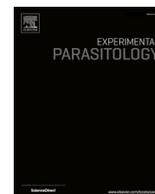




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Cryptosporidium genotyping in Europe: The current status and processes for a harmonised multi-locus genotyping scheme[☆]

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ARTICLE INFO

Keywords:

Cryptosporidium
Multi-locus genotyping
Scheme
Subtyping
Outbreak

ABSTRACT

Due to the occurrence of genetic recombination, a reliable and discriminatory method to genotype *Cryptosporidium* isolates at the intra-species level requires the analysis of multiple loci, but a standardised scheme is not currently available. A workshop was held at the Robert Koch Institute, Berlin in 2016 that gathered 23 scientists with appropriate expertise (in either *Cryptosporidium* genotyping and/or surveillance, epidemiology or outbreaks) to discuss the processes for the development of a robust, standardised, multi-locus genotyping (MLG) scheme and propose an approach. The background evidence and main conclusions were outlined in a previously published report; the objectives of this further report are to describe 1) the current use of *Cryptosporidium* genotyping, 2) the elicitation and synthesis of the participants' opinions, and 3) the agreed processes and criteria for the development, evaluation and validation of a standardised MLG scheme for *Cryptosporidium* surveillance and outbreak investigations. *Cryptosporidium* was characterised to the species level in 7/12 (58%) participating European countries, mostly for human outbreak investigations. Further genotyping was mostly by sequencing the *gp60* gene. A ranking exercise of performance and convenience criteria found that portability, biological robustness, typeability, and discriminatory power were considered by participants as the most important attributes in developing a multilocus scheme. The major barrier to implementation was lack of funding. A structured process for marker identification, evaluation, validation, implementation, and maintenance was proposed and outlined for application to *Cryptosporidium*, with prioritisation of *Cryptosporidium parvum* to support investigation of transmission in Europe.

1. Introduction

Gastrointestinal infections with the protozoan *Cryptosporidium* have clinical, public health, socio-economic, industrial and agricultural impacts of global importance (Korpe and Bartelt, 2015; Santín, 2013). The oocysts can be transmitted directly through the faecal-oral route, and through contaminated food and water (Efstratiou et al., 2017; Robertson and Chalmers, 2013). Two species cause most human cases of cryptosporidiosis: *Cryptosporidium hominis*, which is transmitted anthroponotically, and *Cryptosporidium parvum*, which is zoonotic with a wide host range (Cacciò and Putignani, 2014). Traditional testing and

diagnostics identify the genus (Chalmers and Katzer, 2013), but species discrimination requires molecular assays, for which sequencing part of the small subunit ribosomal RNA (*SSU rRNA* or *18S*) gene provides the benchmark but is rarely undertaken routinely (Xiao, 2010). In 2014, the European epidemiological report on food- and waterborne diseases and zoonoses identified a “need to better understand the epidemiology of cryptosporidiosis in the EU/EEA through increased laboratory testing and speciation/sub-typing of isolates” (European Centre for Disease Prevention and Control, 2014). Sequencing part of the highly polymorphic 60 kDa glycoprotein (*gp60*) gene has been used for intra-species characterisation, but multi-locus genotyping (MLG) would be much

Abbreviations: MLG, multilocus genotyping; *gp60* gene, 60 kDa glycoprotein gene; *SSU rRNA* or *18S* gene, small subunit ribosomal RNA gene

[☆] Authors are those who organised the workshop and were involved in writing; all other participants are mentioned on behalf of the consortium.

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<https://doi.org/10.1016/j.exppara.2018.06.004>

Received 16 February 2018; Received in revised form 11 May 2018; Accepted 12 June 2018

Available online 13 June 2018

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more informative, given the sexual phase of the *Cryptosporidium* life-cycle that enables recombination of genetically dissimilar haplotypes (Widmer and Lee, 2010). However, there has been no international adoption of a standardised MLG scheme (Chalmers and Cacciò, 2016).

