Published in final edited form as:

J Eukaryot Microbiol. 2016 March ; 63(2): 146–152. doi:10.1111/jeu.12262.

Fast Technology Analysis (FTA) Enables Identification of Species and Genotypes of Latent Microsporidia Infections in Healthy Native Cameroonians

Edward S. Ndzi^{a,b}, Tazoacha Asonganyi^c, Mary Bello Nkinin^d, Lihua Xiao^e, Elizabeth S. Didier^f, Lisa C. Bowers^f, Stephenson W. Nkinin^{g,h}, and Edna S. Kaneshiro^{h,1}

^aInstitute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé, Cameroon

^bUniversity of Buea, Buea, Cameroon

^cFaculty of Medicine and Biomedical Sciences, University of Yaoundé-1, Yaoundé, Cameroon

^dNeurology & Physical Medicine Services, Electroencephalograph Laboratory, Central Hospital Yaoundé, Yaoundé, Cameroon

^eDivision of Foodborne, Waterborne and Environmental Diseases, National Center of Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

^fTulane National Primate Research Center, Covington, Louisiana, USA

^gCincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

^hDepartment of Biological Sciences, University of Cincinnati, Cincinnati, Ohio, USA

Abstract

Several enteric microsporidia species have been detected in humans and other vertebrates and their identifications at the genotype level are currently being elucidated. As advanced methods, reagents, and disposal kits for detecting and identifying pathogens become commercially available, it is important to test them in settings other than in laboratories with “state-of-the-art” equipment and well-trained staff members. In the present study, we sought to detect microsporidia DNA preserved and extracted from FTA (fast technology analysis) cards spotted with human fecal suspensions obtained from Cameroonian volunteers living in the capital city of Yaoundé to preclude the need for employing spore-concentrating protocols.

Further, we tested whether amplicon nucleotide sequencing approaches could be used on small aliquots taken from the cards to elucidate the diversity of microsporidia species and strains infecting native residents. Of 196 samples analyzed, 12 (6.1%) were positive for microsporidia DNA; *Enterocytozoon bienersi* (Type IV and KIN-1), *Encephalitozoon cuniculi*, and *Encephalitozoon intestinalis* were identified. These data demonstrate the utility of the FTA cards in identifying genotypes of microsporidia DNA in human fecal samples that may be applied to field testing for prevalence studies.

¹Correspondence: E. Kaneshiro, Department of Biological Sciences, University of Cincinnati, Cincinnati Ohio 45221-0006, USA, Telephone number: +1 513-556-9712; FAX number: +1 513-556-5280; edna.kaneshiro@uc.edu.

Keywords

Encephalitozoon cuniculi; *Encephalitozoon intestinalis*; enteric parasites; *Enterocytozoon bieneusi*; epidemiology; fecal samples; microsporidial DNA; opportunistic pathogens; PCR analysis

MOST epidemiological studies on microsporidia in humans have focused on HIV/AIDS patients since these organisms can cause life-threatening opportunistic infections (OIs) in immune- deficient people (Desportes et al. 1985; Coyle et al. 1996; Samé-Ekobo et al. 1997; Brasil 2000; Wittner and Weiss 2000; Lono et al., 2001; Tumwine et al. 2005; Sarfati et al. 2006; Breton et al. 2007; Nkinin et al. 2007; Samie et al. 2007; Viriyavejakul et al. 2009; Liu et al. 2011; Lono et al. 2011; Akinbo et al. 2012; Heyworth 2012; Lobo et al. 2012; Matos et al. 2013; Maikai et al. 2012; Ojuromi et al. 2012; Saigal et al. 2013; Widmer et al. 2013; Halánová et al. 2013). Publications currently in the literature thus predominantly feature reports of diagnostic studies performed on subject groups such as persons with HIV/ AIDS, infants, children attending schools, outpatients at clinics or hospitals, admitted hospital patients, and those with chronic diarrhea where prevalence values have been as high as 80%. In some of these studies, however, many participants had received medical care and medications prior to collecting their fecal samples for analysis contributing to some lower prevalence values in these highly selected segments of the community populations.

