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

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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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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 standarised 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, turnaround 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