially greater than the Pitsford outbreak with a peak concentration of oocysts almost 40 times greater and the continued presence of oocysts in the water supply being for much longer. For the purposes of this analysis, exposure in the Clitheroe outbreak was assumed to last until 15 days after contamination at which point the water supply was changed. For the Pitsford outbreak it was assumed to last just 4 days. In order to estimate oocyst counts on all days, the distribution of oocysts in the Clitheroe outbreak was then modelled using @Risk (Palisade Co) which fitted an exponential distribution to the data with the parameters β=4.945 and shift=-2.1513. As this equation gives the distribution as a proportion of all oocysts rather than the actual count all counts were multiplied by 22.92 to give estimated counts.

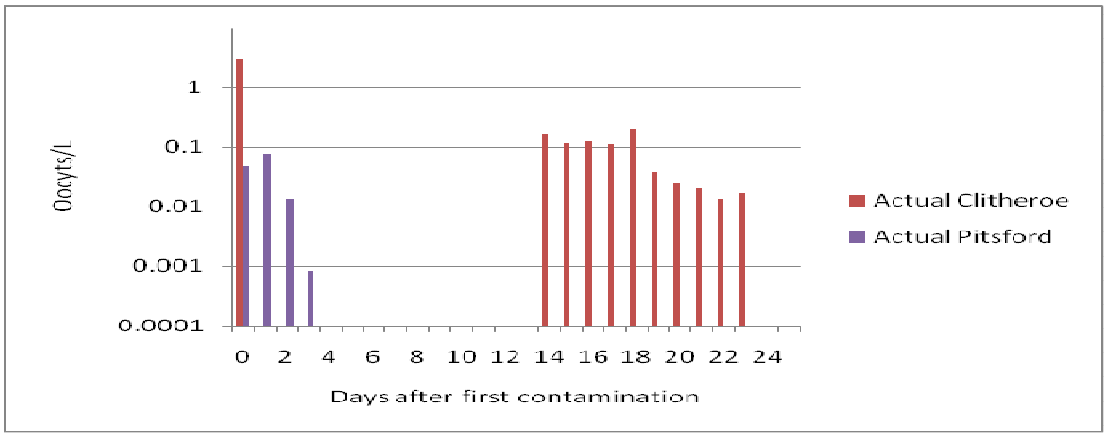

Figure 12. Oocyst counts measured in drinking water from the day of first contamination.

All calculations were done using @Risk (Palisade Co). The overall rates of infection were calculated as described above and the proportion of infecteds being identified given by dividing the attack rate by the infected rates. Attack rates were randomly determined from the number of estimated primary cases for both outbreaks. For the Pitsford outbreak, the last two cases to be identified were assumed to be unrelated to the primary outbreak as they would have had incubation periods of >5 weeks.

The calculated attack rate for the Clitheroe outbreak was 29.6 (95%CI 21.5 to 37.7)/10000 people (Howe *et al.*, 2002) and for Pitsford it was 0.81 (95%CI 0.5 -1.2). Clearly the Clitheroe outbreak had a substantially higher attack rate than the Pitsford outbreak but, as discussed above, there was a much greater exposure to oocysts.

Results and discussion

The results of the Monte Carlo modelling are shown in Table 5. It can be seen that only a very small proportion of the population estimated to be infected in either outbreak are actually identified by the outbreak team, only some 0.3%. It can also be seen that this proportion did not differ significantly between the two outbreaks. So in the absence of evidence of differing immunity levels it would not appear to be the case that the rabbit genotype was substantially less virulent/infectious than the strain of *C. parvum* responsible for the Clitheroe outbreak. In any event any difference in virulence between the two strains in this outbreak would appear to be less than the variation between different strains of *C. parvum* (Messner *et al*., 2001; Teunis *et al*., 2002a). However, if population immunity levels differed substantially between the two populations then this would affect the conclusions. Clearly prior exposure to the rabbit genotype is likely to be low but given the close genetic similarity to *C. hominis* discussed elsewhere in this report it is certainly plausible that prior infection with *C. hominis* at least would reduce the number of clinically relevant infections. In the Clitheroe outbreak it was noted that Clitheroe had one of the lower rates of reporting in the Region prior to the outbreak suggesting that immunity levels in this population were unlikely to be very high.

Table 5. Results of Monte Carlo modelling showing the probability / risk of infection during the contamination period

	Mean	Median	L90%CI	U90%CI
Clitheroe outbreak				
Probability of infection	0.807	0.896	0	0.958
Probability of an infected person being recorded as part of outbreak	0.00352	0.00343	0.00260	0.00473
Pitsford outbreak				
Probability of infection	0.0427	0.0467	0	0.0846
Probability of an infected person being recorded as part of outbreak	0.00239	0.00191	0.00088	0.00564
Ratio of probabilities of being reported Pitsford:Clitheroe	0.701	0.552	0.239	1.693

In conclusion this analysis has shown that:

- In an outbreak setting, the proportion of people infected with *Cryptosporidium* as estimated by QMRA that are identified and reported to the outbreak team is very low, about 0.3%.

- There is no significant difference in apparent infectivity or virulence between the rabbit genotype associated with the Pitsford outbreak and the *C. parvum* associated with the Clitheroe outbreak and is substantially less than the variation in infectivity shown between different *C. parvum* strains.
- The very different attack rates in the Clitheroe and Pitsford outbreaks are explainable by the different concentrations of oocysts and the duration of oocyst contamination of the drinking water.
- In the absence of human volunteer feeding studies, the currently used dose response model for *Cryptosporidium* would appear to be applicable to QMRA analyses of the rabbit genotype.

Further work

- The prevalence and epidemiology of *Cryptosporidium* spp. in wild rabbit populations needs to be investigated, and there is a general lack of information about the role of small mammals in the transmission of *Cryptosporidium*.
- Sympatric character displacement needs to be investigated for complete taxonomic identification but poses difficulties in achieving parasite population parity.
- Molecular markers for sexual recombination between *Cryptosporidium* isolates should be explored and utilised in taxonomic studies.
- The emergence of new pathogenic isolates must be monitored through continual molecular epidemiological surveillance.