A variety of genetic loci, mainly containing microsatellite and minisatellite repeats (also known as variable-number tandem-repeats, VNTR) has been investigated, either by fragment sizing or sequence analysis; a systematic review published in 2012 found 55 VNTR loci used in varying combinations (Robinson and Chalmers, 2012). However, the rationale for the selection of loci used in most studies has not been explained. Furthermore, assessment of nine loci (Chalmers et al., 2016) against key criteria for the selection of VNTR loci (Nadon et al., 2013) revealed that only one was compliant, and may explain the poor correlation of MLGs identified by fragment sizing with sequencing that has been reported (Widmer and Cacciò, 2015). A set of new VNTR loci have subsequently been selected from *C. parvum* whole genome sequences using the Nadon criteria and identified as suitable for evaluation *in vitro* (Pérez-Cordón et al., 2016).

Guidelines for the evaluation and validation of bacterial typing schemes have been published (Van Belkum et al., 2007), and although these are also relevant to *Cryptosporidium*, reports are few. Hotchkiss and colleagues evaluated a multi-locus fragment typing (MLFT) tool for *C. parvum* by application to 140 bovine-derived samples from the United Kingdom (UK) (Hotchkiss et al., 2015). They reported that, using six loci, typeability was 84%, specificity was 100%, discriminatory power calculated by Simpson's Index of Diversity was 0.92, the allelic allocation was repeatable and reproducible, and the MLG results comparable with that obtained by sequencing. However, two loci were found to be mono-allelic among the bovine-derived sample set (Hotchkiss et al., 2015), whereas one of these loci was poly-allelic in a set of 14 human-derived *C. parvum* samples (Chalmers et al., 2016).

One of the conclusions of a workshop on *Cryptosporidium* genotyping held in Berlin in June 2016 was that “a robust, standardised, multi-locus genotyping scheme should be developed, using a defined process to replace or supplement the multitude of genotyping methods used” (Chalmers and Cacciò, 2016). The objectives of this further report are to describe, in the context of surveillance and outbreak investigations, 1) the current use of *Cryptosporidium* genotyping in the European countries represented, 2) the elicitation and synthesis of participants' assessment and opinions of *Cryptosporidium* genotyping, and 3) how agreed processes for the development, evaluation and validation of MLG schemes can be applied to this parasite. In addition, perceived barriers to the implementation of such a scheme were identified.

2. Methods

2.1. Participation and *Cryptosporidium* genotyping

The workshop was conducted as part of the COST Action “A European Network for Foodborne Parasites (Euro-FBP; FA1408)”, a network to promote collaboration between scientists working on foodborne parasites in Europe (<http://www.euro-fbp.org/>; http://www.cost.eu/COST_Actions/fa/FA1408). Participant selection was in two stages: first, by submitting a curriculum vitae and an application form demonstrating knowledge of foodborne parasites through COST Action national coordinators; secondly, by applying to join the activity through the leader of the “analytical and diagnostic methods” working group, indicating their knowledge of *Cryptosporidium* genotyping and/or surveillance, epidemiology or outbreaks. Specific invitation was extended to relevant, active professionals in different regions of Europe where applications were lacking. The processes were considered sufficiently robust to assure participation of knowledgeable specialists only. For financial reasons, numbers were limited to < 25. An external expert from the United States (US) was invited to contribute to the discussions.

The workshop participants' focus of work and opinions on the need for, future direction of, and barriers to *Cryptosporidium* genotyping were

elicited ahead of the workshop through questionnaires administered online (<https://www.surveymonkey.com>) in May 2016. This was conducted first at an individual level (<https://www.surveymonkey.com>) and, then through a selected representative of each participating European country, at a country-level (<https://www.surveymonkey.com>). The closing date for completion of the questionnaires was 6th June 2016.

2.2. Multi-attribute assessment

The first questionnaire asked about the individual participant's workplace, and application of *Cryptosporidium* genotyping, and included a multi-attribute assessment ranking exercise of performance and convenience criteria for an MLG scheme (Van Belkum et al., 2007). The nine attributes investigated were (in alphabetical order): biological robustness, cost of consumables, discriminatory power, hands-on time, level of staff expertise, portability, specialist equipment needed, turn-around time and typeability. The participants ranked each attribute using an ordinal, linear ranking scale (1 was considered the least important and 9 the most important attribute). The second questionnaire asked about the status of *Cryptosporidium* genotyping in each country, barriers to implementation, and potential mechanisms for adoption of such a scheme.

