EXECUTIVE SUMMARY

The species of Cryptosporidium present on Regulatory and non-Regulatory Cryptosporidium slides cannot be determined microscopically because the dimensions of oocysts of those species which are infectious to humans can overlap with those species which are not infectious to humans. Confusion arises from the detection of oocysts which have no significance to human health. Polymerase chain reaction (PCR)-based methods can offer solutions to this dilemma, and also offer the potential for increased sensitivity and specificity for detecting waterborne Cryptosporidium oocysts on Regulatory (and non-Regulatory) Cryptosporidium slides. Currently, for many PCR assays, there is a distinct difference between laboratory and field data.

We developed methods for removing coverslips from slides and oocysts from samples and validated oocyst removal from Regulatory and non-Regulatory sample slides. The methods developed were effective but time consuming. Freeze-thawing is an effective treatment for releasing Cryptosporidium DNA from small number of oocysts for PCR amplification, particularly when conditions to optimise disruption of the oocyst wall and release of sporozoite DNA are met. This method of DNA extraction is simple and reliable and purification is not necessary as long as Tween 20 is added to the PCR mixture. We recommend this method where partially purified oocysts (e.g. following immunomagnetisable separation) are used for DNA extraction.

Most methods for identifying Cryptosporidium species / genotypes are based upon amplifying target DNA by PCR, following which the amplified product is digested using restriction endonucleases which digest the product at defined sites into fragments of varying sizes, dependent upon the frequency of occurrence of the restriction site for the chosen endonuclease. The polymorphic fragments produced are resolved on gels, producing unique patterns which are indicative of defined species of Cryptosporidium. Digestion and resolution in gel provides information on species / genotype, and is named restriction fragment length polymorphism (RFLP). PCR-RFLP generates patterns which are equivalent to molecular signatures for the species.

We used four loci (N18SDIAG, 18SXIAO, STNCOWP and MAS-PCR; see enclosed Report) for amplifying Cryptosporidium DNA from seeded slides as basing results on analysis at 1 locus can be misleading and confirmation from different loci provides greater strength to the analysis. Here, one locus (N18SDIAG) performed consistently better than the other 3 tested.

In our initial studies on PCR assay sensitivity with C. parvum oocysts, N18SDIAG was more sensitive than 18SXIAO which, in turn, was more sensitive than STNCOWP and MAS-PCR, based on the percentage of samples positive and amplicon intensity. The reason for this sensitivity hierarchy is because the N18SDIAG, 18SXIAO loci are on a multicopy (ribosomal DNA) gene, while the STNCOWP locus is on a single copy (COWP) gene. PCR amplification of multi-copy genes is an useful approach to molecular identification as it provides enhanced sensitivity therefore, N18SDIAG, 18SXIAO primers should prove more sensitive than STNCOWP primers for amplifying Cryptosporidium DNA from small numbers of oocysts and probably should be used as the primary choice before considering STNCOWP for amplifying DNA from oocysts on Regulatory and non-Regulatory Cryptosporidium slides.

We used oocysts of human derived C. felis, C. hominis and C. parvum and commercially purchased C. muris to determine the sensitivity of three loci tested (N18SDIAG, STNCOWP and 18SXIAO) primers to detect Cryptosporidium DNA extracted from DWIEQAL Regulatory slides. MAS-PCR did not prove sufficiently sensitive and was excluded from further analyses. Again the N18SDIAG primers generated the most positive results, the 18SXIAO primers performed less well and the STNCOWP primers produced fewest amplicons of lowest intensity. Our data indicate that the recommended hierarchy for amplifying Cryptosporidium DNA from slides of water concentrates
would be N18SDIAG, followed by 18SXIAO then STNCOWP to verify the presence of *C. parvum* / *C. hominis* RFLP patterns.

No Water Company sent us Regulatory slides containing *Cryptosporidium* oocysts. We received non-Regulatory *Cryptosporidium* slides from 2 Water Companies and 1 Water Utility in sufficient time to generate useful data. Extracted DNA was amplified with the N18SDIAG and 18SXIAO primers which recognise more DNA species signatures than STNCOWP primers. Initially, this decision was made empirically because we could not speculate which *Cryptosporidium* species were likely to be present in these non-Regulatory samples. Importantly, a finite volume of template (~40µL) and a theoretical requirement to repeat test a sample also focussed decisions to progress with this sequence of analyses. Should 1 of the 18s rRNA assays fail, sufficient template should be available for amplification with STNCOWP which could corroborate the presence of *C. hominis / C. parvum* DNA, the most important pathogenic species for humans, in a particular sample.

As for the seeded samples, N18SDIAG proved more sensitive (higher percentage positivity and more intense amplicons) than 18SXIAO. One reason why the 18SXIAO primers were less effective in generating visible amplicons than the N18SDIAG primers is that the primary amplicon generated with 18SXIAO (~1325 bp) is larger than that generated with N18SDIAG (655 - 667 bp). In one non-Regulatory sample, an amplicon, smaller than expected, was generated with N18SDIAG, which was not consistent with published data. Sequencing followed by database searching and sequence matching revealed a closest match (95% identity on a fragment length of 312 of 327 bp) with *Cryptosporidium* sp. KLJ-7, a new genotype identified in an isolate from wild geese in Canada.

These findings highlight the fact that many *Cryptosporidium* species / genotypes / subtypes are present in the UK aquatic environment and that a sensitive molecular assay is required to determine the extent of environmental contamination with accepted and ‘novel’ species / genotypes / subtypes of *Cryptosporidium*. Consistently, the N18SDIAG primers were the most effective in detecting accepted (and novel species, based on sequence analysis and matching) of *Cryptosporidium*.

A proportion of *Cryptosporidium* oocyst positive slides tested during this project failed to generate a PCR product at any of the genetic loci tested, but the limit of sensitivity is not based on oocyst number. Such variability is of importance when developing standardised methods for small numbers of oocysts on non-Regulatory and Regulatory *Cryptosporidium* sample slides and requires further investigation.

The PCR-RFLP approach developed to determine the species / genotype of small numbers of *Cryptosporidium* oocysts on Regulatory and non-Regulatory *Cryptosporidium* sample slides may permit identification on a significant proportion of slides however, a proportion of slides will not be amenable to this identification approach.