Cryptosporidiosis in Haiti: surprisingly low level of species diversity revealed by molecular characterization of Cryptosporidium oocysts from surface water and groundwater

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Abstract – The protozoan parasite Cryptosporidium sp. has emerged as one of the most important water contaminants, causing waterborne outbreaks of diarrhoeal diseases worldwide. In Haiti, cryptosporidiosis is a frequent cause of diarrhoea in children under the age of five years, HIV-infected individuals, and people living in low socioeconomic conditions, mainly due to the consumption of water or food polluted by Cryptosporidium oocysts. The aim of this study was to detect and identify Cryptosporidium oocysts present in 12 water samples collected in Port-au-Prince and 4 water samples collected in Cap Haïtien. Initial detection consisted of immunomagnetic separation – immunofluorescence assay (IMS-IFA), which was confirmed by nested PCR, targeting the most polymorphic region of the 18S rRNA gene in 15/16 samples. Genotyping was performed by PCR-restriction fragment length polymorphism (RFLP) analysis and DNA sequencing. Under our working conditions, neither nested PCR-RFLP nor direct DNA sequencing revealed the expected species diversity, as only Cryptosporidium parvum was identified in the water samples studied. This study highlights the difficulty of detecting mixed populations of Cryptosporidium species in environmental samples.

Key words: Haiti, Water, Environmental Pollution, Cryptosporidium oocysts, Genotyping.

Résumé – Cryptosporidiose en Haïti: niveau étonnamment bas de diversité spécifique révélé par caractérisation moléculaire d’oocystes de Cryptosporidium collectés dans des eaux de surface et souterraines. Le protozoaire parasite Cryptosporidium sp. apparaît comme un polluant hydrique majeur engendrant des épidémies diarrhéiques à travers le monde. En Haïti, la cryptosporidiose est responsable de la plupart des diarrhées chez les enfants âgés de moins de 5 ans, les patients VIH, les personnes vivant dans des conditions socioéconomiques difficiles, surtout suite à la consommation de nourritures et d’eau de boisson polluées par les oocystes de cryptosporidies. Le but de cette étude était de détecter et d’identifier des oocystes de cryptosporidies dans 12 prélèvements d’eau effectués à Port-au-Prince et 4 au Cap Haïtien. La méthode de détection initiale a été une séparation immunomagnétique couplée à l’immunofluorescence, confirmée par méthode de PCR nichée, utilisant des amorces spécifiques de la région la plus polymorphe du gène codant pour l’ADN-rRNA, positive dans 15 des 16 prélèvements. Le génotypage a été réalisé par l’utilisation des enzymes de restriction et le séquençage direct de l’ADN. Dans les conditions de notre étude, ces deux méthodes n’ont pas révélé la diversité d’espèces attendue. En effet, seul Cryptosporidium parvum a été identifié dans tous les prélèvements étudiés. L’étude pointe la difficulté d’obtenir une bonne résolution de mélange d’espèces de cryptosporidies dans les échantillons environnementaux.

Introduction

Cryptosporidium sp. is a protozoan parasite that causes diarrhoeal illness via faecal-oral transmission. It has an incubation period of up to 2 weeks. Cryptosporidium parvum was reported for the first time in 1907 in gastric crypts of a laboratory mouse [28]. It has been subsequently found in many other animals (chickens, turkeys, rats, guinea pigs, horses, etc.). The first human case was reported in 1976 [1]. Cryptosporidium has subsequently become a well-known cause of opportunistic
infections among AIDS patients and is responsible for outbreaks of gastrointestinal disease including the memorable outbreak that occurred in Milwaukee, Wisconsin in 1993 involving 403,000 cryptosporidiosis infected individuals, 4,400 of whom were hospitalized and 69 died [20]. This outbreak was due to contamination of drinking water. Five years later, it was reported that residents of Sydney, Australia were infected by Cryptosporidium contamination of the drinking water supply from July to December 1998 [12]. Cryptosporidium-related diarrhea is transient in immunocompetent individuals [13, 16, 29], while diarrhea can become severe, chronic and life-threatening in immunocompromised patients, especially those infected by human immunodeficiency virus (HIV) [14, 19, 23]. No effective treatment of cryptosporidiosis is available at the present time. Cryptosporidiosis generally appears to be closely correlated with environmental causes, mainly in developing countries where most people live in inadequate hygiene conditions. In Haiti, cryptosporidiosis is a frequent cause of diarrhea and is responsible for 17.5% of cases of acute diarrhoea in children under the age of 2 years and 30% of cases of chronic diarrhoea in HIV-infected patients [21, 22]. In Haiti, in the early 2000s, several environmental investigations were conducted in Port-au-Prince and the surrounding region, Les Cayes and Cap Haitien in order to identify sources of human contamination of cryptosporidiosis. These surveys simply consisted of detecting Cryptosporidium oocysts in the environment by screening various types of water (surface water, groundwater, public water supplies) used by the population [3, 4, 24]. The main objective of these preliminary investigations was to evaluate the circulation level of Cryptosporidium oocysts in the Haiti environment. In the present study, the investigation comprised molecular characterization of environmental Cryptosporidium oocysts detected in order to identify Cryptosporidium species present in the survey zones.