2.3. Process for evaluation, validation, and implementation of a harmonised multi-locus genotyping scheme

At the workshop, the results of the questionnaires were presented and used alongside the outcomes of the discussions in four working groups concerning the development, implementation, and maintenance of suitable genotyping resources for *Cryptosporidium* that have been summarised and reported previously (Chalmers and Cacciò, 2016). Here, these are synthesised into a proposed process for evaluation, validation, implementation, and maintenance of a harmonised MLG scheme for *Cryptosporidium*.

3. Results and discussion

3.1. Participants

The workshop provided for the first time a structured assessment of the status and a process for the development of *Cryptosporidium* MLG in Europe for surveillance and outbreak investigations. A total of 23 participants attended from 17 organisations in 12 European countries (Table 1) and the USA. Ideally, professional opinions from all European countries would have been obtained, but participation was limited by a combination of restricted budget and, for some countries, a lack of available, relevant expertise. This has been addressed to some extent by this COST Action through the provision of a training school that included *Cryptosporidium* genotyping in Lisbon, Portugal in September 2017. Further training will be provided through planned activities including webinars and training schools.

The response rate to the individual-level questionnaire, administered to the 22 European participants was 100%. These participants were mainly from health organisations (n = 14, 64%), universities (n = 4, 18%), research institutes (n = 3, 14%) and one federal risk assessment institution (5%). The main focus of the majority of participants was human and public health (n = 16, 73%), animal health (n = 4, 18%) or food, water and environmental testing (n = 2, 9%). With regard to *Cryptosporidium* genotyping, 19 participants were currently active, mostly for human epidemiology (n = 16) and/or animal (n = 14) testing, but fewer participants genotyped food, water and environmental samples (n = 10). Four participants also tested samples for external quality assurance (as part of an informal scheme, in the absence of any formal scheme) and to maintain competency. The joint activity for investigation of human and animal samples was

Table 1
Cryptosporidium genotyping activities for human and animal surveillance and outbreak investigations in twelve European countries, 2016.

Country	Humans				Animals	
	Location and samples tested for species identification	Methods used for species identification	Samples that are genotyped	Methods used for genotyping	Samples tested for species and genotype identification	Methods used for species identification; genotyping
Czech Republic	Not done routinely				Not done routinely	
Denmark	Not done routinely				Not done routinely	
France	Funded centrally. Done in sentinel laboratories	18S sequencing	Sentinel and outbreak cases	gp60 sequencing and other microsatellites	Not done routinely	
Germany	Funded centrally. Done in a reference laboratory on outbreak cases	18S sequencing	Outbreak cases	gp60 sequencing	Not done routinely	
Hungary	Not done routinely				Not done routinely	
Italy	Not done routinely				Not done routinely	
The Netherlands	Funded centrally. Done in a reference laboratory on samples from sentinel laboratories and outbreak cases	Real-time PCR with <i>C. parvum</i> (Lib13) and <i>C. hominis</i> (gp60) primers and probes	A selection of sentinel and all outbreak cases	gp60 sequencing	Yes, done on samples related to human outbreaks	gp60 sequencing
Norway	Funded centrally. Done in a reference laboratory on outbreak cases	18S sequencing	Outbreak cases	gp60 sequencing	Yes, done on samples related to human outbreaks	18S and actin gene sequencing; gp60 sequencing
Slovenia	Not done routinely				Not done routinely	
Spain	Funded centrally. Done in reference centres on outbreak cases	18S sequencing	Outbreak cases are subtyped	gp60 sequencing	Not routinely tested for <i>Cryptosporidium</i>	
Sweden	Funded centrally. Done in reference laboratories on samples from sentinel laboratories and outbreak cases	18S sequencing	Sentinel and outbreak cases	gp60 sequencing (also investigation of actin, hsp70, and cowp genes, and a new multi locus system)	Yes, done on samples related to human outbreaks	18S sequencing; gp60 sequencing (also investigation of actin, hsp70, and cowp genes, and a new multi locus system)
UK	Funded centrally. Done in a reference laboratory on samples from most cases in England and Wales, sentinel laboratory samples and outbreak cases in Scotland; outbreak cases only in Northern Ireland	Real-time PCR with <i>C. parvum</i> (Lib13) and <i>C. hominis</i> (A135) primers and probes (England, Wales and Northern Ireland); gp60 sequencing (Scotland); 18S sequencing (UK)	Sentinel and outbreak cases	gp60 sequencing	Yes, done on samples related to human outbreaks	18S sequencing and <i>C. parvum</i> real-time PCR; gp60 sequencing

