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MULTILOCUS SEQUENCE TYPING AS A USEFUL TOOL FOR THE STUDY OF THE GENETIC DIVERSITY AND POPULATION STRUCTURE OF *CRYPTOSPORIDIUM* SPP.

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ABSTRACT

One of the most important aquatic parasites in industrialized countries, *Cryptosporidium* spp., is a major cause of diarrheal disease in humans and animals worldwide. The contingent evolution of cryptosporidia with hosts, host adaptation, and geographic variation contributed to the creation of species subtypes, thereby shaping their population genetic structures. Multilocus typing tools for population genetic characterizations of transmission dynamics and delineation of mechanisms for the emergence of virulent subtypes have played an important role in improving our understanding of the transmission of this parasite. However, to better understand the significance of different subtypes with clinical disease manifestations and transmission risks, a large number of samples and preferably from different geographical areas need to be analyzed. This review provides an analysis of genetic variation through multilocus sequence typing, provides an overview of subtypes, typing gene markers for *Cryptosporidium parvum, Cryptosporidium hominis, Cryptosporidium muris* and *Cryptosporidium andersoni* genotypes and an overview of the hosts of these parasites.

Key words: *Cryptosporidium*; Multilocus Sequence Typing MLST; PCR

INTRODUCTION

Cryptosporidium spp. belongs to protozoan pathogens that infect the gastrointestinal epithelium of a wide range of vertebrates, including humans. Globally, the average prevalence of *Cryptosporidium* is thought to be ~7.6 %, with rates as high as 4.3 % in developed countries and 10.4 % in developing countries. Some areas report a prevalence of this parasite as high as 69.6 % [14]. In immunocompromised individuals, *Cryptosporidium* spp. are responsible for severe diarrhoea, stunted growth, and cognitive impairment in children in developing countries. It has recently been associated with colon cancer [35, 37, 48]. In infectious stages, oocysts of *Cryptosporidium* spp. are transmitted through the faecal-oral route and are highly resistant to disinfection. For this reason, cryptosporidia are considered the causative agents of waterborne and foodborne diseases [15, 65]. The only drug approved for the treatment of cryptosporidiosis in humans is nitazoxanide. However, treatment failure is common in diseases caused by *Cryptosporidium* spp., and no vaccines are available [6].

Clear and unambiguous descriptions of species are essential for understanding their epidemiology, pathogenicity and the development of effective therapeutics [17]. We currently know that most *Cryptosporidium* spp. are not host-specific. However, the oocysts of many *Crypto-*

sporidium spp. are identical or overlap in size and lack other distinguishing features [16]. Molecular tools are needed to understand taxonomy and epidemiology [61]. In recent years, many new species and genotypes of *Cryptosporidium* spp. have been confirmed, and many *Cryptosporidium* spp. were reclassified based on morphological and biological data.

Cryptosporidium is one of more than 300 genera in the phylum Apicomplexa, which contains more than 6000 species [44]. For a long time, *Cryptosporidium* was classified as a coccidia, a parasite of the order Eucoccidiidae, based on the similarity of its life cycle with another enteric coccidia [29]. However, *Cryptosporidium* was later speculated to be closely related to gregarines, that are parasites which form a diverse and primitive group that mostly parasitizes invertebrates. This similarity has been described based on molecular and biological similarities, including the presence of a "feeding" organelle in developmental stages, based on the absence of sporocysts and, last but not least, based on epicellular localization in host cells [8, 20, 28, 46]. The whole genome sequencing (WGS) of the gregarine, *Ascogregarina taiwanensis*, supported a monophyletic relationship between the gregarine and cryptosporidia but demonstrated significant differences in the metabolic capacity of the cryptosporidia [2]. In 2014, based on 18S ribosomal data, Cavalier-Smith revised the taxonomy, transferred *Cryptosporidium* spp. from coccidia to gregarines, and created a new subclass, called the *Cryptogregaria* within the class *Gregarinomorphea* [9]. In 2019, Adl et al. modified the class to the subclass *Cryptogregarinorida* within *Gregarinasina* [3]. Transcriptomic analysis of a broader spectrum of apicomplexan parasites suggests that *Cryptosporidium* represents a distinct lineage from gregarines [24, 34, 47] and further taxonomic revision is needed.

