Water Services Association *of Australia*

WSAA Strategic Workshop on Viability Testing and Genetic Typing of Cryptosporidium Oocysts

9th and 10th March 2000,
Hyde Park Plaza Hotel, Sydney

Occasional Paper No 4

DISCLAIMER

This research paper/occasional paper is issued by the Water Services Association of Australia Inc. on the understanding that:

1. Water Services Association of Australia Inc. and individual contributors are not responsible for the results of any action taken on the basis of information in this research paper/occasional paper, nor for any errors or omissions.
2. The Water Services Association of Australia Inc and individual contributors disclaim all and any liability to any person in respect of anything, and the consequences of anything, done or omitted to be done by a person in reliance upon the whole or any part of this research paper/occasional paper.
3. The research paper/occasional paper does not purport to be a comprehensive statement and analysis of its subjective matter, and if further expert advice is required, the services of a competent professional should be sought.

© Water Services Association of Australia

ISBN: 1 876088 80 X

FOREWORD

This report describes in detail the outcomes of a strategic workshop held in March 2000 to review, develop and trial suitable methodologies that can determine the viability tests for *Cryptosporidium* oocysts (ie are oocysts alive or dead?).

Development and validation of reliable methods for measuring infectivity is necessary before work on preventative strategies such as detention in reservoirs or disinfection can proceed.

The workshop aimed to further define research strategies in the areas of:

- viability measurement (capability of infecting a human); and
- genetic typing as a tool for use in genotyping (strain identification) and molecular epidemiology (tracking transmission) of Cryptosporidial infections.

Development of guidelines setting out the conditions required to inactivate oocysts would be an outcome of the research.

This project initiated by the Water services Association of Australia (WSAA) is part of its 1999-2000 Business Plan for Drinking Water Quality. The development of viability tests for *Cryptosporidium* is a key component of the Association's *Cryptosporidium* strategy.

WSAA sponsored this workshop to introduce the technique of human cell culture to Australia (particularly for the human genotype) and stimulate multi laboratory collaborative initiatives to accelerate research of viability measurement.

This report has also been presented to the National Cryptosporidium Research Steering Committee as part of WSAA's strategy to be involved in international coordinated research programs.

I would like to thank the workshop presenters, the post graduate students who drafted the report and those who reviewed the report particularly Dr Martha Sinclair from the CRC WQ&T. Support of the Drinking Water Inspectorate DWI (UK) in sponsoring Dr Rachel Chalmers is also gratefully acknowledged.

Dr John Langford

Executive Director, Water Services Association *of Australia*

Workshop presenters: Prof Andrew Thompson, (Murdoch University WA)
Dr George Di Giovanni (American Water Works Services Co USA)
Prof Duncan Veal (Macquarie University & representing NHMRC)
Dr Paul Monis (Australian Water Quality Centre)
Dr Una Morgon, (Murdoch University WA)
Dr Jim McLauchlin (Public Health Laboratories, London)

Authors of proceedings by Macquarie University Post graduate students:
Ms Belinda Ferrari
Ms Hayley Brown
Dr Matthias Dorsch
Ms Michelle Power

TABLE OF CONTENTS

SUMMARY AND OUTCOMES

Context of the Workshop

The Water Services Association of Australia has developed a research strategy to address the issue of *Cryptosporidium* in water supplies. The research strategy is dynamic and continues to evolve as more as knowledge and experience build up.

The strategic workshop held on the 9th and 10th of March 2000 was sponsored by WSAA to further define research strategies in the areas of:

- viability measurement (capability of infecting a human); and
- genetic typing as a tool for use in genotyping (strain identification) and molecular epidemiology (tracking transmission) of Cryptosporidial infections.

Reliable measurement of the viability of oocysts is vital in evaluating preventive strategies to inactivate the oocysts. Such a viability test could be used to compare the viability of oocysts subject to a range of environmental conditions, storage in water and exposure to disinfectants either before, or after environmental exposure. Development of guidelines setting out the conditions required to inactivate oocysts would be an outcome of the research.

Predictive epidemiology is vital to medically based hazard analysis. Is the particular hazard, in this case *Cryptosporidium*, significant or not to public health? Early detection and management of any outbreaks is also based on predictive epidemiology. Genotyping and ultimately genetic fingerprinting are essential tools in molecular epidemiology. Improved hazard analysis, health surveillance and incident management would be another outcome of this research.

The joint workshop sponsored by The Drinking Water Inspectorate, UKWIR, AWWARF and WSAA Workshop held in the UK at Tadley Court concluded that human cell culture represented the best option for reliable measurement of oocyst viability. The techniques for use of cell culture, cell lines and media components are well established for the cattle genotype (Genotype 2) oocysts but not as well established for human (Genotype 1) oocysts.

WSAA therefore sponsored this March 2000 workshop to follow up the findings of Tadley Court, introduce the technique of human cell culture to Australia (particularly for the human genotype) and stimulate multi laboratory collaborative initiatives to accelerate research.

Outcome of the Workshop for Objective 1

The first objective of the workshop was to define the research and technology transfer necessary to refine and establish in vitro procedures for the maintenance of *Cryptosporidium parvum* isolates for both the human and cattle genotypes (genotype 1 and genotype 2 respectively). The outcome of the research would be a routine and reproducible procedure for viability assessment of *Cryptosporidium* genotypes of public health significance.