encouraging and facilitated harmonisation of methods for human, animal, and environmental samples (Table 1), but there was relatively little investigation of food and water samples. Preparation of these samples for genotyping is time consuming and expensive, and not always fruitful (Smith and Nichols, 2010), but can be important in outbreak investigations.

3.2. *Cryptosporidium* genotyping for surveillance and outbreak investigations in Europe

The response rate to the country-level questionnaire was 12/12 (100%) (Table 1). *Cryptosporidium* isolates were characterised to the species level from human samples, mostly for outbreak investigations in 7 (58%) participating European countries (France, Germany, The Netherlands, Norway, Spain, Sweden, UK). Further genotyping was also undertaken on samples from sentinel cases and/or outbreak cases in these countries. Although genotyping *Cryptosporidium* from animals was widespread among participants for research purposes, it was not part of surveillance activities. Four participating countries included genotyping to support the investigation of human outbreaks linked to animals (The Netherlands, Norway, Sweden, and UK) (Table 1).

A total of six countries reported monitoring programmes for *Cryptosporidium* in drinking water (Germany, Hungary, The Netherlands, Norway, Slovenia, and UK) and species identification was undertaken regularly in three of these (Hungary, Norway, and UK) and also in France and Sweden if monitoring was initiated as part of an outbreak response. None of the participating countries had monitoring programmes for *Cryptosporidium* in food, but sampling and genotyping were attempted in Norway and Sweden as part of an outbreak response (Table 1).

The method used for species identification, whatever the sample source, was predominantly sequencing the 18S gene, although one participant used restriction fragment length polymorphisms (Table 1). In two countries, UK and The Netherlands, real-time PCRs had been adopted to test human samples specifically for *C. parvum* and *C. hominis* (Hadfield et al., 2011; Roelfsema et al., 2016). One variation within the UK was the use of *gp60* sequencing for species identification in Scotland. Even where these alternative methods were used, sequencing of the full-length or partial 18S gene was also used to confirm referred *Cryptosporidium*-positive samples not identifiable by the targeted assays. Further genotyping for surveillance and outbreak investigations was undertaken almost exclusively by *gp60* sequencing. Only in Sweden was MLG used, based on sequence analysis of seven loci for single nucleotide polymorphisms (SNPs) (Karin Troell, personal communication). Where samples from food and/or water were investigated, this was also by *gp60* sequencing.

A greater variety of genotyping approaches were used by individual participants for research purposes. At eight of the 17 participating organisations, human and/or animal samples were genotyped using: 1) SNP analysis of seven markers; 2) MLFT of seven loci; 3) *gp60* sequencing and MLFT of more than 12 loci including some newly identified; 4) *gp60* sequencing and other loci (unspecified numbers); 5) various unstated methods; 6) MLFT of seven loci; 7) sequencing *gp60* and occasionally three other loci; 8) sequencing nine loci. None of the participants reported using whole genome sequencing (WGS) routinely, although some participants were actively developing processes for this. Upstream processing is laborious and appropriate pipelines have yet to be validated for public health purposes. Even so, as the costs of WGS continue to decrease, it is becoming increasingly available to routine diagnostic laboratories for investigation of other pathogens. However, during outbreak investigations, rapid results are essential in order to apply appropriate interventions, and a fragment analysis method may provide the most rapid assessment of relationships between *Cryptosporidium* isolates (Nichols et al., 2014).