Before 1971, there were only three valid species within the genus *Cryptosporidium*: *Cryptosporidium muris* [53], *Cryptosporidium parvum* [54] and *Cryptosporidium meleagridis* [49]. Greater interest did not emerge until 1993, when a massive waterborne outbreak affected more than 400,000 residents of Milwaukee, Wisconsin [31]. In the mid-1990s, molecular detection and identification methods appeared, which became the basis for taxonomic identification and epidemiological studies. Interest in cryptosporidiosis has resurfaced after the Global Enteric Multicenter Study (GEMS) of more than 22,500 children in Africa and Asia, conducted from December 1, 2007, to March 3, 2011, revealed that *Cryptosporidium* is the second most crucial diarrheal pathogen after rotavirus in children up to 5 years of age [27]. There are currently at least 44 verified species of *Cryptosporidium* (many of which were initially identified as genotypes and later described as species); six avian *Cryptosporidium* spp., four species of *Cryptosporidium* spp. in fish, one in amphibians, four in reptiles, and 29 species of *Cryptosporidium* spp. in mammals. In addition to the 44 species, more than 120 genotypes have been reported, and perhaps many of these will be formally described as species in the future. Valid names for *Cryptosporidium* spp. are essential to elucidate *Cryptosporidium* taxonomy and understand its transmission dynamics. Genotypes often have multiple names in the literature and GenBank, leading to confusion and inaccuracy. All species and genotypes were summarized in the work by Ryan et al. [46].

The greatest diversity of *Cryptosporidium* spp. and genotypes has been described in rodents; namely 14 species and more than 57 genotypes. This is not surprising, as rodents include more than 2,000 species, representing 40% of all mammals, and inhabit all continents [12]. A wide range of other species have also been reported in rodents, including the human infectious species *C. parvum*, *C. meleagridis* and *C. ubiquitum* [21, 26]. Fourty four species and more than 120 genotypes, 19 species and four genotypes have been reported in humans. However, 95% of diseases are caused by two species, *C. hominis* and *C. parvum*, which are very crucial in public health and they are responsible for several sporadic diarrheal outbreaks [7, 41]. *C. parvum* is a zoonotic species and infects a wide range of animal hosts, including humans, whereas *C. hominis* infects only humans [42]. The genomes of *C. parvum* and *C. hominis* (reference strains IOWA and TU502) have been sequenced [2, 62], and their genome sizes are 9,11 and 9,16 Mb. Genome comparison showed that *C. hominis* and *C. parvum* are very similar and show only 3–5% sequence divergence, without large insertions, deletions or rearrangements. Genomic data for genome representatives are available online [11].

Multilocus analysis

Multi-locus typing has become one of the most valuable strategies to address the genetic diversity or variability of microorganisms such as fungi [50]; protists [10]; and

bacteria [5]. The study of alleles of different loci increases the discriminative power of typing schemes, allowing the detection of genetically distinct lineages. Two main strategies were developed in multilocus analysis. One is based on comparative analysis of the size of amplified fragments of multiple loci (Multilocus Fragment Typing-MLFT) and identification of the length/size of polymorphisms; and the second strategy is focused on the comparative analysis of nucleotide sequences (Multilocus Sequence Typing-MLST), which allows the identification of differences in length and variations of nucleotides [5, 10, 22, 23, 40, 43, 50]. Although MLFT can provide good differentiation and it is relatively fast and inexpensive to perform, MLST is considered the gold standard because it is able to unambiguously identify genomic variations such as single nucleotide polymorphisms (SNPs) or point mutations, small insertions and deletions, inversions, and translocations and deletions or duplications [1, 13, 39, 58].

