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Benefits of high resolution melting analysis for rapid detection and genotyping of selected protozoal and fungal parasites

Abstract

Microsporidiosis, cryptosporidiosis, blastocystosis, etc., of humans and animals are an intestinal diseases caused predominantly by infection with zoonotic species and genotypes of these pathogens. These diseasesare transmitted mainly via the faecal-oral route.Generally, these deseasesare occurs worldwide, but in places with poor sanitation and crowded living conditions is endemic and is associated with source of water and food supply, age, and socioeconomic status.[1] The diagnosis and genetic characterization of the main species and population variants of *Microsporidia*, *Cryptosporidium* and *Blastocystis* infecting humans andanimalsare central to the prevention, surveillance and control of these diseases, particularly as there is presently no cost effective chemotherapeutic regimen or vaccine available. In this Minireview we want to draw attention to the possibility of using HRM analysis for species and genotypic differentiation of these pathogens in clinical samples of humans and animals. This approach is well suited for the rapid screening of large numbers of *Cryptosporidium* oocyst, *Blastocystis* cyst and *Microsporidia* spores DNA samples and, although qualitative, is significantly less time-consuming to carry out than electrophoretic analysis. This method provides a useful tool for investigating the epidemiology and outbreaks of cryptosporidiosis, microsporidiosis and blastocystosis and could be applicable to identification of species of these pathogens.

Keywords: microsporidiosis, cryptosporidiosis, blastocystosis, high resolution melting

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Abbreviations: ELISA, enzyme-linked immuno sorbent assay; PCR, polymerase chain reaction; LAMP, loop mediated isothermal amplification; RAPDs, random amplified polymorphic DNAs; SSU Rdna, small-subunit rRNA genes; RFLP, restriction fragment length polymorphism; ITS, internal transcribed spacer; SSCP, single-strand conformation polymorphism Analysis; DGGE, denaturing gradient gel electrophoresis; TGCE, temperature gradient capillary electrophoresis; SNP, single-nucleotide variants

Cryptosporidiosis and *Cryptosporidium spp.*

Cryptosporidium is an important zoonotic parasite globally. The ecology and epidemiology of this pathogen in rural tropical systems are characterized by high rates of overlap among humans, domesticated animals, and wildlife. Diagnosis of cryptosporidiosis in animals and humans including, microscopic analysis with specific stainingof faeces for the presence of oocysts, detection of *Cryptosporidium* copro-antigens using enzyme-linked immunosorbent assay (ELISA) or molecular methods using DNA analysis.[2] The various molecular analysis such as polymerase chain reaction (PCR) and sequencing, and loop mediated isothermal amplification (LAMP) are widely used to detect the parasite in faecal material and his genetic characterization.[3–5] To date, *Cryptosporidium* isolates have been classified to 30 species, and over 60 have been assigned to a genotype or subtype based on molecular method.[6,7] For identification of species, genotypes or sub-genotypes, either highly variable genes or unique genes are used. Gene 18SSU rRNA serves as an appropriate genetic marker for identification of particular species of *Cryptosporidium*. "Variable" areas that are present in e.g. gene GP60 and loci ML1 and ML2,70 kDa heat shock protein (hsp70), and *Cryptosporidium* oocyst wall protein (cowp) genes, can be used for identification and classification of species, but they are mainly used for description of genotypes and sub-genotypes of *Cryptosporidium*, and thus considerably aid with understanding of transmission and identification of *Cryptosporidium* outbreak.[8,9]

Microsporidiosis and *Microsporidia spp.*

Microsporidia is an opportunistic parasite andto date, are composed of approximately 1300 formally described species in 160 genera.[10] Most of these ubiquitous obligate intracellular parasites infect invertebrates and fish, but 14 species in eight genera infect mammals.[11] Diagnosis usually relies on the identification of the stained microsporidial spores, but these methods lack sensitivity and require highly trained technicians to perform and interpret the results. Molecular diagnosis offers an alternative with both superior sensitivity and specificity as compared to microscopy.In the last years, several methods were developed for molecular detection of microsporidia, by conventional or quantitative PCR. Several PCR-based methods have been published to amplify different regions of the SSU and LSU rRNA gene as well as the intergenic spacer region for diagnosis and species differentiation of microsporidia infecting humans and animals.[12,13] Despite the recent advances, there is still confusion about the reproduction mechanism of *E. Bieneusi*, especially whether meiotic recombination occurs in *E. Bieneusi* like in some other microsporidia.[14] If genetic recombination occurs in its reproductive stage, the use of ITS marker may be inadequate in identifying genotypes.Currently, sequence analysis of the only ribosomal internal transcribed spacer