Outputs

Published papers

Chalmers, R. M., G. Robinson, K. Elwin, S. J. Hadfield, L. Xiao, U. Ryan, D. Modha, and C. Mallaghan, 2009: *Cryptosporidium* rabbit genotype, a newly identified human pathogen. *Emerg. Infect. Dis.* 15, 829-830.

Robinson G. and R. M. Chalmers, 2010: The European Rabbit (*Oryctolagus cuniculus*), a Source of Zoonotic Cryptosporidiosis. *Zoonoses Public Health* [Epub ahead of print, doi: 10.1111/j.1863-2378.2009.01308.x]

Papers in preparation

Robinson G.R. *et al*., The taxonomy of *Cryptosporidium hominis cuniculus*

Chalmers R.M. *et al*., The epidemiology of sporadic human cases of *Cryptosporidium hominis cuniculus*

Puleston R. *et al*., A human waterborne outbreak of *Cryptosporidium hominis cuniculus*

Hunter P. R. *et al*., Risk of waterborne disease from *Cryptosporidium hominis cuniculus*

Presentations

Rachel Chalmers - Challenges in assessing new *Cryptosporidium* risks. Health Protection Conference, Warwick September 2009

Chalmers, R.M., Elwin, K., Puleston, R., Robinson, G., Hadfield, S.J., Modha, D., Mallaghan, C. The *Cryptosporidium* rabbit genotype in human outbreak and sporadic cases. 3rd International Giardia and Cryptosporidium Conference, Orvieto, October 2009

Posters

Chalmers, R.M., Robinson, G., Wright, S., Hunter, P.R., Elwin, K., Bouzid, M., Innes, E.A., Hadfield S.J., Tyler, K., Katzer, F. Comparative biology of *Cryptosporidium hominis* and the *Cryptosporidium* rabbit genotype. 3rd International Giardia and Cryptosporidium Conference, Orvieto, October 2009

Intellectual property or technology transfer

Technology transfer: primer sets and real-time PCR for subtype families Va and Vb have been developed and are currently being evaluated.

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Appendix 1.

Literature review.

Method

Search terms, including wild card characters and Boolean operators, were designed to identify natural or experimental *Cryptosporidium* infections in rabbits, and any human *Cryptosporidium* infections that may be associated with rabbits. International spelling was considered by using the National Library of Medicine's Medical Subject Headings thesaurus.

All retrieved references were entered into Reference Manager 11 for Windows (Thomson ResearchSoft, Carlsbad, CA, USA), duplicates deleted and remaining references screened for relevance. The inclusion criteria for papers were: the investigation of Lagomorphs for *Cryptosporidium* infections; the attempt to infect Lagomorphs with *Cryptosporidium*; or any link between human *Cryptosporidium* infection and Lagomorphs. References cited in each of the included publications were hand-searched. Structured questions were also sent to experts to identify any unpublished information on *Cryptosporidium* in rabbits.

Results

Figure 1.1. Nucleotide differences in the partial SSU rRNA genes of *C. hominis, C. hominis* monkey genotype and rabbit genotype isolates available on GenBank. Dots denote nucleotide identity to the *C. hominis* reference sequence (AJ849462), stars GenBank sequence ended

	Location of nucleotide differences in the partial SSU rRNA gene		
GenBank Accession	nt 420-435	nt 570-580	nt 740-772
AJ849462 *C. hominis*	AAATATTTTGATGAAT	TCTTTTTTATT	GATCAGATACCGTCGTAGTCTTAACCATAAACT
AF112569 Monkey gt	.T..............	....	....
AY120901 Rabbit gt	.T.........AG..	.....C.....	....
AY273771 Rabbit gt	.T.........AG..	.....C.....	*
AY458612 Rabbit gt	.T.........AG..	.....C.....	..........C.................T*
EU437413 Rabbit gt	.T.........AG..	.....C.....	....

Table 1.1. Natural *Cryptosporidium* infections identified in wild European rabbits (*Oryctolagus cuniculus*), unless otherwise stated.

Study location	Study population	Number positive/number sampled (% prevalence)*	*Cryptosporidium* detection method	Published identity following molecular investigation	Reference
Illinois, USA	Wild cottontail rabbit (*Sylvilagus floridanus*), incidental capture, died in a live trap	1/1	Histological examination	Not done	Ryan *et al*., 1986
Chile	No details	0/14 (0.0%; 95%CI 0.0% to 21.5%)	mZN and phenol auramine - rhodamine	Not Applicable	Araya *et al*., 1987
Warwickshire, UK	Wild rabbit droppings from latrines	1/109 (0.9%; 95%CI 0.2% to 5.0%)	F-E sedimentation, IF and mZN staining	Not done	Chalmers *et al*., 1995; Chalmers, 1996
The Netherlands	Wild rabbits, samples pooled	1/4 pooled samples, which represented 6.5% of the animals (equates to 2 rabbits)	Cold sucrose flotation, IF filter staining	Not done	Medema, 1999
Norfolk, UK	Wild rabbits, live-trapped	2/28 (7.1%; 95%CI 2.0% to 22.6%)	F-E sedimentation, IF and mZN staining	Not done	Sturdee *et al*., 1999
Germany	No details	0/232 (0.0%; 95%CI 0.0% to 1.6%)	No details	Not Applicable	Epe *et al*., 2004
New Zealand	Wild rabbit, single shot animal	1/1	IMS-PCR (ITS1, COWP, ß-tubulin, PolyT, RNR-R1 and SSU rRNA genes)	*C. parvum*, host rabbit	Learmonth *et al*., 2004
Sydney, Australia	Individual wild rabbits	1/2	IMS-IF	Not done	Cox *et al*., 2005; Ferguson, 2005
New York, USA	Wild eastern cottontail rabbits (*S. floridanus*)	0/8 (0.0%; 95%CI 0.0% to 32.4%)	FastDNA spin kit DNA extraction and PCR nested SSU rRNA	Not Applicable	Feng *et al*., 2007
Northampton, UK	Wild rabbit, discovered in a treated water tank	1/1	IF staining; saturated salt floatation, freeze-thaw and Qiagen DNA mini kit extraction, PCR (nested SSU rRNA, HSP70, GP60), sequencing	*Cryptosporidium* sp. rabbit genotype	Anon., 2008a
Melbourne, Australia	Wild rabbits	1/23 (4.3%; 95%CI 0.8% to 21.0%)	Flotation and mZN	Not done	Dr Melita Stevens, pers. comm.