There are relatively fewer publications on the occurrences of microsporidia infections in healthy people and interestingly, these reported prevalence rates were also quite high. A prevalence of 67.5% was reported in healthy volunteers in Cameroon, an underdeveloped county in sub-Sahara Africa (Nkinin et al. 2007) and an incidence of 86% was found in the Czech Republic (Sak et al. 2011) in a three-month study of weekly monitoring by calcofluor staining and immunofluorescence microscopy on 15 individuals in a developed country in Europe. High prevalence rates were also reported in healthy aboriginal Malaysians in Southeast Asia (Shahrul et al. 2013) and in Pakistan on the Asian continent (Yakoob et al. 2012). Thus, Sak et al. (2011) concluded that microsporidiosis represents latent ubiquitous infections among healthy carriers, consistent with other immunodeficiency-dependent diseases (IDD; Frankel 1999) also produced by opportunistic infections. It was also suggested that due to poorer sanitation standards, the higher microsporidial prevalence in Cameroon reported earlier using cytochemical and immunochemical techniques and analyzed by fluorescence microscopy by highly trained personnel (Nkinin et al. 2007) might have been related to people ingesting spores more frequently and who have developed high tolerance and resistance to the clinical manifestations of microsporidia infection. The purpose of the present study was to evaluate the use of fast technology analysis (FTA) cards for use in PCR-based surveillance detection of microsporidia in feces from human volunteers living in Yaoundé, Cameroon using this technology as successfully performed in other studies on microsporidiosis (Snowden et al. 2002; Milks et al. 2004; Sokolova et al. 2011; Yan et al. 2014).

MATERIALS AND METHODS

Study Site: Yaoundé, capital city of Cameroon

Yaoundé, with a population of approximately 2.5 million people is divided into 7 subdivisions each with several established neighborhoods. Healthy volunteer donors of fecal specimens for this study were recruited from four of the subdivisions (III, IV, V, and VI) living in 10 different neighborhoods in Yaoundé. Of the 196 participants 77 lived in the neighborhood of Etoug Ebe (Yaoundé VI), 60 in Ekounou (Yaoundé IV), 20 in Odza (Yaoundé IV), 15 in Biyem Assi (Yaoundé VI), 11 in Damas (Yaoundé III), 3 in Château Ngoa Ekele (Yaoundé III), 3 in Anguissa (Yaoundé IV), 3 in Essos (Yaoundé V), 3 in Mimboman (Yaoundé V), and 1 in Manguier (Yaoundé V).

Poor sanitation and the lack of clean and safe drinking water are serious public health problems in cities such as Yaoundé (Fig. 1). Many neighborhoods, like Damas, lack potable water and most use well water (Fig. 1A, 1D, 1E) and spring water (Fig. 1G) for laundering and household cleaning. People are instructed to add 10–12 drops of 12% Clorox per 10 liters of water as disinfectant for use in washing eating utensils, laundering and sometimes for cooking.

Running water taps are absent from many homes but water for drinking and cooking may be purchased from the national water distribution company, Camerounaise Des Eaux (CDE) from neighbors who do have outdoor water taps (Fig. 1C). Some individuals are financially able to construct bore holes to tap underground aquifers and pump water for use in their homes. Most food items for meals are purchased at outdoor markets (Fig. 1G) and there are no government guidelines or formal food inspections.

Fecal samples

Approval from the Cameroon National Ethics Committee (Ethical Clearance #230/CNE/SE/2011) was obtained prior to obtaining fecal samples. Since stool sample collection is not an invasive process, there was no major risk to the participants. Potential participants were given information about the goals, importance of the study, and procedures, including the risks and benefits involved; only those who gave their informed consent were recruited. For participants below 15 years of age, informed consent was obtained from their parents. Participants were to benefit from free diagnosis of microsporidiosis and those testing positive were to be informed of the results on their stool samples. Specimens were coded to protect identification of the participants during testing.

Fecal samples were collected from March to May, 2011. Some out-patients were recruited at the Ekounou Baptist Health Centre (Yaoundé). Nineteen of the 196 participants recruited for this study had also participated in a previous study conducted for detection of microsporidia (Nkinin et al. 2007). Demographic information (name, gender, age, location or neighborhood, HIV status, profession, region of origin and source of water) was obtained from each participant and recorded on standardized questionnaires.

Demography

Participants included 89 males and 107 females; 35 were infants (0–5 years old); 26 children (6–11 years old), 32 teenagers (12–19 years old), 97 adults (20–50 years old), and 6 elderly people (>50 years old). The HIV status of participants was either negative or unknown, with the exception of one individual who was positive and healthy. The majority of the population in these neighborhoods was poor and unemployed. Apart from infants and children, individuals in this study included 22 students, 23 public service workers, 4 military personnel, 24 housewives, 16 small-scale traders (hawkers or peddlers), 6 unemployed individuals, and 3 retired civil servants. A total of 73 participants were from 21 different households; as many as 8 or more people were commonly found living in a single apartment.