Various molecular tools are available to distinguish between different *Cryptosporidium* spp., pathogenic for humans. Molecular typing studies based on a single locus are limited in their ability to capture population structure, which is better explored using a multilocus approach. To better understand transmission dynamics, genetic diversity and population structure of *Cryptosporidium* spp., it is, therefore, necessary to study markers with high resolution. Multilocus sequence typing (MLST), widely used to study the population biology of many microorganisms [36, 55], has been used for subtyping primarily for *C. hominis* and to a lesser extent for *C. parvum* [32, 51]. Studies from Scotland and the United States have shown that *C. hominis* exhibits a clonal population structure, as it exhibits a lower number of subtypes and a much stronger linkage disequilibrium (LD) compared to *C. parvum* [51]. Significant variation at minisatellite loci is in the form of SNPs that may be missed by typing techniques based on length polymorphism alone. MLST is based on both the length of polymorphisms and SNPs and therefore increases the discriminative power of typing methods. Sequence length polymorphism was studied at loci GP60, CP47, TP 14, MS5, MS9, and MSC6-7, while loci CP56 and Mucin-1 were monomorphic in size. Among all markers studied, GP60 showed the highest gene diversity [41, 43]. Many subtypes in the GP60 family were of the same length, even though the sequences were different. In 2017, Y a d a v et al. [63] discovered that MLST based on the length of polymorphism and SNPs showed that only the CP47 gene is monomorphic, while the study by G a t e i et al. [19] described all other loci as polymorphic except for HSP70. G a t e i et al. [19] identified six subtypes of CP47 and six subtypes of GP60. In a study by Y a d a v et al. [63], up to seven subtypes of GP60 were listed based on the length of polymorphism, and all subtypes were different, except the 847 bp. Although three subtypes were observed in both studies based on the length of polymorphism at the MSC6-7 locus, these were distinct from each other. The Mucin-1 locus had 11 copies of a 63 bp minisatellite repeated in both studies. In both studies, three subtypes were observed based on SNPs. Based on this analysis, the UPGMA tree of Calcutta isolates generated twenty multilocus subtypes, and the Bayesian-derived MLST phylogram showed four major subtype groups. The UPGMA tree generated nine multilocus subtypes, and the MLST phylogram derived by the neighbour-joining method also showed nine groups of subtypes [63]. Although MLST is a better tool for studying transmission dynamics of *Cryptosporidium* subtypes due to its high resolution, to further understand the significance of different subtypes with clinical disease manifestations and transmission risks, it will be necessary to analyze a large number of samples and preferably from different geographical areas. It follows that the 60-kDa glycoprotein (GP 60) is one of the predominant typing gene markers for *C. parvum* genotypes [59]. So far, 15 subtypes of *C. parvum* have been described, including IIa to IIi and IIk to IIp, in which families IIa and IId are defined as zoonotic pathogens [45, 59].

While GP60 showed the highest resolution in MLST, other markers including CP47 and MSC6-7 also showed high gene diversity. The CP47 locus has a classic microsatellite gradual mutation and offers a good alternative for *C. hominis* and *C. parvum* typing. Unlike GP60, CP47 has a high resolution for both length of polymorphism-based and MLST-based typing and discriminates *C. hominis* and *C. parvum* by size without overlap in TAA repeat number. Similarly, MSC6-7 had 28 polymorphic sites and strong LD to both GP60 and CP47; making it a useful minisatellite locus for both *C. hominis* and *C. parvum* typing [19].

Subsequently, multilocus sequence typing (MLST) tools based on four minisatellite and microsatellite loci were developed to analyze genetic variation in *C. muris* and *C. andersoni* [18]. For example, until now the iden-

tification of *C. andersoni* isolates from humans has been based only on the analysis of the SSU rRNA gene or the COWP gene. Epidemiological data from different hosts document the host range or host specificity of *C. andersoni*. Cattle are considered the main animal host of *C. andersoni*, although it is occasionally found in other animals such as dromedary camels, sheep and hamsters [57, 64]. A search for microsatellite and minisatellite sequences in the *C. muris* genome was performed on March 8, 2007. The first 2,500 sequences (tracks 1649410657 to 1649413156) in the *C. muris* whole genome sequencing database were obtained from the Institute for Genomic Research (TIGR, now the J. Craig Venter Institute) [25]. Microsatellite and minisatellite sequences in the sequences were identified using Tandem Repeat Finder software [52].

A search for tandem repeats in 2,500 short sequences obtained from the *C. muris* genome-wide sequencing project identified 101 sequences with microsatellites and minisatellites. Based on the nature (mostly absence of imperfect repeats) and length (>6 copies for minisatellite targets and >10 copies for microsatellite targets) of the repeats and the availability of suitable sequences for primer design (except sequences with short or AT-rich 5'- or 3'- adjacent nucleotide sequences) 13 loci were selected from 101 potential targets, including 6 microsatellite loci and 7 minisatellite loci. Primers for nested PCR were designed for each locus (Tab. 1).