(ITS), a unique genetic characteristic of microsporidian, is regarded as the standard technique for genotyping of *E. Bieneusi* isolates.[15] Among over 100 *E. Bieneusi* ITS genotypes identified in humans and animals thus far, several unique groups (Groups II–V) of genotypes are found in specific groups of animals, thus have less public health importance. Nevertheless, a large group (Group I) of ITS genotypes have been found in both humans and animals.[16,17] These observations need to be substantiated by sequence characterization of other genetic markers.[18]

Blastocystosis and *Blastocystis spp.*

Blastocystis spp. is a controversial unicellular microorganism which lives in the intestinal tract of the humans and other mammals, birds, amphibians, reptiles, insects, etc.[19] There are many morphological forms of *Blastocystis* including vacuolar (multivacuolar, avacuolar and granular), amoeboid and the form of infectious cyst.As other intestinal parasites, transmission occurs by fecal oral route with the cyst form, although this has not been confirmed experimentally.[20,21] Diagnosis techniques of blastocystosis currently in use include microscopy, xenic *in vitro* culture, andmolecular detection.The traditional molecular methods in the diagnosis of *Blastocystis* species and genotypes including PCR analysis with seven pairs of STS primers derived from random amplified polymorphic DNAs (RAPDs) developed and described Yoshikavwa et al.[21,22] These primers correspond to phylogenetically different clades inferred from the small-subunit rRNA genes (SSU rDNA).[23] Moreover recently, polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP),[24] single-strand conformational polymorphism,[19] and pyrosequencing[25] were developed.Currently, sequence analysis of the small subunit ribosomal RNA (SSU rRNA) has shown, a unique diversity of *Blastocystis* subtypes and 17 subtypes (STS), have been recognized.[26]

Benefits of high resolution melting analysis

Early and correct diagnosis of disease is necessary for the beginning of rapid and effective actions that are essential for managing epidemiologic and epizootologic situation. Molecular identification and genotyping of pathogens is important part of this process, because response speed is decisive for minimization of final impacts from emerging outbreaks.[27] Therefore, methods used for molecular identification and typing must be fast, credible and easily reproducible. Currently, various DNA analysis methods for detection of causal gene mutation or polymorphism associated with disease are used. Even though Sanger sequencing is still considered as a "golden standard" for detection of unknown mutations, there is a need to develop and use new methods which can reduce cost and time needed for individual examinations. There are many methods that are capable of detecting changes in nucleotide sequence of DNA such as: SSCP (single-strand conformation polymorphism analysis),[28] DGGE (denaturing gradient gel electrophoresis),[29,30] TGCE (temperature gradient capillary electrophoresis).[31] Until now high-resolution analysis of melting curves seems unparalleled (High Resolution Melting, HRM).[32] This method enables high-performance mutation sequencing of single-nucleotide variants (SNPs, single nucleotide polymorphisms) smaller deletions and insertions, genotyping[33] or methylation analysis.[34] HRM is fast, simple, cost effective and at the same time high-performance sensitive method. It is a closed-tube method; i. e. amplification and analysis are done in the same tube without the need of further post-PCR manipulations, which lowers the risk of contamination.[35] This method was used in bacteriology,[36] virology [Steer], mycology[37] and protozoology.[33] This type of DNA analysis has a potential for fast identification and simultaneous genotyping of medically important bacteria, viruses, protozoa or fungi in culture or in clinical or environmental samples. HRM can achieve a high rate of small amplicons (100-250 bp) mutations detection and it is also able to provide quantitative or qualitative results. This analysis is appropriate for fast screening of large number of infective stages (oocysts, cysts or spores) of above mentioned pathogens.[38] Current diagnosis of these pathogens is based on use of genetic markers (from 100 to 450 bp), which allows us to detect sequencing variations in ITS and SSU rRNA regions of genes in individual species or within species as identification of genotype or subtype of *Cryptosporidium* spp, *Microsporidia* spp a *Blastocystis* spp. HRM should become a practical method for diagnostics, for detection of infection caused by *Cryptosporidium*, *Microsporidia* and *Blastocystis* in small geographic area and for monitoring outbreaks of these pathogens. This method was used for diagnostics of *Cryptosporidium* infection by authors Pangasa et al.,[33] where species *C. hominis*, *C. parvum* and *C. meleagridis* were identified. The use of this methods is also promising for identification and genotyping of other *Cryptosporidium* spp. (unpublished results), *Microsporidia* spp. - especially for detecting genotypes of species *Enterocytozoon bieneusi* or for determination of 17 *Blastocystis* spp. genotypes. Construction of primers and likewise characterization and size of sequence variations in ITS or SSU rRNA regions of gene are very important.[39] Research of this issue is only at its beginning but promising results point out that the use of HRM method in diagnostics of diseases will be a significant progress.

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Conflict of interest

Author declares that there is no conflict of interest.

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