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Report on Nordic workshop on *Cryptosporidium* and *Giardia* in water and on vegetables/berries, Uppsala 26-27/9-13

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Summary

In 2012, the issue of an NMKL method for detection of *Cryptosporidium* and *Giardia* in water was raised and a referent and contact persons appointed. After the first contact, the group agreed that a workshop should be held with one or several additional experts present to discuss the matter. The workshop was consequently held in Uppsala in September 2013 with eight participants from five different countries. The outcome of the workshop was

- There is currently no need for a special NMKL method as there are several published standard methods that are used all over the world.
- The analytical capacity in the Nordic countries is sufficient in relation to the needs of today.
- In outbreak situations, it is possible to send samples to neighboring countries to enable analysis of a higher number of samples in a short time.
- The analytical capacity in the Nordic countries has increased since 2007.
- Meeting such as this are very useful for exchange of information and discussions.

Background

In 2004, the issue of an NMKL-method for detection of *Cryptosporidium* and *Giardia* in water was first raised. This resulted in a technical report "*Cryptosporidium* and *Giardia* in drinking water: Discussion document for selection and evaluation of an NMKL method" by Lucy Robertson, Norwegian School of Veterinary Science, Norway, and published in 2007. The document compared the situation in the Nordic countries to other parts of the world where validated standard techniques were implemented. At the time, three standard methods were available, US EPA 1622 (*Cryptosporidium* only) and 1623 (*Cryptosporidium* and *Giardia* simultaneously) and the UK (DWI) Method. It was concluded in that report that implementation of a proscribed NMKL method at that time was inappropriate. However, implementation of guidelines was recommended and stipulated inclusion of QA procedures.

Since then the standard methods available have been updated, and in 2006 the ISO-method 15553 "Water quality – Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water" was published. In 2012, the issue of an NMKL-method was once again raised and money from NMDD (Nordic Council of Ministers) granted for the work. However, the project group agreed that the methods present today are also appropriate to the of Nordic countries and that it is questionable whether a translation into a Nordic language would make them more accessible to new users. Therefore, a modified application was submitted to NMDD asking whether part of the grant could be used for a workshop for the project group and one or several invited experts. In early 2013, the application was granted and the workshop was held at Livsmedelsverket (National Food Agency) in Uppsala, Sweden, September 26-27.

The aims of the modified project were:
- To evaluate the need for an NMKL-procedure for detection of *Cryptosporidium* and *Giardia* in water.
- To compile and evaluate the Nordic analytical capacity in relation to the need today.
- If there was a need, translate the present standard methods, only available in English, to one or several Nordic languages, or, alternatively, provide support material to complement the existing methods.

Participants

NMKL-project group:
Karin Jacobsson (referent), National Food Agency, Sweden
Åsa Rosengren (co-referent), National Food Agency, Sweden (not present at workshop)
Lucy Robertson, Norwegian School of Veterinary Science, Norway
Eeva Klemettilä-Kirjavainen, MetropoliLab, Finland
Matthías Eydahl, Keldur Institute, Iceland
Jógvan Páll Fjallsbak, Faroese Food- and Veterinary Authority, Denmark (not present at workshop)

Others:
Rachel Chalmers, UK Cryptosporidium Reference Unit, Wales (invited expert)
Anette Hansen, Swedish Institute for Communicable Disease Control (SMI), Sweden
Karin Troell, National Veterinary Institute (SVA), Sweden
Jimmy Kjellén, National Food Agency, Sweden

Water

The situation in UK
Rachel Chalmers presented the situation in UK. In 1999, new water supply regulations stipulated continuous monitoring for *Cryptosporidium* in certain water supplies. The analyses performed should be of a quality that enabled use of the results in criminal courts, if required. In 2000/2001 came requirements for risk assessment for *Cryptosporidium* in all water treatment plants that should take into consideration the source water, catchment characteristics and the water treatment. Sites identified as "at significant risk" were then required to treat their water to ensure that less than 1 oocyst was present in 10 liters of finished drinking water, and this should be measured by continuous monitoring of a minimum of 40 liters per hour. Since 2007, there is no legislative demand for continuous monitoring but is still done by many plants as part of their risk assessment.
To meet the initial demand for analyses, training programs were initiated. Today there are 18 water laboratories in UK that are accredited for analysis of *Cryptosporidium* in water and an informal group of water testing personnel, *Cryptosporidium* Analyst Group (CRAG,) has been formed that meets to discuss, share information etc.