Healthy participants in households were recruited during evening visits to these neighborhoods. Participants were given instructions on safe procedures to obtain stool without contaminating the samples. Each participant was given a labelled clean glass cup with lid into which they were to provide 2–3 g of fresh stool the following morning. The stool samples were collected from participants early in the morning, just after they were excreted and then transported on ice to the Parasitology Laboratory of the Institute of Medical Research and Medicinal Plants Studies (IMPM) in Yaoundé.

After arrival to the laboratory, one third of each stool sample was transferred to a 5-ml test tube and homogenized with 2 ml of deionized water. Approximately 200 µl of each suspension was then withdrawn by pipette, spotted onto Whatman FTA® cards (Sigma Chemical Co, St. Louis, MO), and labelled with the participant's identification number. After being air-dried for 24 h, the cards were packed in paper envelopes, stored at room temperature, and then placed into Ziploc® plastic bags, sealed, and shipped to the University of Cincinnati for further analysis.

Processing FTA cards for PCR

FTA cards that had been spotted with fecal homogenate were shipped from the University of Cincinnati to Tulane University in New Orleans, LA. There two discs of 2 mm² each were punched from FTA cards and placed into a microfuge tube containing 200 µl of FTA purification reagent (Qiagen, Valencia, CA) that was then gently tapped to mix the disc in the reagent. After 5 min incubation at room temperature, the purification reagent was replaced with fresh FTA purification reagent and this washing procedure was repeated for a total of three washes.

After removing the final FTA purification reagent wash, the discs were similarly washed two times using 200 µl of Tris-EDTA (TE) pH 8.0 buffer. After the second wash, the TE buffer was removed and the discs were allowed to air dry at room temperature for at least 1 h.

PCR analysis and genotyping

The washed and dried discs were then added directly to the PCR master mix. Nested PCR was applied using MSP primers that target the internal transcribed sequence (ITS) and

flanking region of the large and small subunits of the rDNA genes of *Encephalitozoon*, *Enterocytozoon*, *Vairimorpha* and *Nosema* species of microsporidia as previously described (Katzwinkel- Wladarsch *et al.* 1996). The reaction utilized the Illustra PuReTaq Ready-To-Go Beads (GE Healthcare Life Sciences, Piscataway, NJ USA) that are each comprised of 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, stabilizers, and BSA to which were added 1 µM of each primer and 1 µl of template DNA in a final reaction final volume of 25 µl. The PCR first-round primers were 5'-TGA ATG KGT CCC TGT-3' (MSP-1), 5'-TCA CTC GCC GCT ACT-3' (MSP-2A) and 5'-GTT CAT TCG CAC TAC T-3' (MSP-2B). The second- round primers were 5'-GGA ATT CAC ACC GCC CGT CRY TAT-3' (MSP-3), 5'-CCA AGC TTA TGC TTA AGT YMAARG GGT-3' (MSP-4A) and 5'-CCA AGC TTA TGC TTA AGT CCAGGG AG-3' (MSP-4B). These primers detect all the *Encephalitozoon* species and *Enterocytozoon bienersi*. Thermal cyclor conditions were 5 min at 95 °C followed by 36 cycles of: 1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C and then a final primer extension at 72 °C for 10 min. Amplicons were electrophoresed in a 1% agarose gel and stained with ethidium bromide.

PCR amplicons were excised from the gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, Catalog #A9281). Products were submitted to the GeneLab at Louisiana State University School of Veterinary Medicine in Baton Rouge, LA USA for bi-directional sequencing using the secondary forward and reverse primers.

The nucleotide sequences obtained from the amplicons were assembled using the ChromasPro (version 1.5) software (http://technelysium.com.au/?page_id=27) and aligned with each other and reference sequences using ClustalX (<http://www.clustal.org/>) to determine the microsporidia species and *E. bienersi* genotypes.

RESULTS

Of the 196 fecal samples from healthy people living in Yaoundé, Cameroon that were analyzed, 12 (6.1%) were positive for microsporidia (*Enterocytozoon bienersi*, *Encephalitozoon intestinalis* and *Encephalitozoon cuniculi*) (Table 1). The most prevalent species and genotype was *E. bienersi* Type IV, which is consistent with its abundance and known broad geographical and host ranges. The genotype KIN-1 (GenBank accession number JQ437573) identified in a native Cameroonian might represent a more regional isolate that was also previously reported in an individual from Kinshasa, Democratic Republic of the Congo (Wumba *et al.*, 2012).