All countries identified at least one barrier to including *Cryptosporidium* genotyping routinely in surveillance and outbreak

investigations. The barriers were: lack of funding (5 countries); the low prevalence or lack of reported outbreaks and therefore low level of knowledge, interest or awareness of *Cryptosporidium* (4 countries); lack of expertise (2 countries); lack of standardised procedures (2 countries); lack of regulations or requirements for testing (2 countries); lack of communication between laboratories (2 countries); lack of surveillance for the parasite (1 country); different administration systems for testing and surveillance (1 country). Only two countries considered they had an official mechanism for the promulgation of a genotyping programme, through a microbiology Standards Group or association.

Just over half of the countries represented at the workshop had a structured process to genotype isolates from cases for surveillance purposes, but this was usually done for outbreaks. *Cryptosporidium* is investigated and reported variably; it was reported by the European Centre for Disease Control that in 2014, of the 23 EU/EEA countries included, 15 reported 0–10 cases and nine countries did not report any data at all (ECDC, 2018). Two of the most commonly cited reasons for not including *Cryptosporidium* genotyping routinely in surveillance and outbreak investigations were the low prevalence or lack of reported outbreaks and lack of awareness of *Cryptosporidium*, which undoubtedly impacted the other most common reason, lack of funding.

3.3. Multi-attribute assessment

There was no obvious association between scores and participants' organisation type or focus of work (data not shown). The most important features of a MLG scheme identified by the multi-attribute scores were portability, biological robustness, typeability and discriminatory power (Table 2). Nevertheless, despite the variety of multi-locus tools being used by participants and reported in the literature, these attributes have been little investigated. One of the outcomes of the workshop was a structured process for this (Fig. 1), and existing markers could also be re-evaluated within this framework. Although cost was ranked lower in importance (Table 2), the lack of funding was one of the three most commonly cited barriers to implementation.

3.4. Process for evaluation, validation, and implementation of a harmonised multi-locus genotyping scheme

The participants agreed that a standardised MLG scheme for zoonotic *C. parvum* would support a “One Health” approach for the surveillance and investigation of this zoonotic parasite, providing the capability to compare across Europe. Following the discussions of the four working groups during the workshop as described previously (Chalmers and Caccio, 2016), a process was proposed (Fig. 1) whereby the identification and selection of markers, evaluation, validation, implementation, and sustenance of a developing MLG scheme would follow published criteria (Van Belkum et al., 2007; Nadon et al., 2013)

Table 2

Ranking of key performance and convenience attributes by workshop participants for developing a multi-locus genotyping scheme for *Cryptosporidium* (scale 1–9, least to most important).

Attribute	Ranked scores range; mode	Median ranked score
Specialist equipment and infrastructure needed	1 to 5; 1	3
Hands on time	1 to 7; 1	3
Cost of consumables	1 to 7; 3	3
Level of staff expertise	1 to 7; 3	3
Turnaround time	1 to 7; 2	4
Portability (for standardisation and comparability)	3 to 9; 6	6
Biological robustness	1 to 9; 7	7
Typeability (proportion of samples typed)	6 to 9; 7	8
Discriminatory power	6 to 9; 8.5	8