Summary of the methodology today
Today there are three main published standard methods;

- ISO 15553:2006(E) Water quality – Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water,
- US EPA 1623.1: *Cryptosporidium* and *Giardia* in water filtration/IMS/FA and
- UK Environment Agency: Drinking Water (2010) - Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts.

All three methods consist of the same three basic steps; 1) concentration, 2) isolation of (oo)cyst by immunomagnetic separation (IMS) and 3) detection and enumeration by fluorescence microscopy (immunofluorescent antiobody testing, IFAT). However, the methods differ regarding the concentration techniques that can be used, transportation and storage of samples, requirements for

quality controls etc. Some of the differences are summarized in table 1. For example, for concentration Method 1623.1 allows only capsule filtration and continuous flow centrifugation (unless laboratory equivalence can be demonstrated) while other two also contains protocols for membrane filtration and flocculation, thus offering cheaper alternatives for analysis of small volumes of water. The ISO-method contains the least detailed protocol while the UK method explains the different steps in detail, including precautions that should be taken, color pictures of what should be seen under the microscope (to a lesser extent also present in 1623.1) etc. An important difference is quality control where the ISO-method just states that "seeding and recovery tests using the combined concentration and separation method at regular intervals (e.g. 1 in 20) samples but a minimum of one per month" should be performed and gives examples of indicative performance data for various concentration techniques and IMS kits. US EPA 1623.1 on the other hand contains a detailed eight page section and some additional tables on quality control.

Food

The coming "ISO/DIS 18744 Microbiology of the food chain – Detection and identification of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruit" is mainly based on three papers by Cook *et al*; Cook, N., Paton, C. A., Wilkinson, N., Nichols, R. A., Barker, K. and Smith, H. V. 2006. Towards standard methods for the detection of *Cryptosporidium parvum* on lettuce and raspberries. Part 1: development and optimization of methods and Part 2: validation, International Journal of Food Microbiology 109: 215-21 and Cook, N., Nichols, R.A.B., Wilkinson, C.A., Paton,C.A., Barker, K. and Smith, H.V. 2007. Development of a method for detection of *Giardia duodenalis* cysts on lettuce and for simultaneous analysis of salad products for the presence of *Giardia* cysts and *Cryptosporidium* oocysts. Applied and Environmental Microbiology, 73: 7388-7391. The latter describes a method where (oo)cysts are extracted from salad products in a 1 M glycine buffert, pH 5.5, followed by centrifugation and immunomagnetic separation. Detection and quantification are by microscopy in the same manner as in the standard methods for detection in water. The authors suggest that ColorSeed (Texas Red-stained (oo)cyst preparation from BTF) could be included as a process control. In the study, recoveries of the process control varied between 3 and 70% for *Cryptosporidium* and 5 and 65% for *Giardia* in different food samples.

The method is currently at stage 40, enquiry stage. The same problems as with the water method can be foreseen when it comes to costs (consumables, especially if ColorSeed is included, and salaries) and the experience required for the microscopy. However, most likely less method specific equipment will be required. Considering the nature of the reagents used, e.g. monoclonal antibodies used both in the IMS step and for detection, and the limited number of suppliers, no big reduction in costs can be expected in the immediate future.