Unidentified genotypes of microsporidia were also detected but were not further analyzed. These probably originated from people consuming infected fish (Fomina *et al.* 1992), because they exhibited 85–98% sequence similarities to a reference sequence from a *Spraguea* sp. strain that commonly infects the greater amberjack (GenBank accession number AB623034).

DISCUSSION

A relatively high prevalence (~68%) of microsporidia was reported in healthy residents of Yaoundé using calcofluor staining to detect the chitinous spore wall of microsporidia and immunofluorescence antibody staining using monoclonal and polyclonal antibodies to *Enterocytozoon bieneusi* or *Encephalitozoon intestinalis* on fecal specimens (Nkinin et al. 2007). In that study, PCR and genotyping of microsporidia species was not performed. In contrast, DNA extraction and nucleotide sequence analysis employed in the present study enabled the identification of different microsporidia species and genotypes but results showed lower overall prevalence of microsporidia in this native population. The higher prevalence in the earlier study compared to the current report may have been due to lower specificity of microscopic approaches. It is also possible that because samples applied onto the FTA cards were not processed for spore concentration and because only small discs were excised from the filters, there was a loss of sensitivity.

More likely, the higher prevalence of microsporidia in the earlier report on healthy Cameroonians (Nkinin et al. 2007) may have resulted from calcofluor staining and immunofluorescence microscopy of organisms lacking amplifiable DNA due to germination of spores. The high prevalence data is comparable to that which were reported by Sak et al. (2011) who used similar reagents.

Calcofluor binds cellulose and chitin with intense fluorescence emission but lacks specificity for microsporidia and although there are stains such as a Gram-chromotrope protocol (Moura et al. 1997) that are more specific for microsporidia, these again bind to the spore walls. Thus, the combination of these staining methods with indirect immunofluorescence also provided identification based on specific binding of the antibodies to the walls of spores even after they had germinated. Both microscopic approaches would identify intact spores as well as spores after cytoplasmic contents had been ejected and reflects the sum of organisms that had been present in the host.

The results obtained in the present study analyzing prevalence of microsporidial nucleic acid sequences were instead based on analysis of DNA material presumably present in spores and could partly account for the lower prevalence reported here. Also, our epidemiological data are more similar to those reported in other developing countries (Matos et al. 2012) based on DNA analyses. For example, Subrungruang et al. (2004) tested FTA cards and other extraction methods using the same primer pair (MSP3-MSP4B) as that in the present study. These workers concluded that FTA cards offered excellent sensitivity and specificity and reported a prevalence of 4.1% of *E. bieneusi* in children living in an orphanage in Bangkok, Thailand (Leelayoova et al. 2005). In the study reported here, however, nested PCR was performed compared to a single round of PCR in the Thai study and could explain the slightly higher prevalence data we obtained. Subsequent to the data in the present study, microsporidia spores were concentrated from fecal samples obtained from a comparable population of healthy Cameroonians. DNA was extracted and subjected to PCR amplification; preliminary results indicated prevalence was 6.7% (Ndzi, unpubl. data), which is in close agreement with the 6.1% obtained by using the FTA cards reported here.

Because of poor sanitation conditions in communities within underdeveloped regions of the world, it would not be surprising that a high proportion of healthy people and animals, especially those living in close proximity to each other, can readily transmit infections with enteric parasites (Abe and Kimata 2010; Ye et al. 2014). This is consistent with a recent report of a foodborne *E. bienersi* outbreak among guests in a hotel in Sweden (Decraene et al. 2012).

Most previous studies on enteric microsporidia focused on cross-sectional prevalence data while reports on longitudinal studies are sparse in the literature. However, there are indications that if several large-scale longitudinal studies are performed, they are likely to reveal high percentages of microsporidia infections (carriage) among human populations (Munghthin et al. 2005; Sak et al. 2011). Since microsporidia spore shedding can be sporadic (Munghthin et al. 2005; Sak et al. 2011) it would be important that future epidemiological studies include longitudinal studies on the same individuals.