The outcomes are:

1. given the unique Australian environment Australian research should concentrate on development of human cell culture integrated with PCR (CC-PCR) as a viability test for environmental studies;
2. a multi laboratory trial should be initiated to transfer CC-PCR to Australia from the American Water Services Company (water utility). The Company has special skills in applying the technique to the human genotype, and is willing to collaborate to support such a multi laboratory trial. CC-PCR is an emerging technology and all involved should benefit from application of the technology in new environments;

3. participating laboratories should be selected for the initial technology transfer trial on the basis of technical capability, willingness to collaborate and support for transferring the technology to others;
4. collaborative links with other overseas laboratories should be sought through the Drinking Water Inspectorate (DWI), the American Water Works Association Research Foundation (AWWARF) and New Zealand contacts to stimulate wider use of CC-PCR and encourage collaboration between research centres;
5. Initially the IOWA Strain (genotype 2 or cattle strain) should be used as a reference strain to transfer the CC-PCR technology allowing comparison of the results from participating laboratories. Collaboration between the laboratories will depend on their experiences in applying the technology to a consistent source of oocysts (consistent strain and clean-up procedure). The use of a consistent source of oocysts should accelerate the development of CC-PCR.
6. Following successful application of CC-PCR to the IOWA Strain by the participating laboratories, a human strain should be exchanged between the collaborating laboratories and the collaborative approach repeated;
7. A strategic workshop should be held in October 2001 in Perth to review experiences in the multi laboratory collaborative trial, and conclude the technology transfer and development phase of CC-PCR. This workshop will form part of the scientific program of the forthcoming international conference on Cryptosporidium – Cryptosporidium from Molecules to Disease. This workshop will be a satellite meeting of the Australian Society for Microbiology (ASM) Perth meeting in October 2001, taking advantage of the international expertise attending the ASM meeting. WSAA has decided to be a major sponsor of both the Conference and workshop.

Outcome of the Workshop for Objective 2

The second objective of the workshop was to develop strategies for evaluation of RT-PCR and FISH procedures for their value as a routine procedure for assessment of oocyst viability. The results obtained with RT-PCR and FISH could be compared directly with *in vitro* and *in vivo* models of viability. Strategies could include the concept of multi laboratory trials and comparison of the various techniques. A more widely applicable, reliable and cost effective method is the desired outcome.

The outcomes were:

1. a multi laboratory trial to compare of a number of potential viability methods including FISH and RT-PCR against CC-PCR as a reference method;
2. variables for the trials should include: laboratory skills; viability measurement techniques; strain of oocyst, and storage time of oocysts in water. Such a trial should build knowledge of the techniques in a range of situations;
3. the design of the trial should be prepared with input from a statistician, an epidemiologist and a parasitologist with expertise in *Cryptosporidium*. The final design could be agreed at the October 2001 workshop concluding transfer of CC-PCR technology.

Outcomes of the Workshop for Objectives 3 and 4

Objectives 3 and 4 relate to detection and genetic typing and have been considered together.

The third objective of the workshop was to develop strategies for a multi laboratory trial within Australia and overseas to compare currently available PCR based detection procedures for

Cryptosporidium with the aim of evaluating broad applicability in terms of specificity and sensitivity.

The fourth objective of the workshop was to develop strategies for a multi-centre trial within Australia and overseas to compare currently available PCR based genotyping techniques in order to determine the most useful genetic loci.

The outcomes were:

1. development of reference sets of HSP 70 sequences and sequences of the variable region of 18S through establishment of an international data base on polymorphism of *Cryptosporidium* HSP 70 and the most polymorphic part of 18S;
2. Use of cloned DNA as standard reference in the multi-laboratory trials. Potentially clones of the complete HSP 70 and 18S genes could be obtained from Lihua Xiao at CDC for both the human and cattle genotypes;
3. use of standard methods, that is use of standard primer sets and PCR conditions, for PCR amplification of parts of HSP 70 and 18S;
4. a standard primer is available for 18S through Una Morgan of Murdoch University; a review is required to agree on a standard primer for HSP 70;
5. while all participants in the multi-laboratory trial would be free to continue investigations of their own genotyping ideas, all participants should use the reference methods to allow results to be compared;
6. development of standard protocols for genetic fingerprinting should be considered at the October 2001 Workshop. Progress on finger printing will require access to abundant sources of DNA from environmental and clinical samples.
7. The use of differential display proteomics of different strains of *Cryptosporidium* should be investigated as a future tool to identify new loci for genotyping or fingerprinting Cryptosporidium, and for correlating phenotypic with genotypic differences.

Outcomes of the Workshop for Objective 5

The fifth objective of the workshop was to explore strategies for determining the prevalence and distribution of human infectious and non-infectious genotypes in different water sources, and in populations of human and animal hosts. Identification of genetic markers for infectivity and virulence would be one long-term goal of such strategies.

Genetic fingerprinting of *Cryptosporidium* oocysts is dependent on the availability of a diverse collection of DNA from oocysts taken from environmental, and clinical samples of both animal and human origin. DNA can be taken from aged faecal samples and there was the suggestion that it could be isolated from archived laboratory slides.

The outcomes were:

1. **Human Sources** The cooperation of the both the Public Health Laboratory Network (of Australia), and commercial pathology laboratories should be sought. A suitable laboratory should be identified in each state to genotype the oocysts and store them for future fingerprinting studies.
2. **Environmental Sources** Current or proposed research projects should provide a diverse set of environmental samples with comprehensive documentation (where, when and how the sample was taken and prepared).
3. **Management of the Collection** A protocol should be prepared for cooperating laboratories, and users of the collection to ensure maximum scientific value can be gained.

WSAA STRATEGIC WORKSHOP ON VIABILITY TESTING AND GENETIC TYPING OF CRYPTOSPORIDIUM OOOCYSTS

9th and 10th March 2000, Hyde Park Plaza Hotel, Sydney

DAY 1 Thursday 9th March 2000

Introduction to Workshop and overview of WSAA's *Cryptosporidium* Research Strategy. - John Langford WSAA

Dr Langford reported that WSAA's strategy is to develop cost effective approaches to achieve public health outcomes.

For *Cryptosporidium* the strategy was to first identify if a public health risk exists - does *Cryptosporidium* exposure from drinking water supplies contribute significantly to illness in the community?

WSAA is also involved in assessing health risks from algal toxins, aluminium and copper.

The approach to assessing the health risks from aluminium in water was used demonstrate WSAA's approach. A study of intake of aluminium by humans demonstrated that water is not the most significant source.

WSSA's innovative approach to in regulation based on hazard principles was also described.

WSAAs *Cryptosporidium* Strategy.