*95% confidence intervals calculated for studies of wild rabbits

IMS - immunomagnetic separation; IF – immunofluoresence microscopy; mZN - modified Ziehl-Neelsen staining; PCR - polymerase chain reaction; ITS-1 - internal transcribed spacer region 1; COWP - *Cryptosporidium* oocyst wall protein; PolyT - poly threonine repeat motif; RNR-R1 - ribonucleotide reductase R1 subunit; GP60 – 60kDa glycoprotein; HSP70 – 70kDa heat shock protein; F-E - formol-ether.

Appendix 2.

Morphology

Table 2.1. Comparison of *C. parvum*, *C. hominis* and rabbit genotype oocyst morphology

	Mean Length (SD) (µm)	Length Range (µm)	Mean Width (SD) (µm)	Width Range (µm)	Length:Width ratio (SD)
C. parvum (Upton & Current, 1985)	5.0	4.5 to 5.4	4.5	4.2 to 5.0	1.1
C. hominis (Morgan-Ryan *et al.*, 2002)	5.2	4.4 to 5.9	4.9	4.4 to 5.4	1.07
Rabbit genotype from the outbreak rabbit in suspension	5.98 (±0.18)	5.55 to 6.40	5.38 (±0.24)	5.02 to 5.92	1.11 (± 0.06)
Rabbit genotype from human clinical case after fixation	5.08 (± 0.23)	4.25 to 5.76	4.28 (± 0.20)	3.90 to 4.75	1.19 (± 0.08)
Rabbit genotype from exp. infection in a rabbit	5.21 (± 0.24)	4.74 to 5.75	4.69 (± 0.22)	4.32 to 5.21	1.11 (± 0.07)
Rabbit genotype from exp. infection in a gerbil	5.06 (± 0.22)	4.48 to 5.58	4.59 (± 0.20)	4.19 to 4.94	1.10 (± 0.05)
Rabbit genotype from exp. infection in an adult mouse	5.06 (± 0.29)	4.49 to 5.83	4.46 (± 0.18)	4.08 to 4.91	1.14 (± 0.07)

Appendix 3.

Animal Infectivity.

Details of immunosuppression, animal husbandry, sampling and sample preparation

To immunosuppress the gerbils and adult mice, dexamethazone was administered in the drinking water at a rate of 8mg/litre, for 7 days prior to inoculation. Rabbits and gerbils were 7 and 6 weeks old respectively at the start of the experiment (weaned), adult mice were 10 weeks old, and the neonates were 5 days old. Monitoring of infection was undertaken by the microscopical screening of faecal smears. Animals were moved to clean cages daily, and faecal pellets collected at random from the cage floor to give a pooled daily sample. As it was not practicable to obtain faecal material from the neonatal mice daily, pairs of mice were serially sacrificed at 3 day intervals until day 21 post infection (PI), and their intestines removed to provide material for screening. After 21 days, it was possible to harvest faecal pellets from the cages of the remaining neonates. A faecal sample was removed from the last centimetre of the terminal ileum of each sacrificed mouse pup to prepare a faecal smear. In addition, the remainder of the intestine was homogenised in 1ml of water, using an Ultra-Turrax homogeniser (IKA Werke GMBH & Co. Staufen), diluted 1/5 in an aqueous solution of 0.2%malachite green/1% sodium dodecyl sulphate, and an aliquot examined microscopically in an improved Neubauer haemocytometer for the presence of oocysts or endogenous stages of *Cryptosporidium.*

Faecal samples were mixed with distilled water, smeared on a microscope slide, air dried and fixed with formalin before staining with auramine phenol (AP), then counterstained with potassium permanganate prior to screening using an Olympus BX 50 fluorescence microscope. A random selection of samples were also stained using a commercially available specific monoclonal antibody stain (CryptoCel, Cellabs) to confirm the AP results.

Appendix 4.
Initial Cryptosporidium Surveillance Questionnaire

Cryptosporidium Enhanced Surveillance

East Midlands Health Protection Unit – North Local Authority _ _ _ _ _ _ _ _ _ _ _ _ _
N.B. Please note if you are answering on behalf of a child all questions relate to the child.

1. Age   Sex   F   M

2. Main occupation (or school for child) _
_ _

3. Address of workplace/ school/ nursery
_ _
_ _

Postcode __ __ __ __   __ __ __ __

4. Home Postcode __ __ __ __   __ __ __ __

5. Date of first diarrhoea (DD/MM/YY): __ __/__ __/__ __ (If none please leave blank)
6. Date of first vomiting(DD/MM/YY): __ __/__ __/__ __(If none please leave blank)

7. How long did your diarrhoea last for: _

8. Have you passed blood in your diarrhoea: Yes ___ No ___ Don't know ___

9. Date of first attending GP/ hospital (DD/MM/YY): __ __/__ __/__ __

10. Please give your GP's name & address: _
_ _

11. Were you admitted to hospital? Yes ___   No ___

Please give details of hospital & dates of stay: _
_ _

12. How long did you need to take time off work/school (or regular duties)? _ _ _ _ _ _ _ _

13. Have you been abroad in the 2 weeks prior to becoming ill? Yes ___   No ___

Please give details of countries visited and dates to stay: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
_ _

14. Have you stayed overnight in any of the following in the 2 weeks prior to becoming ill?
Tent ___   caravan ___   B&B ___   hotel ___