FTA technology has previously been employed for detecting various microbes such as viruses, bacteria and parasites (Beck et al. 2001; Lampel et al. 2000; Snowden et al. 2002; Milks et al. 2004; Sokolova et al. 2001; Yan et al. 2014). As verified in the present study, FTA cards are useful for preserving parasite DNA for subsequent amplification and sequencing and for safe transportation or shipment of specimens for subsequent testing. These DNA stabilization cards are relatively effective in reducing the need for highly trained technicians and cost for expensive reagents. Future development of reagents and methods for studying microsporidia in fecal as well as environmental samples would also be strengthened if quantitative methods for determining viability and infectivity of these organisms become available.

Acknowledgments

Supported in part by a grant from the National Institutes of Health, OD011104, to the Tulane National Primate Research Center.

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Fig. 1. Environment and sources of water and food in Cameroon. **A.** Walkway between houses in Yaoundé showing wooden planks over standing water. **B.** Inside a community well from which a bucket filled with water is being lifted. **C.** Outdoor tap; water available for purchase. **D.** Transfer of water from a community well shown in E where a jug is used to get the water in the well then transferred into buckets for transport to homes. **E.** Another neighborhood community well. **F.** Washing used plastic bottles to recycle for storing other liquids such as oil, water, local drinks. **G.** Children collecting spring water for household use. **H.** Latrines or pit toilets commonly used by the lower class neighborhoods in the city of Yaoundé. **I.** Local open market where most food is purchased. Note a vender advertising fish (poissonnaire).

Table 1
Stool samples from healthy Cameroonians preserved on FTA cards: Microsporidia gene sequencing data.

Sample Code	Gender ^a	Age	Neighborhood	Primer (Eb ^b =500 bp; Non-Eb=300 bp)	Notes	Microsporidia Species	<i>E. bienersi</i> Genotype
20	F	12	Etoug Ebe	MSP-3 Forward	Eb size 500 bp	<i>E. bienersi</i>	KIN-1
20				MSP-4B Reverse (Eb)	Eb size 500 bp	<i>E. bienersi</i>	Unknown
65	F	4	Ekounou	MSP-3 Forward	Non-Eb size 300 bp	<i>E. intestinalis</i>	
65				MSP-4A Reverse (Non-Eb)	Non-Eb size 300 bp	<i>E. intestinalis</i>	
66	F	16	Ekounou	MSP-3 Forward	Non-Eb size 300 bp	Not identified ^c	
66				MSP-4A (Reverse (Non-Eb)	Non-Eb size 300 bp	Not identified ^c	
70	M	27	Ekounou	MSP-Forward	Eb primer but band at 300	Not identified ^c	
70				MSP-4B Reverse (Eb)	Eb size 500 bp	Not identified ^c	
74	M	16	Ekounou	MSP-3 Forward	Eb primer but band at 300	<i>E. cuniculi</i>	
74				MSP Reverse (Eb)	Eb size 500 bp	<i>E. cuniculi</i>	
83	M	37	Odza	MSP-3 Forward	Non-Eb size 300 bp	Not identified ^c	
83				MSP-4A Reverse (Non-Eb)	Non-Eb size 300 bp	Not identified ^c	
99	F	2	Ekounou	MSP-3 Forward	Non-Eb size 300 bp	Not identified ^c	
99				MSP-4A Reverse (Non-Eb)	Non-Eb size 300 bp	Not identified ^c	
106	F	38	Ekounou	MSP-3 Forward	Eb size 500 bp	<i>E. bienersi</i>	IV
106				MSP-4B Reverse (Eb)	Eb size 500 bp	<i>E. bienersi</i>	IV
107	F	23	Etoug Ebe	MSP-3 Forward	Eb size 500 bp	<i>E. bienersi</i>	IV
107				MSP-4B Reverse	Eb size 500 bp	<i>E. bienersi</i>	IV
107				MSP-4A Reverse (Non-Eb)	Non-Eb size 300 bp	No sequence	
108	M	23	Etoug Ebe	MSP-3 Forward	Eb size 500 bp	<i>E. bienersi</i>	IV
108				MSP-4B Reverse (Eb)	Eb size 500 bp	<i>E. bienersi</i>	IV
119	F	13	Etoug Ebe	MSP-3 Forward	Non-Eb size 300 bp	Not identified ^c	
119				MSP-4A Reverse	Non-Eb size 300 bp	Not identified ^c	
156	F	12	Awae	MSP-3 Forward	Eb size 500 bp	<i>E. bienersi</i>	IV
156				MSP-4B Reverse (Eb)	Eb size 500 bp	<i>E. bienersi</i>	IV

^aF, female; M, male

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^bEb, *Enterocytozoon bienersi*

^cUnknown microsporidia related to those in fish; probably consumed in diet.