WSAA's *Cryptosporidium* Strategy involves:

1. Hazard analysis - Clinical trials, case control study, genotyping and fingerprinting to determine if we have a problem before investing money to solve the 'problem.' Of particular note is the trial in Melbourne comparing health outcomes with real and sham point of use filtration devices.
2. Risk assessment in catchments – what are the most important sources.
3. Prevention – how is *Cryptosporidium* removed by catchment management, water treatment, etc. Perhaps the key issue here is the development of reliable measures of oocyst viability.
4. Verification - third party accreditation and improved health surveillance.
5. Revision of drinking water guidelines to be based on hazard analysis and risk assessment.

Introduction to Viability Testing and Genetic typing of *Cryptosporidium* oocysts – a brief overview of the Tadley Court Workshop. - Andrew Thompson, Murdoch University

Prof. Thompson noted there are a large number of genotypes in water that are not relevant to human health. Whilst some genotypes are clearly zoonotic others are probably not. Genotyping and Viability determination are key issues. There was a note of caution in that some species thought non-infectious to humans (eg *C. felis*) have recently been found infectious to immuno-suppressed individuals. Thus we need to consider the susceptibility of sensitive groups in the community.

The Tadley Court workshop recognised the need not only to detect oocysts but also to determine genotypes and viability.

- The gold standard for determining viability (animal infection) only worked for Genotype 2.
- Require a simple PCR test, which is both quick and cost effective.

OBJECTIVE 1:

Define the research and technology transfer necessary to establish in vitro procedures for the maintenance of Cryptosporidium parvum isolates for both human and cattle genotypes (genotypes 1 and 2). Dr George Di Giovanni - American Water Works Services Company.

Cell Culture PCR for Cryptosporidium – presentation by Dr George Di Giovanni America Water Works Services Company

The USA has invested substantial amounts in *Cryptosporidium* research. This has been largely in response to the Milwaukee outbreak which had a major impact on the US water industry. Milwaukee was of particular significance as there were a very large number of individuals who became ill and a number of fatalities. The Milwaukee outbreak also prompted changes in the regulation of water treatment which have had substantial cost impacts on the water industry.

The current US method for the detection of *Cryptosporidium* (Method 1622 / 23) depends on concentration by filtration, recovery by immunomagnetic separation, followed by detection by microscopy IFA, DAPI and DIC. The major limitation of this method is that it does not provide viability or species discrimination. It was pointed out that Method 1622 initially assessed viability through DAPI / PI but this was dropped due to problems with reliability of PI for determining viability. DAPI is still used for (oo)cyst confirmation.

To address water utilities needs new *Cryptosporidium* methods should:

- Detect all strains capable of being pathogenic to humans.
- Determine viability / infectivity of oocysts detected
- Provide information on the species and genotype,
- Provide also source of oocysts
- Sensitive 1 oocyst
- Rapid (results in 1 day) and easy to use
- Inexpensive

Integrated Cell culture - PCR (CC-PCR)

The advantages of cell culture PCR are:

- Specificity of molecular methods
- Infectivity determination
- Sensitivity: amplification through growth in cell culture
- Species and strain identification
- Speed

The disadvantages of cell culture PCR are:

- The requirement for a skilled analyst.
- The data is semi-quantitative based largely on presence / absence in a volume of water.
- Expensive?

Integrated Cell culture - PCR Assay protocol

Overall method

1. Filtration (Envirochek™ filters)
2. IMS recovery and purification
3. IMS dissociation
4. Detection of infectious *C. parvum* oocysts using CC-PCR
5. Genotype ID using DNA sequence analysis (mainly product confirmation)

Assay time - 72 hours. Time required for maximising target (HSP70) for DNA techniques. Optimum incubation time may vary depending on the type of target (ie DNA or mRNA).

Oocyst Dissociation

In method 1622/3 0.1N HCl used to dissociate magnetic beads from (oo)cysts. There were concerns that this would have an undesirable effect on oocysts viability. An alternative dissociation method using of HBSS pH 2.0 - 2.75 and 1% trypsin incubation 37^{o}C for 1 hour has been developed. This method acts both to dissociate oocysts and as an excystation trigger.

Selection of cell line

A number of cell lines have been trialed for cell culture of *Cryptosporidium* oocysts. American Water Works Company has selected HCT8 on the following basis:

- Robust
- Rapid growth
- Firmly adherent (wash easily)
- Resistant to debris. Can cover monolayer with environmental sample with no detrimental effect to cell growth
- HCT8 can support human genotype

Cell culture method

- Cell culture media is based on the formulation Upton et al
- Maintenance for cell line RPMI 1640 with 5% FBS
- Cell growth media consists of RPMI, 10% FBS, vitamins and antibiotics
- There have been some reports that FBS inhibits cell culture growth of Cryptosporidium (Arrowood *et al*)

Detection

- The cell monolayer washed to remove intact oocysts to prevent PCR contamination as few as 20-30 non-excysted oocysts could give false positive. Although generally environmental samples contain small oocysts numbers <5, washing is considered a useful precaution against detecting non-excysted oocysts.
- CC-PCR has a sensitivity of 1 infectious oocyst although infection with 1 oocyst produces various levels of cell infectivity

CC-PCR field applications

CC-PCR has been used in field applications including.

- Recycled backwash.
- Source water assessment
- Finished water
- Evaluation of treatment plant performances

Results of field applications

Currently, finished waters from 80 treatment plants are being monitored over a 24 months period using CC-PCR.

Prior to undertaking the program protocols were put in place with relevant state health authorities to deal with any positive detection results.

Of 350 samples tested (100 litres /sample), 4 were found to be positive for cell culture infectious *C. parvum*. On re-sampling all results were negative and no outbreaks of Cryptosporidiosis were identified. On all occasions when *C. parvum* was found the plants appeared to be operating well with no obvious problems in the treatment process.

Of 500 environmental samples analysed 43 were found to be positive. Seven different genotypes were found in the 43 positives. The Bovine genotype was found most commonly (*c*50% of samples). Unknown genotypes were also found.

Relative to method 1622/3 CC-PCR resulted in approximately 50% positives. This could indicate that approx. 50% of oocysts are potentially infectious to humans.

Future Research required on CC-PCR

- More research on a reliable method to culture the Human genotype is required.
- More research on excystation triggers is required
- There is a need to optimise and standardise cell culture conditions
- More work on single oocysts through micromanipulation.
- Quantitation is important and there is a need to develop reliable quantitative CC-PCR
- A number of new genotypes are being identified.The cell culture and human infectivity of these genotypes needs to be determined.