Table 1. A comparison of differences in the three standard methods available today

	ISO 15553 (2006)	UK Environment Agency (2010)	1623.1 (updated 2012)
Concentration	Filtration using: Pall Envirocheck™ STD Pall Envirocheck™ HV IDEXX Filta-Max® Membrane filtration ≤ 2 µm Calcium carbonate flocculation Iron (II) sulfate flocculation	Filtration using: Pall Envirocheck™ HV IDEXX Filta-Max® IDEXX Filta-Max xpress™ Flat membrane filtration 1-3 µm Calcium carbonate flocculation	Filtration using: Pall Envirocheck™ HV IDEXX Filta-Max® Portable Continuous flow centrifugation Other procedures and products may be used if demonstrated to a have an equivalent or superior performance in a multi-laboratory validation study.
Recommended volumes	10-1000 l depending on the purpose of the analysis	1-1000 l depending on water quality	10 or 50 l
Transportation	In the dark at ambient temperature but then stored at 5±3 °C	In the dark at ambient temperature but then stored at 5±3 °C	At 1-10 °C, preferably under monitoring. Store at 1-10 °C
Centrifugation speed and time	1100 x g for 15 minutes	1100 x g for 15 minutes	1500 x g for 15 minutes
IMS	Brands not specified. Used according to manufacturer´s recommendation. Suppliers listed	Brands not specified but procedure described.	Dynabeads GC-Combo
FITC-labelled antibodies	Not specified Suppliers listed	Not specified. Used according to the manufacturer´s instructions but also briefly described.	MeriFluor® Cryptosporidium/Giardia Aqua-Glo™ G/C Direct FL Crypt-a-Glo™ and Giardi-a-Glo™ EasyStain™
Quality Control	Water seeded with 100 (oo)cysts from commercially available flow cytometry flow sorted preparations or diluted stock. Examples of recoveries are given. Regular intervals (e.g. 1 in 20 samples with a minimum of one per month).	Recommendation given e.g. that recoveries should be determined for the full range of sample matrices examined routinely using the same volumes that are normally analyzed. All new materials and reagents used in (oo)cyst recovery should be verified before being used routinely.	Very extensive QC requirements for which the reader is referred to the method description (Table 2 with details in the text). Also states quality control criteria for several of the requirements.

Alternative detection techniques

None of the three methods offers any alternative to detection than IFAT. Techniques such as flow cytometry, microarrays and qPCR have been tested in several publications, but results are variable and are not yet considered sensitive and selective enough to replace IFAT in analysis of water samples where, also after concentration, few or even single (oo)cysts must be detected. Detection by microscopy has the additional advantage of identifying also (oo)cysts lacking DNA. This is important, especially in drinking water, where identification of empty (oo)cysts tells the water producer that particles of this size have passed through the purification steps and thus, viable *Cryptosporidium* and *Giardia* may be present in the water.

Molecular techniques for species identification of *Cryptosporidium* isolates remain important. Species identification is normally done by removing the (oo)cysts from the slide, lysis of the cells followed by PCR. In 2010, Water research Foundation published a method for genotyping from microscope slides (web report #4099) that distinguish human-pathogenic species (*C. hominis*, *C. parvum* and *C. meleagridis*) from those associated with animals. The differentiation is made by a single-round multiplex PCR that targets the heat shock protein 70 (hsp70) and 18S rRNA-genes. Either conventional or real-time PCR can be used, but an instrument that enables high resolution melting (HRM) is recommended for full resolution. The method was only tested on single (oo)cysts while in practice, several (oo)cysts of different species may be present on a slide which may make the results difficult to interpret. When using (oo)cysts from slides for molecular typing, it is important not to use mounting medium not containing formalin as formalin interferes with the PCR-reaction causing false negative results.

The analytic capacity today

In the report from 2007, information on the analytic capacity was compiled. At the time there were four laboratories, two in Norway and one in Finland and Sweden respectively, that analysed *Cryptospordium* and *Giardia* in water. In connection to workshop, questionnaires were sent to the laboratories known or believed to perform the analysis but were few answered except from those represented at the workshop. Laboratories known to perform the analysis today are shown in Table 2. However, details are not available from Alcontrol and Eurofins in Sweden, both of which accept samples and forward them to their British branches for analysis. According to their webpage, Analysesenteret in Trondheim, Norway, analyse according to US EPA 1623 but this analysis is not on their list of accredited analyses. In 2007, University of Helsinki performed the analysis but has not replied, so the current situation is unclear.