Because DNA sequencing rather than fragment length measurement was used to determine the polymorphism, the final PCR product was larger than that normally used for microsatellite and minisatellite analysis, with expected PCR products ranging from 307 bp to 751 bp. Four loci, CM-MS1, CM-MS2, CM-MS3 and CM-MS16, were selected for sequence polymorphism evaluation using a total of 44 DNA extractions from *C. muris* and *C. andersoni* samples. Of these, 41, 39, 24, and 38 DNA preparations were amplified at the CM-MS1, CM-MS2, CM-MS3, and CM-MS16 loci, respectively. These PCR products were successfully sequenced [18].

Sometimes the difference was only a slight modification of the repeat, such as one of the two minisatellite regions in the *C. muris* variant at the CM-MS1 locus, while sometimes the repeat sequences were completely different, such as those at the CM-MS3 locus. In total, there were 10, 5, 5, and 6 subtypes for *C. muris* and 2, 3, 4, and 1 subtypes for *C. andersoni* at loci CM-MS1, CM-MS2, CM-MS3, and CM-MS16, respectively (Tab. 2). *C. andersoni* also had repetitive sequences distinct from those of *C. muris* at the CM-MS2 and CM-MS3 loci (Tab. 3).

Currently, 18 MLST subtypes have been found in *C. andersoni* isolates from cattle [18, 30, 38, 56, 66]. By providing additional genetic data, it will help in solving the problems of tracing the source of infection/contamination and in elucidating the transmission dynamics of human cryptosporidiosis caused by *C. muris* and *C. andersoni*.

Sequence polymorphism in *C. muris* and *C. andersoni* was predominantly in the form of differences in copy number of microsatellite and minisatellite repeats. In contrast, both *C. hominis* and *C. parvum* have extensive single nucleotide substitutions in the nonrepetitive regions of most microsatellite and minisatellite targets. The coding nature of the targets was probably not responsible for the differences observed between gastric and intestinal *Cryptosporidium* spp., as most microsatellites and minisatellites in *Cryptosporidium* occur in the coding regions of protein genes due to the presence of few introns and short intergenic regions. This difference may reflect intrinsic biological and genetic differences between gastric and intestinal *Cryptosporidium* species, as suggested by previous data on phylogenetic relationships and G/C content in SSU rRNA genes between the two groups of *Cryptosporidium* spp. [60]. Biological differences are known to exist in *C. andersoni*. *C. andersoni* isolates in Japan, the so-called Kawatabi strain, differ from *C. andersoni* isolates in other areas in their ability to infect SCID mice [33]. The genetic difference between the two biological types of *C. andersoni* is not yet clear. In order to better understand the geographic distribution of *C. andersoni* and the genetic determinants of host specificity, it is again necessary to include more genetic loci and a large number of samples from different geographic areas.

CONCLUSIONS

Our understanding of the extent of genetic diversity and zoonotic potential of *Cryptosporidium* species and genotypes has been greatly improved by the development of molecular detection and typing tools, particularly the gp60 typing tool, as it showed the highest genetic diversity of all markers studied. We recorded the lowest number of subtypes of the species monitored by us in *C. hominis*,

Table 1. Primers used in multilocus sequence typing, nature of genetic diversity, annealing temperatures used in PCR, and expected sizes of PCR products for *C. hominis* and *C. parvum*