Human genotype

The Human genotype appears to comprise of 5 HSP70 sub genotypes. The human genotype appears to be only infective to humans and is thus difficult to maintain in animal culture. Sources of the human genotype are clinical samples. These can be difficult to obtain in a state suitable (viable) for cell culture. At present there is very limited information on the human genotype.

Cell culture of Human Genotype

It is possible to infect both HCT8 and CACO2 cell lines with the Human genotype but it is not possible to maintain the infection. The ability to infect cell culture with the human genotype is highly variable between laboratories for unknown reasons. The oocysts of the human genotype also seem to have short viabilities on storage (c6 weeks).

No mouse model exists for the human genotype. Recently infections have been achieved in Gnotobiotic piglets, infected with high levels (10^6) of oocysts and recoveries of 10^8 oocysts have been achieved. This is an expensive and highly specialised method of producing oocysts that requires large animals to be reared in germfree conditions.

CC- PCR Conclusions

1. CC-PCR can detect many genotypes and strains of *Cryptosporidium parvum*
2. Data provide information for public health
3. Basis for improving water treatment
4. Molecular analysis provides a better understanding of what genotypes are present.

General Discussion

Dr Di Giovanni offered to share the detailed methodology with Australian Researchers on the CC-PCR method.

There was concern that contamination with large number of dead or intact oocysts could result in false positive results due to residual DNA. Washing the cell culture mono-layer and cell culture-selective DNA extraction apparently overcomes this problem according to Dr Di Giovanni.

Work with micro-manipulation indicates that infection of the cell culture with a single oocyst is possible although there is variation in infection. It may be possible to automate selection of individual oocysts using flow cytometry. This would speed up the process and allow individual oocysts to be sorted from environmental samples and cultured in individual wells of a micro-titre plate.

A major consideration is the likely survival time of *Cryptosporidium* oocysts in catchments. This is of particular importance in Australia where the water storages are often very large and retention times long. A major area of interest would be the lengths of time oocysts remain infectious in Australian water and any variations in survival between genotypes.

There was discussion about why only some groups are able to get the human genotype to infect cell cultures. The problems may relate to the age of materials, methods of purification, and biological variation and cell culture conditions.

There was also comment that the infectivity of oocysts may change during an infection ie oocysts produced soon after onset of an infection may differ from those produced at the end of an infection.

The need for comparative studies using a single strain comparing cell culture infectivity with animal infectivity and other methods of determining viability (eg FISH, RT-PCR, excystation was noted. It was pointed out that Paul Rochelle et al in the US are currently comparing cell-culture with animal infectivity.

The issue of potential cytopathic effects of water concentrates was raised – do water concentrates adversely affect the monolayers. Dr Di Giovanni reported that this was not a problem in his experience with IMS purified samples.

The recent report that oocysts that do not excyst under artificial conditions may still cause infections was raised as a possible limitation of the CC-PCR method for determining infectivity.

There was a concern that the HSP70 primer may not be *C. parvum* specific but may also amplify *C. wrairi*.

The issue of obtaining adequate supplies of oocysts for comparative experiments was discussed. Some workshop participants had experienced difficulties with importing supplies, however others had not found significant problems. The expense involved in large scale oocyst production (eg from cattle) makes it unlikely that any Australian lab would set up such a facility.

Summary Workshop Objective 1:

- In Australia CC-PCR for environmental monitoring in the first instance. It was agreed that for Australia the highest priority for application of the CC-PCR method was for environmental detection of oocysts rather than to assess water treatment efficiency. A considerable amount of research on disinfection studies is occurring around the world that could be directly applicable to Australia. AWWARF is currently assessing animal models and cell-culture for disinfection studies and it seemed most appropriate to wait for these results.
- An open process should be used to request expressions of interest from Australian laboratories wishing to acquire the technique. Laboratories should be selected for initial technology transfer from the American Water Works Services Company based on their technical capabilities and willingness to collaborate and transfer technology to other labs. New Zealand labs should also be invited to collaborate.
- The method should be initially established using the IOWA isolate as reference strain to enable comparisons between laboratories.
- Once the technique was reliably established with the IOWA isolate, isolates of the human genotype will be used and exchanged between collaborating laboratories to ensure comparability of results. It was estimated 18 to 24 months would be required to reach this stage.
- A workshop should then be held to transfer the technology to other labs wishing to acquire the technology. Such a workshop could be associated with the ASM Cryptosporidium Conference scheduled for October 2001.
- Collaborative links with AWWA and DWI in the project will be sought.

OBJECTIVE 2:

Develop strategies for evaluation of RT-PCR and FISH procedures for their value as a routine procedure of viability (and strains/species?)

Introduction - Duncan Veal, Macquarie University

Considerations for Viability methods

1. Ease/Speed- How does the method fit in with existing analytical protocols.
2. Do oocysts need to be maintained viable?
3. Quantification- is this important? Why are we measuring for viability and how will that information be utilised?
4. Is species determination important?
5. What do we mean by "infectivity" and does this relate to what we are using the data for?

Fluorescent In Situ Hybridisation

What is FISH?

- F-fluorescence tagging,
- I-In
- S-situ,
- H- Hybridisation – often to ribosomal RNA.

Why Target Ribosomal RNA?

- Multiple copies of targets are present thus amplification is not required
- RNA has been found in (recently) viable cells from environmental samples
- Regions of rRNA range from highly conserved to highly variable this enables probes to be designed of various specificities.

Advantages of FISH:

Rapid assay (90 minutes), robust reliable method, provides a conservative estimate of viability, easy to enumerate, does not suffer from inhibitors found in environmental samples. FISH also provides information on species and viability. FISH can provide a retrospective indication of viability – once the sample is fixed, it can be stored before FISH testing. This has practical and biosafety advantages.

FISH is a 5 Step Process:

1. Oocyst wall permeabilisation
2. Probe hybridisation to target oocyst rRNA
3. Wash away excess unbound probe
4. IFA stain
5. Epifluorescence microscopy

There is a need for inter-laboratory validation of FISH. Kits are available upon request from Duncan Veal.