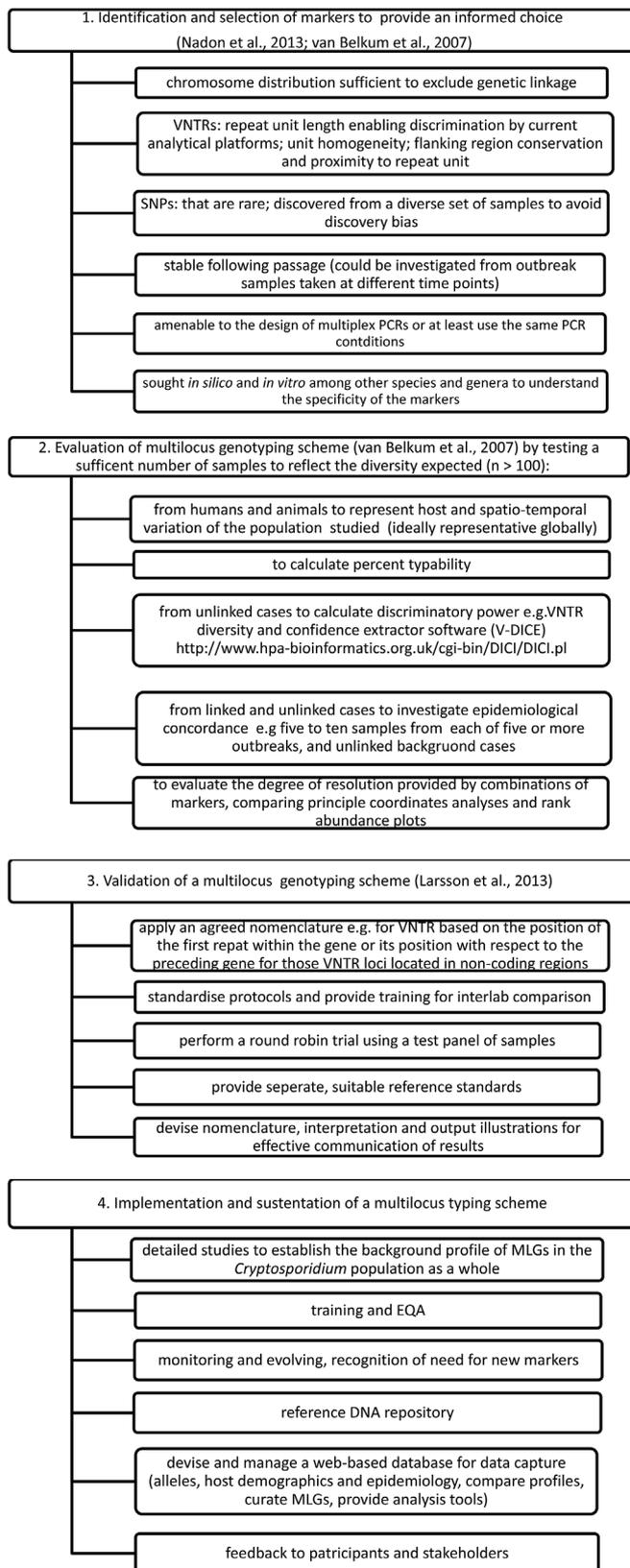


Fig. 1. Proposed process for the identification, evaluation and validation of markers for a multi-locus genotyping scheme.

and be supplemented by *post hoc* pairwise investigation of loci for both genetic linkage disequilibrium and exploration of the genetic dissimilarities by a principle coordinate analysis (PCoA) (Widmer and Lee, 2010). Multivariate methods such as PCoA have advantages over

classical clustering methods, such as Bayesian clustering, as the former do not require strong assumptions about an underlying genetic model, such as the absence of linkage disequilibrium (Jombart et al., 2009). In addition, the use of rank abundance plots would enable visualisation of MLG abundance distributions and establish how informative are individual, or combinations of, markers, thereby allowing evaluation of the economic efficiency of a developing scheme. The process could be used for the review of existing and new markers.

The nature of the loci and analysis was debated. Fragment sizing could be done in more laboratories relatively cheaply compared with sequencing, supporting the adoption of a VNTR approach. If VNTR markers are chosen carefully (Fig. 1, step 1), the MLG outcome could be comparable to sequencing (Nadon et al., 2013; Chalmers et al., 2016). Participants with in-house sequencing facilities favoured adopting sequence analysis of fragments or of SNPs, but this is expensive if a commercial provider needs to be used for multiple loci, whereas the cost of fragment sizing is much cheaper. However, there is the potential for SNP analysis to be undertaken by alternative methods, such as mini-sequencing, real-time PCR melt curve analysis, or even commercialised in microarrays, although this is unlikely while the market remains small. Either way, evaluation and validation are still needed (Fig. 1, steps 2 and 3), and schemes should be compared on the same sets of samples (Fig. 1, step 3). The use of sequenced reference standards has been shown to enable reproducible allelic allocation across different analytical platforms (Hotchkiss et al., 2015; Chalmers et al., 2016). Prior consideration of implementation strategies, such as streamlining workflow by designing primers and PCRs to use same PCR conditions, and using differently labelled primers to enable multiplexed PCR reactions were identified as being of value.