15. Do you have any pets? Yes ___   No ___

Please give details: _
_ _

Cryptosporidium Enhanced Surveillance

16. Had any of the pets been ill? Yes ___ No ___

Please give details: _

17. Had you visited a farm in the 2 weeks prior to becoming ill? Yes ___ No___

Please give details, including dates if known: _

18. Have you had any other animal contact? Yes ___ No ___

Please give details: _

19. Have you swam in any swimming pools in the 2 weeks prior to becoming ill?
Yes ___ No ___

Please give details of pools visited, including dates if known: _

20. Have you used any fresh water for recreational purposes in the 2 weeks prior to becoming ill? Yes ___ No ___

Please give details of places visited, including dates if : _

21. Have you walked in the countryside, hills etc in the 2 weeks prior to becoming ill?

Yes ___ No ___ If yes please give details: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

_ _

22. Did you have any contact with any person with diarrhoea or vomiting in the 2 weeks prior to becoming ill? Yes ___ No ___

Please give details: _

_ _

23. Did you drink water from any of the following sources? (Tick all that are applicable)
well ___ stream ___ river ___ spring ___
bottled water ___ bore hole ___ other non-mains ___

Please give details of location/ supplier: _

Cryptosporidium Enhanced Surveillance

24. Did you drink milk from any of the following sources? (Tick all that are applicable)
    milk from a bottle with a damaged top ___
    unpasteurised milk (from farm or green top) ___

Please give details of supplier: _

25. On average how many cups of unboiled water (including squash, etc.) do you drink each day? ___ ___

26. Please list any medicines (including regular drugs) taken in the 2 weeks prior to becoming ill. _

27. Please give details of any serious illness (current or past) or previous operations:
_ _

Date questionnaire completed (DD/MM/YY): __ __/__ __/__ __

More information on Cryptosporidium is available in the leaflet, “Your guide to Cryptosporidiosis”. If you require any more advice contact your Health Protection Unit on tel 01623 819000 or your local Environmental Health Office.

If you are aware of any other people who have had similar symptoms, please tick the box below. This will assist us in the investigation process.

Appendix 5.
Additional Cryptosporidium Questionnaire for the Water Incident, Northamptonshire

(Note to interviewer - make sure you have a calendar with you and please ensure your are familiar with this questionnaire before you visit the patient)

Brief introduction of the purpose of the additional questionnaire and clarify they completed the previous version. Advise the patient this is voluntary and is confidential information which will not be shared with other parties i.e Anglian Water. Name and address will be removed before the information is processed)
Date……...

Name of person Completing the Questionnaire ……

Name……..

Date of birth…….. Male / Female…….

Addresss……
……
……Postcode……..

Telephone number…….

GP……
……..
Contact number Ask for Day time and evening ……...

Can we ask one more question we didn't previously ask

Are involved in changing nappies? Yes / No

Are you involved in helping children in toileting? Yes / No

Are you involved in cleaning after children incontinence of faeces? Yes / No

We want to ask a few questions about your recent symptoms

Have you had Diarrhoea? Yes / No / Not sure
If yes
I will now ask you some questions about the type of diarrhoea that you had - we are interested in if it was watery, mucoid/slimy, or bloody.

Was it watery? ☐

Date of onset _____ Was this - definite date / approximate date

Time of onset _____

Date and time of last diarrhoea ? _____/still ongoing

Can I ask you about the pattern of diarrhoea? Did it occur on one or more consecutive days or recur after one or more clear days ?
Please circle answer below

1 day / over consecutive days / reoccurred after clear days

How many times in a day on average did you have it? _____

How severe was the diarrhoea overall? - Severe / moderate / mild

If severe did it get less severe over time? - Yes / No

Please describe the diarrhoea - explosive / copious / smelly / other

Please describe the daily pattern e.g worse in a morning etc

_____

_____

Was it Mucoid/Slimey ☐

Date of onset _____ Was this - definite date / approximate date

Time of onset _____

Date and time of last diarrhoea ? _____ _____/still ongoing

Can I ask you about the pattern of diarrhoea? Did it occur on one or more consecutive days or recur after one or more clear days ?
Please circle answer below

1 day / over consecutive days / reoccurred after clear days

How many times in a day on average did you have it? _____

How severe was the diarrhoea overall? - Severe / moderate / mild

If severe did it get less severe over time? - Yes / No

Please describe the diarrhoea - explosive / copious / smelly / other

Please describe the daily pattern e.g. worse in a morning etc

_____

_____

Was it Bloody ☐

Date of onset _____ Was this - definite date / approximate date

Time of onset _____

Date and time of last diarrhoea ? _____ _____/still ongoing

Can I ask you about the pattern of diarrhoea? Did it occur on one or more consecutive days or recur after one or more clear days ?
Please circle answer below

1 day / over consecutive days / reoccurred after clear days

How many times in a day on average did you have it? _____

How severe was the diarrhoea overall? - Severe / moderate / mild

If severe did it get less severe over time? - Yes / No

Please describe the diarrhoea - explosive / copious / smelly / other

Please describe the daily pattern e.g worse in a morning etc

_____

_____

Did you take any medication for the diarrhoea? Yes/No
If Yes
What did you take ……. …….
How many days did you take it? ……. days

How often do you normally open your bowels?

Several times a day ……..
Once per day ……..
Every second day …….
Other ……. Per week

****

There are now some questions on nausea and vomiting

Did you have Vomiting? Yes / No
If yes

Date of onset _____

Time of onset _____

How many days did it last for? _____

How many times in a day on average did you have it? _____

Did you take any medication for the vomiting? Yes / No
If yes
What did you take ……. ……
How many days did you take it? ……. days

Have you had Nausea (*feeling sick but did not vomit***)?** Yes / No / Not sure
If yes

Date of onset _____

Time of onset _____

How many days did it last for? _____

How many times in a day on average did you have it? _____

Did you take any medication for the nausea? Yes / No
If yes
What did you take ……. ……
How many days did you take it? ……. days

****

I will now ask you some questions about the type of abdominal pain, cramps or discomfort that you may have had.

Did you have abdominal pain Yes / No / Not sure

How would describe your abdominal pain? *Sharp / dull ache /cramping*

If sharp or dull abdominal pain (please complete section below - if cramps please go to next section)

Date of onset_____.