There was concern about the stability post-death of oocysts. The half life of rRNA in oocysts following heat inactivation is 55 hours. The addition of RNAse results in immediate loss of the RNA signal.

RT-PCR - Paul Monis Australian Water Quality Centre

Reverse transcriptase polymerase chain reaction

- Targets RNA
- 2 step process:- convert RNA →DNA, amplifies target DNA
  - species/strain specific
  - Presence / abscence test
  - -Non-quantitative at present
  - The assay is a marker for viability depending on the stability of RNA after cell death

Published methods: Stinear 1996, Rochelle 1997-both target HSP 70 mRNA

Widmer 1999 targets β-tubulin, and an unknown mRNA

Inhibition problems may need to be overcome in environmental samples for RT-PCR. For routine analysis the sensitivity and robustness in environmental samples needs further optimisation.

The targets for RT-PCR also need investigation. 44% of *Cryptosporidium* has been sequenced. This sequencing may aid in identifying genes involved in infectivity and may aid in determining targets for RT-PCR.

PCR inhibition is no longer a problem in environmental samples. Quiagen has a PCR clean-up kit suitable for this purpose

The major disadvantage of RT-PCR is that it is not capable of quantification and provides a yes/no answer only.

Validation of FISH and RT PCR

Inter-laboratory validations of FISH and RT PCR were considered to be important. For such validations:

- Standard procedures and probes need to be available.
- Need to have standard strains or DNA clones.
- Need to have access to a range of strains/species to test for specificity.
- Need to correlate FISH and RT PCR with cell culture infectivity.
- Need to test with a wide variety of sample types.

Validation of FISH has been difficult to establish due to protected intellectual property associated with this method and thus lack of take-up in the water industry. The lack of availability of a variety of species and strains has made validation of species specificity difficult.

Cell culture-PCR. CC-PCR may aid research validation of FISH and RT-PCR.

Water Industry Needs

The water industry needs to determine what inactivates oocysts and other microbial contaminants in water. For example what disinfection conditions are capable of microbial inactivation. Before this question can be answered we need reliable indicators of oocyst viability and how to interpret these indicators in terms of their significance to human health.

It was suggested that the various FISH and RT-PCR are compared to CC-PCR in blind trials using the IOWA strain.

Currently a USDA blind study incorporating FISH at Macquarie University is underway.

A coordinated approach to such trial is needed both in Australia and Worldwide.

The laboratories involved in RT-PCR and FISH could be recruited into such a trial.

A key issue is that a central and reliable source of oocysts is needed for all laboratories involved in objective 1 and 2.

Approach for Achieving Objective 2:

AIM: To determine a reliable method for determining human infectivity.

Compare the various viability methods to the cell culture based method adopted in objective 1.

1. Select a number of laboratories including those chosen in Objective 1 to be involved in the trial.
2. Select the most suitable technique and defined protocols for viability determination. A quantitative assessment (percentage viability) is only available for the FISH technique.
3. The methods to be evaluated are: CC-PCR, FISH and RT-PCR.
4. Obtain sources of fresh oocysts covering a range of genotypes. At least Genotype 1 (Human) and genotype 2 (Bovine) should be used in the trial.
5. Send viable and heat-treated (eg 65° C for 15 minutes) to kill oocysts to the laboratories for viability determination in blind trials. Heat-killed and viable oocysts in various mixtures would also be sent to laboratories to determine their ability to determine the percentage viability.
6. The effects of storage on oocyst viability, determined using the various different methods would be determined. Oocysts will be stored in water and samples sent to participating laboratories over a time course. Oocysts will lose viability as they age and the ability of the various laboratories and various laboratories to determine this will be determined.
7. There is a need to employ a bio-statistician to help in the design of this experiment
8. The feasibility of carrying out the survival experiment (see 6 above) in a catchment should be investigated.

The difficulties in obtaining oocysts of particular strains were again discussed. The IOWA strain can be obtained from Sterling's Lab at the University of Arizona in US. NATA is gaining experience at importing and suppling oocysts.

Summary Workshop Objective 2:

- After establishment of the cell culture PCR technique in Australia, a multi-laboratory study should be carried out to compare this with FISH and RT PCR methods.
- Comparisons should be made in a blinded manner with fresh oocysts, killed oocysts and aged oocysts (measured over time)
- Therefore comparing: laboratory skills,viability techniques, sources of oocysts and treatment over time for inactivation. Results will provide data on variation within labs, oocyst strains and techniques.
- Need to have reliable sources of oocysts, preferably both genotype 1 and 2.
- In order to ensure that the study would yield reliable results, a feasibility assessment by a statistician/epidemiologist and a *Cryptosporidium* expert should be carried out initially to establish the required number of replicate tests etc. This would generate a proposal for further discussion and costing prior to conducting the study.

Day 2 Friday 10th March 2000

OBJECTIVE 3:

Develop Strategies for a multi-centre trial within Australia and overseas to compare currently available PCR based detection and genotyping techniques in order to determine the most useful genetic loci.

Introduction: Dr. Una Morgan - Murdoch University

What are we looking for in a detection method?

- high sensitivity
- reproducibility
- speed
- detect all genotypes or just the human (H) and cattle (C) types? (genotypes I and II respectively)
- indicates public health outcomes
- genotyping capabilities

Una pointed out that for most PCR based techniques, inhibition of the test by environmental contaminants is no longer a major problem

It was reinforced that immunocompromised humans can be infected by *C. felis* and *C. meleagridis* as well as *C. parvum* genotypes I and II. Although genotypes I and II represent the majority of human infections, there may be aneed for tests that can detect all the different genotypes and species capable of infecting immunocompromised individuals. To date, the development of detection techniques has been very biased towards the detection of genotypes I and II – other genotypes may go unrecognised.

The current state of knowledge on *Cryptosporidium* genotyping was summarised. The taxonomy of this organism is currently under review as increasing knowledge from molecular characterisation reveals details of phylogenetic relationships. For *C. parvum* it is likely that some of the currently recognised genotypes will eventually be designated as separate species. At present 7 distinct major genotypes are classified within *C. parvum*; Type 1 (human), Type 2 (cattle), marsupial, pig, mouse, ferret, and dog. A minor variant of Type 1 occurs in monkeys. The dog genotype is soon likely to be reclassified as a separate species (C. canis), while the cat genotype is already recognised as such (C. felis).