As shown in Table 2, recoveries vary between laboratories as most state that it varies depending on water quality, it is difficult to draw any conclusions from this. As all participate, or will participate in the FAPAS Leap external QC scheme, it may be possible to compare the results in the future.

Table 2. Laboratories known to analyse *Cryptosporidum* and *Giardia* in water in November 2013

	Finland		**Norway**		**Sweden**			
Labs that analyse water for Crypto-sporidium and Giardia?	THL*	Metropolilab	NVH	Analyse-senteret - Information not available	SMI	NFA	Alcontrol – Information not available	Eurofins - Information not available
Accreditations	No	No	Yes		Yes	No		
Approximately how many samples per year?	Two so far	10	Ca. 70 (mostly raw water), also concentrates or slides from other labs for checking		30-40 drinking water and 210 raw water	None so far		
Concentration techniques used?	Filtration – Envirochek HV	Filtration - membrane filter	Membrane filtration		Filtration – Envirochek HV or membrane filtration	Filtration – Envirochek HV		
How often are spiked controls run? Spikes made in lab or purchased? What are the recovery efficiencies	At least once Purchased 50-60%	Appr. once a year EasySeed 10-50%	6 times per year EasySeed 50-70 % (depends on water quality)		Every second month EasySeed 40-80% depending on water quality	Every second month EasySeed 20-80% depending on water quality		
For how long do you store the slides?	At least 6 months	At least some months	6 months		Do not store	Not relevant yet		
What mounting medium do you use? With or without formalin?	EasyStain kit	Crypto CEL and Giardia CEL/Cellabs, without	AquaGlo standard, M101-No Fade With formalin		Citifluor Without formalin	AquaGlo standard M101-No Fade With formalin		

		formalin						
Is there participation in external QC schemes?	FAPAS Leap from 2014	FAPAS Leap once a year since 2010	FAPAS Leap, annually		FAPAS Leap 3-4 times per year	FAPAS Leap once a year		
Cost?	€ 480	€ 500	4250 Nkr (approx. €520)		5700 kr (appr € 640)	-		
Turn-around-time?	1,5 working days	2-3 working days	1 working day		Normally one week but 1-3 days when required	2 working days		
Do you foresee any changes in the future?	May change to using co-concentration of viruses and bacteria and use molecular detection	More spiked samples per year	Probably – but hard to second guess what the changes may be…..					

*THL = National Institute for Health and Welfare, Finland

Conclusions from the workshop

- There is currently no need for an NMKL-version of the standard methods available today. Supportive material can be found in "UK Environment Agency: Drinking Water (2010) - Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts " that is available on http://www.environment-agency.gov.uk/research/commercial/32874.aspx free of charge .

- Today the analytical capacity is sufficient in the Nordic countries. To ensure that competence is maintained in any laboratory, samples must be analyzed on a regular basis. This in turn requires that enough samples are available for analysis, i. e. there is request from drinking water producers etc. for having the analysis performed. Therefore, too many labs may be counterproductive if not enough samples are available to maintain individual laboratory competence. However, the situation may change for example following changes in legislation or recommendations in one or several Nordic countries, and then the capacity in relation to the need will have to be reevaluated.

- The lack of laboratories doing the analysis in countries such as Iceland and Faroe Islands could be solved by sending samples abroad. It is also possible to perform the initial steps of the analysis on-site and send the slides to a laboratory with more qualified personnel for identification of (oo)cysts.

- In an outbreak situation where many samples have to be analyzed, the representatives in NMKL-group agreed on supporting each other, as has been done on previous occasions.

- All participants agreed that regular meetings and workshops, such as the one in Uppsala, are invaluable for discussing problems and obtaining insights from labs in similar positions. Meetings or workshops at regular intervals would mean that all countries could be kept up-to-date with advances in this field, and have a good opportunity to exchange information and ideas. One issue that needs to be discussed is the need for quality controls other than analysis of spiked samples and participation on external quality schemes.