Time of onset_____.

How many days did it last for? _____

How many times in a day on average did you have it? _____

How bad was the pain? - Severe / moderate / mild

If severe did it get less sever over time? - Yes / No

Did the pain occur - All the time / just with the diarrhoea / fluctuated

If fluctuated
How long did the pain last for -
1- 5 mins / 6- 30 mins / 31-59 mins / 1-2 hrs / 3-5hr / >5hrs

Did the abdominal pain occur each day? Yes / No / Not sure
If no

Please describe pattern _____

_____

Did you take any medication for the pain? Yes / No
If yes
What did you take ……. ……
How many days did you take it? ……. days

****

Did you have abdominal cramps? Yes / No / Not sure

If you had abdominal cramps

Date of onset_____.

Time of onset_____.

How many days did it last for? _____

How many times in a day on average did you have it? _____

How bad were the cramps? - Severe / moderate / mild

If severe did it get less sever over time? - Yes / No

Did the cramps occur - All the time / just with the diarrhoea / fluctuated

If fluctuated
How long did the cramps last for -
1- 5 mins / 6- 30 mins / 31-59 mins / 1-2 hrs / 3-5hr / >5hrs

Did the abdominal cramps occur each day? Yes / No / Not sure
If no
Please describe pattern _____

_____

Did you take any medication for the cramps? Yes/No
If yes
What did you take ……. ……
How many days did you take it? ……. days

****

Did you have any other abdominal discomfort? e.g. trapped wind /flatulance
Yes / No / Not sure

If yes

Date of onset_____.

Time of onset_____.

How many days did it last for? _____

How many times in a day on average did you have it? _____

How bad was the discomfort? - Severe / moderate / mild

If severe did it get less sever over time? - Yes / No

Did the discomfort occur - All the time / just with the diarrhoea / fluctuated

If fluctuated
How long did the discomfort last for -
1- 5 mins / 6- 30 mins / 31-59 mins / 1-2 hrs / 3-5hr / >5hrs

Did the abdominal discomfort occur each day? Yes / No / Not sure
If no
Please describe pattern _____

_____

Did you take any medication for the discomfort? Yes/No
If yes
What did you take ……. …….
How many days did you take it? ……. days

****

I will now ask questions on feeling hot – fever, high temperature or hot flushes

Did you have hot flushes Yes / No/ Not sure
If yes

Date of onset_____.

Time of onset_____.

How many days did it last for? _____

How many times in a day on average did you have it? _____

How often did the hot flushes happen - all the time / just with the diarrhoea / fluctuated

Did the hot flushes happen each day? Yes / No / Not sure
If no
Please describe pattern _____

_____

Did you have a Fever (increased temperature) Yes / No/ Not sure

If yes

Date of onset_____.

Time of onset_____.

How many days did it last for? _____

How many times in a day on average did you have it? _____

Did the fever happen - all the time / just with the diarrhoea / fluctuated

Did the fever occur each day? Yes / No / Not sure

If no

Please describe pattern _____

_____

Did you take any medication for the fever ? Yes/No

If yes

What did you take ……. ……

How many days did you take it? ……. days

****

Have you had other symptoms Yes / No / Not sure

If yes

Please describe any other symptoms you have including any medication that you took

……

……

……

Have you had a recurrence after the symptoms initially resolved? Yes / No / Not sure

If yes, please describe

……

……

……
……

****

How does this episode compare to previous bouts of sickness and diarrhoea

you may have experienced?
Please circle below

Less severe / same / more severe

……

****

Have you had to take time off work or school? Yes / No
If yes

How many days? _____

Has the illness stopped you from carrying out your usual activities? Yes / No
If yes
For how many days _____

Please describe what the problem has been
……

……

During the time span from 23rd June - onset date can you think about all your water consumption (at home, at work , at leisure activities)

1 litre = 1.75 pints 1 pint = 0.6 litres
Tell them how many pints/litre are in a mug (about 3 mugs = 1 pint)

Do you use a water treatment device at home?

- A filter on the tap Yes / No
- Jug filterYes / No
- Water softening deviceYes / No
- Other……..

How many litres / pints of the following did you have in a day normally?

- Plain cold tap water unfiltered from the tap……
- Cold unfiltered tap water for preparation of cold drinks……
- Cold unfiltered tap water for preparation of food ……

- Plain cold tap water that is then filtered by a jug filter ……
- Jug filtered water for the preparation of cold drinks …….
- Jug filtered water for the preparation of food …….

- Cold water from a fridge dispenser _____

- Cold Water from a dispenser machine using mains …….
  (Please provide the venue and address) _____
  _____

  _____

- Bottled water – from a bottle purchased over the counter …….
- Bottled water – from a large communal bottle (e.g. in a work place/sports centre) with a tap…….... (Please provide the venue and address)
  _____
  _____
- Other cold drinks……

- Hot drinks made with tap water that has been boiled in a kettle ……...

- Hot drinks made with water heated in an urn
  (Please provide the venue and address)
  _____
  _____

- Warmed or hot water to make drinks from a dispenser machine using mains water ……
  (Please provide the venue and address)
  _____
  _____
- Hot drinks e.g. tea / coffee from a vending machine……
- (Please provide the venue and address)
  _____

- Any hot drinks from a flask
  _____

- Other source of cold water – please specify below

Do you have ice (i.e.crushed ice/ice /ice cubes) in drinks? Yes / No

If yes
Between* 26th June– onset date on *how many days did you have ice at home ?

_____***days***

Which of the following did you use?

- Ice made from tap water Yes / No
- Ice made from bottled water Yes / No
- Pre-prepared ice cubes i.e. purchased from a shop Yes / No

***Between* 23rd June– onset date - did you go to pub/social club/night club etc ?**

Yes / **No**

If yes, on how many days?_____

Which "social" club(s) did you go to? _____
_____

Did you drink any of the following?