Which locus ?

Random Amplified Polymorphic DNA (RAPD) using PCR

- useful when no other polymorphic information was available on other loci
- often difficult to reproduce results across different labs
- template DNA needs to be extremely clean, there can be no bacterial contamination
- in general – RAPD is not particularly useful

Acetyl Co-enzyme A

- useful for discriminating human and cattle genotypes

Thrombospondin – related adhesive protein (TRAP)

- *C. muris* and the *C. parvum* cattle genotype are identical (U. Morgan)

Cryptosporidium oocyst wall protein (COWP)

- same problem are experienced as with TRAP (U. Morgan)

Dihydrofolate reductase (DHFR)

- there is some difficulty in detecting the different genotypes at this locus (U. Morgan)

β - tubulin

- sequence variation within the *C. parvum* human genotype was found at this locus (Widmer *et al*)
- Sulaiman *et al* did not detect this variation when only using PCR-RFLP (PCR- Restriction Fragment Length Polymorphism) at this locus instead of sequencing
- no recombination in the human genotype and would only differentiate H and C genotypes and not other types
- some published DNA sequences are incorrect

Internal transcribed spacers I and II (ITS I and ITS II)

- PCR at this locus can discriminate H and C genotypes
- as there are multiple copies of ITS I and II, can perform PCR-RFLP on very small numbers of oocysts taken from a water sample utilising a nested PCR technique

Microsatellites

- very few have been found; only 2 different H microsatellites and 4 different C microsatelites have been found (Caccio *et al*; Xiao *et a*l)
- C. parvum genome is 90% A-T rich in non-coding regions, therefore it is difficult to find polymorphisms using microsatelites
- need to scan for existence of more microsatellites as C. parvum genomic sequence becomes available
- currently, 44% of the *C. parvum* sequence is available

18S rRNA

- sequence variation can discriminate between species and genotypes
- choice of PCR primers is important when targeting this locus as 18S
- primers can cross react with everything in a sample
- U. Morgan's group has sequenced the entire length of the 18S of all the known genotypes
- 18S sequence information has been deposited at GenBank and CDC

Heat Shock Protein 70 (HSP 70)

- very useful locus to investigate
- both H and C genotypes have been fully sequenced (Lihua Xiao *et al*)
- Lewar *et al* have used HSP 70 in combination with 18S rRNA sequence information to suggest the existence of 6-8 different sub-genotypes within the human genotype

PCR – product sequence analysis versus PCR-RFLP

- sequencing is recommended over RFLP as the data obtained is fuller and clearer than that obtained by RFLP

The two main goals of genotyping

1. to discriminate the C. parvum human and cattle genotypes
2. to fingerprint oocysts found in water and in faecal samples to reveal clues on how individuals become infected (reveal sources of contamination)

Currently the NIH is funding a project to sequence the entire bovine *C. parvum* genotype (Iowa strain) and the partial sequence of a strain of the human genotype (NEMC1)

- From sequence data other possibly useful loci for genotyping may be found
- however, at the moment, sequence databases are not user – friendly
- better bioformatics are required

General discussion

Do we want the detection methods to focus more specifically on the H and C genotypes, or to be more applicable to other genotypes?

- in most catchment situations, the H and C genotypes will cover at least 90% of Cryptosporidium present (J. Ongerth).
- however, we need a test to detect any genotypes that are a public health issue (eg a minor, less abundant strain may be particularly virulent to immunocompromised individuals and it may be carried by a ferral or native sources).
- therefore, need to practice more routine monitoring of genotypes present in catchment against a reference species.
- if there is a mixed infection in an environmental sample, the less prominent type may be missed. The ability, therefore, to sort individual oocysts using flow cytometry may be desirable. This would involve sorting a single oocysts then infecting a cell line.
- PCR based detection protocols can pick up genotypes in mixed samples where the minor genotype is 10 times less abundant than the predominant type

Do we need to define the particular genotypes we are targeting or just try to develop a technique that will identify and discriminate all the different genotypes ?

- one suggestion toward the more straight forward screening of a catchment involved the use of a universal set of primers to detect all genotypes present in the catchment, followed by the use of more genotype specific probes on the universal product
- need to be sure that all the different genotypes behave the same in the environment (eg similar survival times) before basing our analyses on a single genotype
- what are the antibodies on the IMS beads reacting with and detecting? Is it possible that IMS may be selecting genotypes before PCR genotyping?

The main issues:

- public health and management
- detection and sources of contamination:
  - human
  - native animal
  - domestic animal
- the effectiveness of barriers designed to remove pathogens needs to be monitored
- source animals of pathogens that are infectious to immunocompromised individuals need to be identified
- also need to investigate the human sources of Cryptosporidium eg) collect samples from daycare centres
- by an understanding of the genetic diversity using molecular epidemiology techniques, could we rank the importance of the different sources of contamination ie)- human (childcare centres for example) or animal ? – John Langford

How can we improve the development of methods ?

- we need to identify reference strains for method comparisons
- probably need to have a panel of reference strains – easier said than done
- need to use standard loci for comparisons across different labs
- perhaps could use one locus for discriminating genotypes and one standard locus for discriminating within genotypes
- general opinion was that the **18S rRNA** and **HSP 70** genes would provide the best basis for comparison of strains

- however, Jim McLauchlin recommends the use of the COWP locus over the use of the 18S rRNA locus

Advantages of using the 18S and HSP 70 loci for genotyping

- there is an enormous amount of full length sequence information available
- the 18S from all the different genotypes has been sequenced
- the 18S and HSP 70 genes from both the H and C genotypes have been cloned (Lihua Xiao *et al*; CDC)

The aim is to compile worldwide data base on polymorphism of the Cryptosporidium 18S rRNA and HSP 70 genes

To do this we need:

1. to use standard methods ie)- use of standard loci (18S, HSP 70)
2. to establish standard reference samples ie)- the use of clones
3. the data compiled can be used as a reference for comparison and placement of any already known or unknown genotype or species
4. the use of cloned DNA as reference material is easier than the use of standard strains of oocysts, due to propagation, storage and transport considerations
5. to standardise the use of primer sets