Gene	The nature of polymorphism	Primer	Sequence (5′ - 3′)	Annealing temperature [°C]	Locations of fragments [bp][a]	Citation
HSP70 in chromosome 2	Minisatellites – 12 bp repeats and SNPs	F1	ACCTATGAAGGTATTGATT	55	1030–1100	[63]
		R1	TTAGTCGACCTCTTCAACAGTTGG			
		F2	CAGTTGCCATCAGTAGAG	45		
		R2	CAACAGTTGGACCATTAGATCC			
Chom3T in chromosome 3	SNP	F1	TTGCAGGAATGGTCGTATTT	50	480–680	This review
		R1	TCTGAGTCTTCCCTCAATTTCT			
		F2	CGGTAAAGTTACCAGTTG	50		
		R2	GGCAAAACTATCACTGAGAA			
Gp60 in chromosome 6	Microsatellites – TCA, TCG, TCT repeats and SNPs	F1	ATAGTCTCCGCTGTATTC	50	800 – 850	[4]
		R1	GGAAGGAACGATGTATCT			
		F2	TCCGCTGTATTCTCAGCC	50		
		R2	GCAGAGGAACCAGCATC			
CP47 in chromosome 6	Microsatellites – TAA, TGA/TAG	F1	GCTTAGATTCTGATATGGATC TAT	43	380 – 500	This review
		R1	AGCTTACTGGTCCTGTATCAGTT			
		F2	ACCCCAGAAGGCGGACCAAGGTT	55		
		R2	GTATCGTGGCGTTCTGAATTATCAA			
CP56 in chromosome 6	SNP	F1	CTCACAGAGTAAAAATCACTT	50	660–750	This review
		R1	GAACGCAAATATTAAGAAAAATTGAG			
		F2	TTGGCAATGTTGTCTTTTTCCA	50		
		R2	ATATGTAATCTGGCGCCAAAG			
Mucin 1 in chromosome 6	Minisatellites – 63 bp repeats and SNPs	F1	ACTGATGTGTCAAGTGGCAATC	58	650 – 900	This review
		R1	TTACAGTTATGAGTTGCTGGT			
		F2	TTGATGATTCAGAATCATCTGACT	55		
		R2	GTGAGTTCTTCTTCATCTGTATAG			
MSC6-7 in chromosome 6	15 bp minisatellites and SNPs	F1	ATTGAACAAACGCCGCAAATGTACA	55	545–564	This review
		R1	CGATTATCTCAATATTGGCTGTTATTGC			
		F2	GCTATTTGCTATCGTCTCACATAACT	55		
		R2	CTACTGAATCTGATCTTGCATCAAGT			

which shows the clonal structure of the population with much stronger linkage disequilibrium. Continuous addition of new data on subtypes of *Cryptosporidium* contributes to the understanding of the role of genetic recombination, the mechanism of host adaptation and interspecies transmission. They will also be useful in understanding the significance of the unique distribution of these subtypes in some geographic areas, such as the dominance of *C. parvum* subtypes in humans, or *C. andersoni* in cattle.

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Table 2. The nature of microsatellite and minisatellite repeats in four genetic loci

Locus	Repetition		
	C. muris	Variant *C. muris*	*C. andersoni*
CM-MS1	$(TAAAGGGAGAGA)_{3}$ $(GAACGAGATAGG)_{14-18}$	$(TAAAGAGAGAGA)_{6}$ $(GAACGAGATAGG)_{14}$	$(TAAAGGGCGAGA)_{3}$ $(GAACGAGATAGG)_{13-14}$
CM-MS2	$(CCATATCCC)_{3-4}$ $(CCATACCTC)_{3}$	$(CCCATTCCT)_{4}$	$(CCATACCTC)_{10-11}$
CM-MS3	$(TGTTGG)_{8-9}$; $(GCTGCA)_{6}$	$(TGTTGG)_{7}$; $(TGC)_{10}$	$(TGTTGGTGTTGCTGT)_{2}$ $(TGCTGCAGCTGC)_{2-3}$
CM-MS16	$(CTTCTTCAT)_{9-2}$	$(CTTCTTCAT)_{8}$	$(CTTCTTCAT)_{14}$

Numbers in subscript indicate number of nucleotide repeats

Table 3. MLST subtypes of *C. andersoni* from different hosts around the world

The host	Subtype number	MS1	MS2	MS3	MS16	Reference
Sheep	1	A2	A4	A2	A1	[56]
	1	A2	A5	A2	A1	[56]
Hamster	2	A3	A4	A2	A2	[56]
Camel	1	A6	A4	A2	A1	[56]
	1	A6	A5	A2	A1	[56]
Cattle	3	A1	A2	A4	A1	[18]
	1	A1	A3	A4	A1	[18]
	34	A1	A4	A4	A1	[56, 66, this review]
	2	A2	A1	A2	A1	[18]
	1	A2	A1	A3	A1	[18]
	1	A2	A3	A1	A1	[18]
	1	A2	A3	A2	A1	[18]
	3	A2	A3	A4	A1	[15]
	6	A2	A4	A2	A1	[56, 66, this review]
	6	A2	A4	A4	A1	[56, 66, this review]
	1	A3	A4	A4	A1	[56]
	3	A4	A4	A2	A1	[66, this review]
	82	A4	A4	A4	A1	[56, 66, this review]
	6	A5	A4	A4	A1	[56, this review]

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