- Beer/larger/cider from a dispenser tap/pump Yes / No
- Soft drinks from a dispenser tap Yes / No
- Tap water Yes / No
- Other (specify)…….. Yes / No

Did you have ice in your drink? Yes / No

***Between* 23rd June– onset date. - did you go to a restaurant or food outlet ?**

Yes / **No**

If yes, on how many days ?_____

Which restaurants /food outlets did you go to?
…….
_____

Did you drink any of the following?)

- Beer/larger/cider from a dispenser tap/pump Yes / No
- Soft drinks from a dispenser tap Yes / No
- Tap water Yes / No

- Other (specify)……. Yes / No

Did you have ice in your drink? Yes / No

***Between* 23rd June– onset date. - did you go to a fitness venue/club etc ?** **Yes / No**

If yes, on how many days? _____

Which fitness venue/club did you go to?

_____

_____

Did you drink any of the following?

- Beer/larger/cider from a dispenser tap/pump Yes / No
- Soft drinks from a dispenser tap Yes / No
- Tap water form the venue Yes / No
- Water form a drinking fountain Yes / No
- Water from a water dispenser Yes / No
- Other (specify)…….

Did you have ice in your drink? Yes / No

***Between* 23rd June– onset date. - did you go to a venue where you drank or took ice e.g. parties or meetings?** **Yes / No**

If yes, on how many days ?_____

Where did you go to? …….

Did you drink any of the following?

- Beer/larger/cider from a dispenser tap/pump Yes / No
- Soft drinks from a dispenser tap Yes / No
- Tap water Yes / No
- Water form a drinking fountain Yes / No

- Water from a water dispenser Yes / No
- Other (specify)…….

Did you have ice in your drink? Yes / No

Do you have any of the following medical problems?

Digestive problems

- Irritable bowel syndrome Yes/ No
- Inflammatory bowel disease Yes/ No
- Coeliac disease Yes/ No
- Other bowel problem _____

- Acid reflux Yes/ No
- Indigestion Yes/ No
- Other digestive problem _____
  _____
- Gastric surgery Yes/ No

  If yes please give details _____
  _____
  _____
- Gastric investigations…… Yes/ No

  If yes please give details _____
  _____
  _____

Diabetes

Type 1 Yes/ No
Type 2 Yes / Borderline / No

Rheumatological problems Yes/ No

Disease affecting your immune system Yes/ No

If yes, please describe…….

……

Are you pregnant Yes / No / Not sure / Not applicable

Other medical condition – please describe

……..
……
……
……..
……
……

Have you ever had radiotherapy or chemotherapy? Yes / No

If yes – when was it

In the month before your infection with cryptosporidium, have you had

- Antibiotics? Yes / No / Not sure
  If yes please write the name(s) below
- Steroids? Yes / No / Not sure
  If yes please write the name(s) below
- Other drug that may affect your immune system? Yes/No / Not sure
  If yes please write the name(s) below
- Proton Pump Inhibitors (e.g. Losec) - Yes/No / Not sure
- Acid suppressants - ranitidine (e.g. Zantac) Yes/No / Not sure
- Other antacids (Gaviscon, Rennies etc) Yes /No / Not sure

What medications are you currently taking?

……
……
……
……

Appendix 6.
Patient data from the Northampton outbreak cases

Number of cases

	All	Male	Female
Number of cases	23	7	16
Proportion	-	0.3	0.7

Date of onset of diarrhoea

Date	All	Male	Female
Earliest	24th June	27 June	24th June
Latest	14th July	5th July	14th July
Mode	1st July	1st July	1st July
Median	1st July	1st July	-

Duration diarrhoea

Days	All	Male	Female
Shortest	2	2	2
Longest	39	22	39
Mode	-	5	-
Median	13	-	13.5

Age ranges

Year	All	Male	Female
Youngest	10	18	10
Oldest	60	54	60
Median	29	29	29

Age distribution

Age band	All	Male	Female
10-19	3	1	2
20-29	9	2	7
30-39	4	2	2
40-49	4		4
50-59	1	1	
60-69	1		1

Although there were 23 confirmed cases, there was data on the extended questionnaire for only 22 (one case presumed lost to follow up). The following tables therefore only relate to these 22.

Self reported condition or treatment	Number of cases	
On medication of any sort*		
	Yes	10
	No	12
Antacid treatment		
	Yes	6
	No	16
Any history of a medical condition		

	Yes	15
	No	7
History of bowel only problems**		
	Yes	10
	No	12
History of other (not bowel) only conditions***		
	Yes	10
	No	11
History of bowel and other medical conditions combined		
	Yes	5
	No	17

*Includes oral contraception

**Includes reflux, indigestion, previous surgery

***For example includes prior history of cancer, endocrine disorders (e.g. diabetes / thyroid problems), skin and mental health issues.

Water consumption

Plain cold water from mains

Litres / day	All	Male	Female
Minimum	0	0	0
Maximum	3.6	2.5	3.6
Median	1.8	1.9	1.63
Mode	0	1.8	3

Plain hot water from mains

Litres / day	All	Male	Female
Minimum	0	0	0
Maximum	1.2	1	1.2
Median	0.6	0.43	0.6
Mode	0.6	2	0.6

Total mains water hot and cold

Litres / day	All	Male	Female
Minimum	0	0	0.6
Maximum	4.8	3.3	4.8
Median	2.2	2.5	1.9
Mode	-	-	-

Clinical symptoms

- Majority of diarrhoea cases described as severe (only one as moderate)
- 5 diarrhoea mucousy but also all described it as watery
- Diarrhoea frequency ranged from 2 to 25 + times per day with a median of 8 times per day. None had bloody diarrhoea.
- Although the majority were nauseated (14) which lasted for between 1 and 21 days (mode 1, median 3), vomiting was not a prominent feature, occurring only in 4 of the cases, lasting a maximum of one day.