Primers and target regions of the 18S and HSP 70 genes

- need to target regions of maximum polymorphism
- the 18S has only 1 or 2 regions that have reasonable polymorphism for discriminating genotypes while HSP 70 has polymophism throughout its sequence
- U. Morgan has already established the most polymorphic region of the 18S, spanning a 350-500 bp stretch and can provide the corresponding primer sequences for amplification of this region
- the HSP 70 primer sets are yet to be decided

Avenues for identifying new polymorphic markers

Despite extensive research efforts in a number of labs, relatively few markers have been identified which allow discrimination of strains within the major *Cryptosporidum parvum* genotypes. The workshop participants agreed that all obvious candidate markers had already been tested. Dr Duncan Veal suggested that the science of proteomics might offer a means of identifying new markers. This methodology involves the automated analysis of differences in protein content between isolates. Proteins are extracted and separated on two directional gels and analysed by computerised scanning. Gel patterns from many isolates can be rapidly compared to detect differences. Once proteins are detected that differ between isolates, their amino acid sequence can be determined and used to design DNA probes for the corresponding genes.

This approach would enhance the probability of finding strain-specific markers in comparison to testing randomly chosen genomic DNA sequences. It was agreed that a preliminary research proposal for this approach should be developed.

Summary of Objective 3/4:

- Collaborating laboratories will undertake to use a standard set of markers, target material (DNA clones of the IOWA isolate) and primers when characterising strains so that results can be readily compared between different laboratories.
- The standard markers will be the 18S and HSP70 genes.

- Efforts will be made to obtain the full length clones of the 18S and HSP70 genes from both the Human and Cattle C. parvum genotypes from Lihua Xiao at CDC for use as the standard target material.
- Standard primers for 18S and HSP70 will be agreed by discussion between labs.
- All groups would also be free to pursue their own genotyping ideas
- The possibility of establishing a common database of verified sequence information should be explored
- Sequence information should also be deposited in GENBANK in the usual manner
- Development of reference sets of HSP 70 sequences and sequences of the variable region of 18S through establishment of an international data base on polymorphism of *Cryptosporidium* HSP 70 and the most polymorphic part of 18S;
- Genetic characterisation to discriminate strains at a finer level (fingerprinting) will require the use of several loci, and developments in this area should be discussed at a future date (possibly the ASM October 2001 workshop).
- Proteomics may offer a means of identifying new markers for fingerprinting - a preliminary project proposal for this approach should be developed.

OBJECTIVE 5:

Explore Strategies determining the prevalence and distribution of human infectious and non-infectious Genotypes in different water sources and in populations of human and animal hosts. Identification of genetic markers for infectivity and virulence would be one goal

Naturally, investigations into the epidemiology are the no. 1 priority for the water industry regarding *Cryptosporidium.* Priority no. 2 is how to kill the organism in our drinking water.

Introduction Prof Andrew Thompson – Murdoch University

Two genotypes of *Cryptosporidium parvum* have been identified in previous studies and we need to establish a procedure to conduct sampling in Australia in order to investigate the distribution of the two genotypes within specimen samples/infected persons. To achieve this goal we need to:

- Identify ongoing projects
- Establish new projects
- Identify relevant expertise
- Identify sources of funding

Epidemiological Studies - Jim McLauchlin Public Health Laboratory Service UK

International Collaboration

Genotyping study collaboration employing Polymerase Chain Reaction (PRC) techniques was initiated between laboratories in the Czech Republic, Italy, Portugal and the UK. Twenty samples of DNA and two samples each containing positive and negative controls and a cloning kit were distributed. Also provided were dilution series of the DNA to establish the sensitivity of the PCR in different laboratories.

Good results were obtained, only one 'false negative' and two 'false positive' reactions were reported.

Identification of genotypes in fourteen samples reached from 11 – 14, a result considered at least satisfactory. However, sensitivity of PCR as estimated by dilution series of the target DNA varied greatly between laboratories.

Significant differences in the sensitivity of PCR conducted in different laboratories is not necessarily due to different laboratory methods but could be caused by a certain degree of degradation of DNA occurring during shipment. To avoid results being influenced by the 'potential degradation factor' it could be beneficial to spot DNA samples on filter paper and dry prior to shipment. The method as proposed here is also likely to reduce the risk of contamination/cross contamination of the nucleic acid shipped to different laboratories.

The overall agreement of the trial presented here ranges from 71 – 100 % and was evaluated a promising approach for international collaboration in genotyping and epidemiology of *Cryptosporidium.*

Epidemiology Studies in the UK

- COWP (*Cryptosporidium* Oocyst Wall Protein) sequence analysis as determined per RFLP (Restriction Fragment Length Polymorphism) applied to 2000 specimen samples revealed genotype 1 (human) and genotype 2 (cattle), a small number of isolates that had to be designated genotype 3 (from 6 samples), and an insignificant number of samples that did not yield any amplification product. The reason for these negative results is subject to speculation.
- Results of epidemiological case studies of several outbreaks were presented:

- Analysis of isolates from eight drinking water related *Cryptosporidium* outbreaks in the UK demonstrated that five outbreaks were due to Type 1 isolates (indicating human faecal pollution) while three were due to Type 2 isolates (human or animal faecal pollution).
- For five swimming pool related outbreaks examined, two were of Type 1, two were of Type 2 and one was mixed Types 1 and 2.
- Investigations into *Cryptosporidiosis* detected in residents of the UK having traveled abroad clearly showed a prevalence of genotype 1 (human).
- Data on farm animal infections demonstrate a consistent peak of infection in sheep during springtime (lambing season), while in cattle infection peaks occur both in spring and in autumn. Human infections by genotype 2 also appear to peak in springtime, althought they are present all year round. Genotype 1 infections appear to be very rare during the winter/spring season.
- The majority of areas investigated in the UK show a significant prevalence of type 2 infection, only in the South Thames region are the numbers of infection by genotype 1 and 2 approximately equal.

Question (Q): Were any (receiving/drinking?) water samples examined?