The continued role of *gp60* sequencing was discussed. However, the use of this marker has become embedded in custom and practice, so it would be hard to exclude (Xiao, 2010). In addition, although *gp60* might be useful as an epidemiological marker, it should be subjected to the same evaluation as other markers, indeed, the locus does not meet all of the selection criteria for fragment sizing (Nadon et al., 2013; Chalmers et al., 2016). If maintained, the sequence could be converted to numerical coding to feed into MLG analysis. Sequencing of a single additional marker may not be satisfactory in the MLFT laboratory workflow. The human and technical resources required for MLFT are no greater than for *gp60* sequencing, and multiple loci can be fragment-sized on commercial platforms for less than the cost of bi-directional sequencing of a single locus.

The importance of correct coding for null alleles in a MLG scheme was discussed; software should code as a real allele rather than dropping that marker from the dataset (Van Oosterhout et al., 2004). Nomenclature would need to be robust across different coding platforms and communicate the predicted relationships among isolates (Fig. 1, steps 3 and 4). Any nomenclature must allow for good communication with non-specialists, and here a visual approach was suggested, such as the minimum spanning tree or minimum spanning network (Salipante and Hall, 2011), based on the same principles as the eBURST algorithm (<http://eburst.mlst.net/>).

Interpretation of VNTRs in evolutionary terms remains uncertain; can it be assumed that repeat numbers evolve stepwise/incrementally? Is an isolate with five copies of a repeat more closely related to an isolate with four copies than one with three? If so, then distance methods that take the repeat number into account can be applied. The development of *in vitro* systems for continuous propagation of *Cryptosporidium* might provide the means to investigate parasite evolution in the laboratory (DeCicco RePass et al., 2017).

The workshop participants agreed that the next steps are to seek funding to validate, implement, and maintain an MLG scheme; further discussions are needed to establish whether delivery is most appropriate regionally, nationally or at a centralized European facility. Although technological advances such as whole genome sequencing are being implemented for the surveillance of some pathogens, establishing

a harmonised MLG scheme for *Cryptosporidium* will launch the collaborative working to take forward developments as they become realistic. As identified in the previous report, greater diversity has been identified in *C. parvum* than *C. hominis* among cryptosporidiosis patients in Europe and, should the development of a standardised multi-locus genotyping scheme for surveillance and outbreaks be prioritised in this region, then it ought to focus initially on *C. parvum* (Chalmers and Cacciò, 2016).

Declarations of interest

None.

Acknowledgements

This work is based on activities within the framework of the COST Action FA1408

“A European Network for Foodborne Parasites: Euro-FBP”. We acknowledge all members of

this COST Action for their contributions, and especially the *Cryptosporidium* genotyping workshop participants:

Anton Aebischer, Christian Klotz; Unit for Mycotic and Parasitic Agents and Mycobacteria, Robert Koch Institute, 13353 Berlin, Germany.

Claire L. Alexander, Lisa Connelly; Scottish Parasite Diagnostic and Reference Laboratory (SPDRL), Glasgow, UK.

Simone M. Cacciò; European Union Reference Laboratory for Parasites, Istituto Superiore di Sanità, Rome, Italy.

David Carmena; Parasitology Service, National Centre for Microbiology, Majadahonda, Madrid, Spain.

Rachel Chalmers, Guy Robinson, Gregorio Pérez-Cordón; National *Cryptosporidium* Reference Unit, Public Health Wales, Swansea SA2 8QA, UK.

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Laetitia L Kortbeek; Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

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Judit Plutzer; Department of Water Hygiene, National Public Health Centre, Budapest, Hungary.

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C. Rune Stensvold; Statens Serum Institut, Copenhagen, Denmark.

Egbert Tannich; Department of Molecular Parasitology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany.

Karin Troell; National Veterinary Institute, Uppsala, Sweden.

Giovanni Widmer; Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine at Tufts University, North Grafton, MA, USA.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.exppara.2018.06.004>.

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