- 17 had abdominal pain with 16 describing abdominal cramps (but not necessarily both). Of the 17 who had abdominal pain, 12 also had nausea. The abdominal pain varied in duration from 1 to 22 days with the mode being 7 days, whereas cramps lasted for between 1 and 7 days, with the mode being 2 days. 9 cases described having other abdominal discomfort, including flatulence for between 2 and 26 days, the mode being 7 days
- 10 cases had flushes / fever (again not necessarily both). Flushes where present occurred for between 2 and 18 days and fever for less time – between 1 and 7 days

Symptoms other than diarrhoea (e.g. fever, nausea, vomiting) started in most cases either on the same day as the diarrhoea or within a couple of days. There were some exceptions to this, for example a case whose abdominal cramps started 12 days before the diarrhoea / another where cramps didn't start until 5 days after initial diarrhoea.

Appendix 7.

MonteCarlo modelling of exposure to Cryptosporidium through drinking water in the Northampton outbreak

Where the date of exposure was sampled from the distribution of oocysts leaving the treatment works plus a random sample from the time in distribution. This was then matched with the date of onset of one of the cases randomly chosen. For the exposure times rather than use the actual data, the data were sampled from two distributions fitted to the existing data. These are shown in Figures 7.1 and 7.2

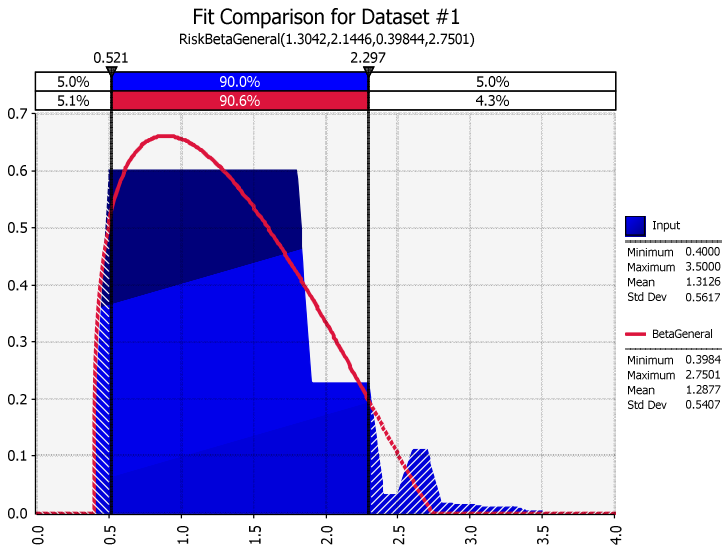

Figure 7.1. Distribution of oocyst concentrations in finished water at plant with fitted Beta General Distribution

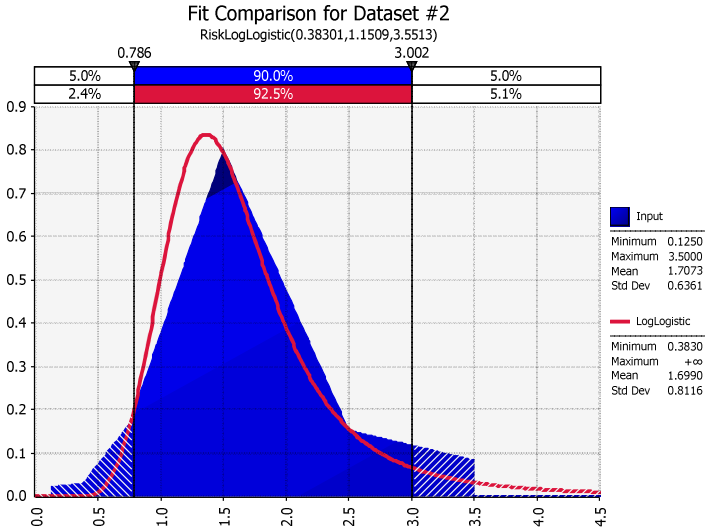

Figure 7.2 Distribution of time from water leaving plant to being delivered to consumer with fitted Log Logistic curve.

Appendix 8.
Sporadic *Cryptosporidium* case demographics in England, Wales and Scotland, 2007 and 2008.

	Rabbit genotype n=37	***C. hominis* n=1383**	***C. parvum* n=1506**
Year			
2007	23 (62.2%)	461 (33.3%)	716 (47.5%)
2008	14 (37.8%)	922 (66.7%)	790 (52.5%)
Age (years)			
0 to 9	6 (16.2%)	581 (42.0%)	665 (44.2%)
10 to 19	7 (18.9%)	195 (14.1%)	275 (18.3%)
20 to 29	4 (10.8%)	190 (13.7%)	200 (13.3%)
30 to 39	9 (24.3%)	223 (16.1%)	180 (12.0%)
40 to 49	5 (13.5%)	96 (6.9%)	95 (6.3%)
50 to 59	2 (5.4%)	33 (2.4%)	41 (2.7%)
60 to 69	2 (5.4%)	35 (2.5%)	23 (1.5%)
70+	1 (2.7%)	10 (0.7%)	9 (0.6%)
Not known	1 (2.7%)	18 (1.3%)	18 (1.2%)
Sex			
Male	22 (59.5%)	616 (44.5%)	704 (46.7%)
Female	14 (37.8%)	736 (53.2%)	781 (51.9%)
Not known	1 (2.7%)	31 (2.2%)	21 (1.4%)
Month			
January	1 (2.7%)	52 (3.8%)	53 (3.5%)
February	1 (2.7%)	34 (2.5%)	38 (2.5%)
March	0	20 (1.4%)	78 (5.2%)
April	1 (2.7%)	26 (1.9%)	178 (11.8%)
May	1 (2.7%)	30 (2.2%)	206 (13.7%)
June	0	29 (2.1%)	133 (8.8%)
July	2 (5.4%)	79 (5.7%)	110 (7.3%)
August	11 (29.7%)	190 (13.7%)	133 (8.8%)
September	6 (16.2%)	372 (26.9%)	171 (11.4%)
October	8 (21.6%)	280 (20.2%)	171 (11.4%)
November	3 (8.1%)	182 (13.2%)	148 (9.8%)
December	3 (8.1%)	89 (6.4%)	87 (5.8%)
Country			
England	24 (64.9%)	888 (64.2%)	858 (57.0%)
Wales	1 (2.7%)	188 (13.6%)	246 (16.3%)
Scotland	12 (32.4%)	307 (22.2%)	402 (26.7%)
Travel			
Yes	2 (5.4%)	333 (24.1%)	90 (6.0%)
No	35 (94.6%)	1050 (75.9%)	1416 (94.0%)

Appendix 8 (continued).
Sporadic *Cryptosporidium* case demographics in England, Wales and Scotland, 2007 and 2008.