Answer (A): No.

Q: What is the similarity between *Cryptosporidium* and *Giardia* infections? In the US *Giardiasis* is 5-fold more common than *Cryptosporidiosis.*

A: The question cannot be answered, different regulations in Australia regarding pathology laboratories do not require routine testing for *Cryptosporidium.* Further, *Cryptosporidiosis* is not a reportable disease in some Australian states. To generate epidemiological data in Australia it might be valuable to attempt tracking down unpublished data.

Q: What are the prospects of processing 'old' samples such as frozen or otherwise stored faeces samples or even slides with smears of stained samples.

A: Ethics involved in the (unauthorised) examination of specimen are an important issue that needs to be clarified. We (Dr Geoff Hogg) will contact reference pathology laboratories to investigate the possibility of processing 'old' samples in order to establish an epidemiology data base of *Cryptosporidium*.

Q: What actually is the frequency of detection of *Cryptosporidium* oocysts in water samples.

A: Frequency of detection depends on the sensitivity of the detection method, the volume of water examined and the source of water examined. It reaches from 'a few oocysts in 100 liters of water to some more'.

Q: What is the typical recovery rate of oocysts from routine water samples.

A: Estimates range from 70 % to a lot less than that. Methods applied in University laboratories may have the highest recovery rate as QC (Quality Control) standards in these laboratories are probably higher than in commercial laboratories.

Q: Can (positive) routine water samples be analysed for the genotype of *Cryptosporidium* present.

A: (Chris Saint**)** No, we detect Cryptosporidium in low numbers in raw water samples. We often find nil detections in a duplicate sample taken at the same time. This means that if Cryptosporidium is confirmed by staining and microscopy in a sample it does not mean that the duplicate would give a positive PCR, as duplicates sometimes give nil detections. This creates problems for genotyping of environmental isolates. The ability to PCR material taken from slides previously confirmed by staining and microscopy is therefore important.

Q: What are the possibilities of investigating 'old' specimen samples. Suggestion: Genotyping of sewage can provide all the answers that we can obtain from specimen samples.

A: The potential of processing 'old' samples will be investigated. Analysing sewage samples is not the tool of choice as genotype composition can be influenced by factors beyond our knowledge and may severely distort the picture we get, ie may be totally unrelated to the 'true' distribution of genotypes.

Q: How can old slides be processed.

A: Jim McLauchlin gives a brief description of the method applied in his laboratory involving scraping the specimen off the slide prior to lysis and DNA isolation.

Q: What about the animals.

A: The issue is considered most important as a goal of this workshop. It is agreed upon that genotyping of *Cryptosporidium* spp. present in faecal samples needs to commence as soon as possible to provide a data base that can be compared with genotyping data obtained from clinical specimen.

David Roser from the CRC for Water Quality and Treatment offered support from a current project monitoring water quality in several catchments in Victoria, South Australia, Canberra and possibly in the Sydney area. Investigations of the CRC project include pathogen analysis (bacterial and protozoan) and a number of other factors such as presence of certain bacteriophages, turbidity etc.

Summary of Objective 5:

A diverse collection of strains from animal, human and environmental sources is needed in order to gain the maximum knowledge from genotyping studies. The following avenues for obtaining a wider range of such isolates were identified:

- Human sources Pathology laboratories should be requested to forward *Cryptosporidium*-positive faecal specimens to researchers after routine pathogen testing had been completed. Such specimens would normally be discarded unless the lab was participating in a research project. Dr Geoff Hogg of Melbourne University suggested that the cooperation of the Public Health Laboratory Network should be sought initially, and then commercial pathology labs should be approached. A suitable laboratory would need to be identified in each state to initially genotype specimens (type 1 and 2) and store them for future fingerprinting analysis.
- Environmental sources Research projects currently underway will provide some isolates - Dr Duncan Veal noted that the Sydney Catchment Authority had agreed to forward positive samples from their sampling program to him for genotyping, and Dr David Roser from the University of NSW undertook to seek similar cooperation from water authorities participating in the CRCWQT Pathogens in Catchments Project.
- Dr Chris Saint and Dr Paul Monis from the Australian Water Quality Centre agreed to carry out preliminary experiments to see if a reliable technique to recover oocyst DNA from stained slides could be developed. Such a technique would allow the genotyping of positive samples following routine testing by water authorities.
- Animal sources Participants with expertise in this area reported that tests for *Cryptosporidium* are rarely done in routine veterinary practice. Most isolates of this nature have to be obtained from specific research projects. Some Australian water authorities are supporting research programs to examine the carriage of *Cryptosporidium* in catchment animals.

APPENDIX 1: WSAA Strategic Workshop on Viability Testing and Genetic Typing of Cryptosporidium Oocysts Attendees

Attendee	Company
Dr Jerry Ongerth	University of New South Wales
Dr George DiGiovani	American Water Works Service Company
Dr Paul Monis	Australian Water Quality Centre
Dr Chris Saint	Australian Water Quality Centre, CRC for Water Quality Treatment
Dr Raj Shanker	AWT Ensight
Mr Tim Stinear	AWT Melbourne
Mr Simon Toze	CSIRO – Perth
Prof Robin Gasser	University of Melbourne
Dr Una Morgan	Murdoch University
Prof Andrew Thompson	Murdoch University
Dr Nick Sangster	University of Sydney
Ms Belinda Ferrari	Macquarie University
Ms Hayley Brown	Macquarie University
Dr Matthias Dorsch	Macquarie University
Ms Michelle Power	Macquarie University
Dr Duncan Veal	Macquarie University
Dr Melita Stevens	Melbourne Water Corporation
Dr Martha Sinclair	Monash Medical School
Mr Ian Smalls	NATA
Dr Jim McLauchlin	PHLS Food Safety Microbiology Laboratory, London
Dr Helen Stratton	Griffith University
Dr Rachel Chalmers	Swansea Public Health Laboratory
Dr Daniel Deere	Sydney Catchment Authority
Dr Judi Hansen	Sydney Water Corporation
Prof Geoff Hogg	University of Melbourne
Dr David Roser	University of New South Wales
Dr John Langford	Water Services Association *of Australia*