	Rabbit genotype n=37	***C. hominis* n=1383**	***C. parvum* n=1506**
Government Office Region (England and Wales)			
Eastern	9 (24.3%)	154 (11.1%)	154 (10.2%)
East Midlands	3 (8.1%)	252 (18.2%)	129 (8.6%)
London	0	13 (0.9%)	2 (0.1%)
North East	0	22 (1.6%)	7 (0.5%)
North West	3 (8.1%)	142 (10.3%)	137 (9.1%)
South East	3 (8.1%)	71 (5.1%)	85 (5.6%)
South West	3 (8.1%)	70 (5.1%)	149 (9.9%)
Wales	1 (2.7%)	188 (13.6%)	246 (16.3%)
West Midlands	2 (5.4%)	88 (6.4%)	146 (9.7%)
Yorkshire and Humber	1 (2.7%)	76 (5.5%)	49 (3.3%)
Health Board (Scotland)			
Ayrshire & Arran	0	20 (1.4%)	6 (0.4%)
Borders	0	3 (0.2%)	29 (1.9%)
Dumfries & Galloway	1 (2.7%)	17 (1.2%)	50 (3.3%)
Fife	0	9 (0.7%)	15 (1.0%)
Forth Valley	0	25 (1.8%)	16 (1.1%)
Glasgow & Clyde	3 (8.1%)	55 (4.0%)	52 (3.5%)
Grampian	4 (10.8%)	49 (3.5%)	81 (5.4%)
Highland	1 (2.7%)	7 (0.5%)	34 (2.3%)
Lanarkshire	0	23 (1.7%)	12 (0.8%)
Lothian	3 (8.1%)	53 (3.8%)	45 (3.0%)
Orkney	0	2 (0.1%)	8 (0.5%)
Tayside	0	44 (3.2%)	54 (3.6%)

Description of temporally and geographically excluded rabbit genotype cases.

A case from Ireland was identified as GP60 subtype VbA30 in September 2008, from a 23 year old female, with an unknown travel history. No further details were available for this patient as she relocated abroad.

Two further cases were identified in the UK between January and July 2009, both in February. One was from a 26 year old female, gp60 subtype VaA21, from the Eastern Region of England, travel history not known. The other was from a 10 year old male, gp60 subtype VbA25, from Grampian Health Board, Scotland, who had not travelled abroad.

Appendix 9

Quantitative Microbial Risk Assessment

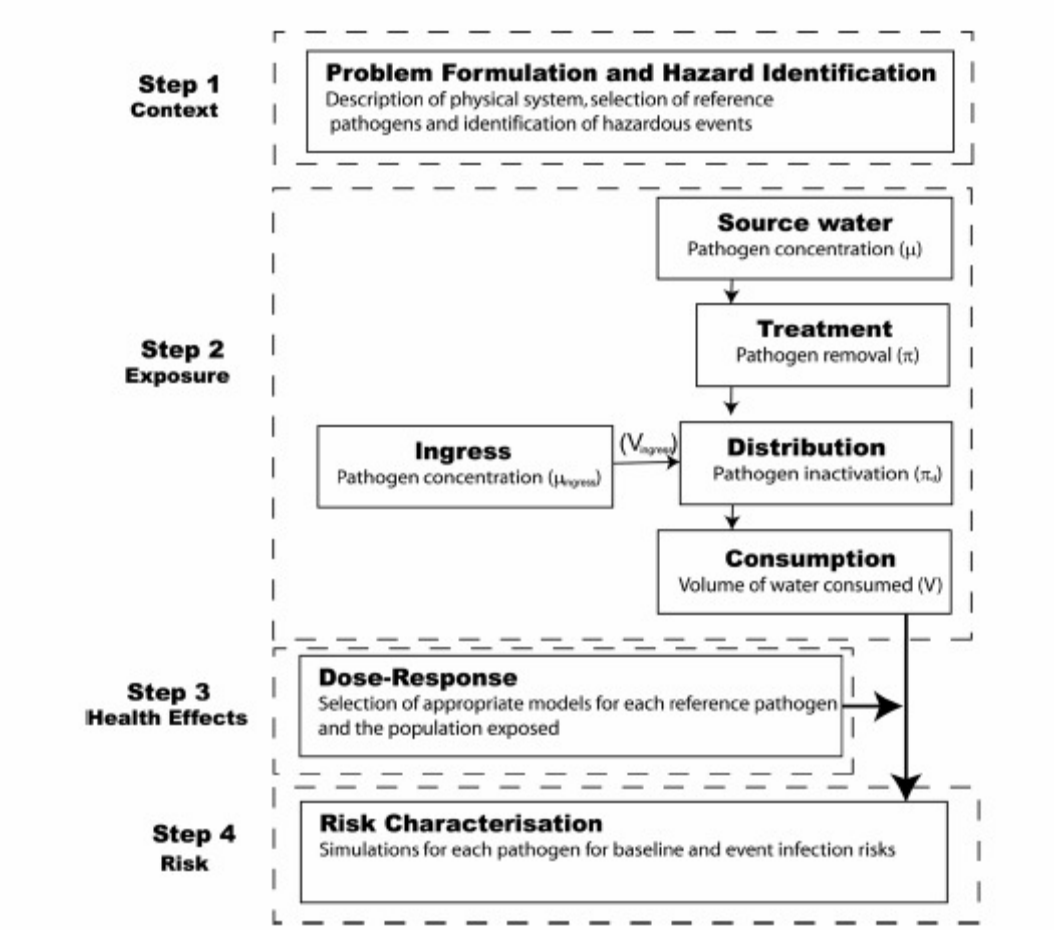

Figure 1 General framework for calculating microbial risk from drinking water

Figure taken from Petterson *et al*. 2006.