### Materials and methods

#### Collection of water samples

Cross-sectional surveys of domestic drinking water were conducted in Port-au-Prince and Cap Haitien in October 2010 and January 2011, respectively. This study was therefore conducted after the earthquake in January 2010. In Port-au-Prince, water sampling was performed in the districts of Pe´tion Ville (No. 1; 2), Delmas (No. 3), Carrefour (No. 4) and Tabarre (No. 5–12). Regarding Cap Haitien, water sampling was performed in the municipalities of Petite Anse (No. 13, 14) and Bande du Nord (No. 15, 16), (Table 1). To minimize cross-contamination in the field, new water sampling equipment (bucket, tumbler and funnel) was used at each site. A sample of at least 100 L of water was collected and immediately filtered using a polyethersulphone capsule (Environchek/C210, Pall Gelman, Saint Germain en Laye, France). Capsules were stored at 4°C until the elution step.

#### Purification of Cryptosporidium oocysts

Capsules were processed according to the Association Française de Normalisation (AFNOR) standard operating procedures [2]. Briefly, capsule filters were rinsed with 240 ml of a detergent elution buffer (phosphate-buffered Saline, pH 7.4 with 0.1% (v/v) Tween 80). Specimens were concentrated by centrifugation at 2000g for 20 min. The final sediment

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**Table 1.** Characteristics of water sampling sites in Port-au-Prince and Cap Haitien and results of Cryptosporidium oocysts detection and molecular characterization.

| Site number | Sampling sites | Source of water samples | Number of litres filtered | Number of oocysts/100 L of water | Results of PCR detection | Cryptosporidium species identified | District/* Population served |
|-------------|----------------|-------------------------|--------------------------|----------------------------------|--------------------------|-----------------------------------|------------------------------|
| **Port-au-Prince** |
| 1 | Turgeau | Reservoir | 340 | 67 | + | C. parvum | Pétion-ville/342,451 |
| 2 | Plaisance | Spring | 255 | 33 | + | C. parvum |
| 3 | Delmas 71 | Reservoir | 170 | 53 | + | C. parvum |
| 4 | Dikini 63 | Spring | 272 | 145 | + | C. parvum |
| 5 | Clercine F2 | Bore | 340 | 17 | + | C. parvum |
| 6 | Clercine F7 | Bore | 340 | 233 | + | C. parvum |
| 7 | Rivière Grise | Surface water | 204 | 19 | Unsuccessful | Unsuccessful |
| 8 | Tabarre 25-F5 | Bore | 255 | 118 | + | C. parvum |
| 9 | Tabarre 41-T4 | Bore | 170 | 20 | + | C. parvum |
| 10 | Tapage-T8 | Bore | 255 | 6 | + | C. parvum |
| 11 | Bureau Central CAMEP | Bore | 340 | 32 | + | C. parvum |
| 12 | Tapage-T6 | Bore | 272 | 28 | + | C. parvum |
| **Cap Haitien** |
| 13 | Zone de Balan1 | Bore | 119 | 6,088 | + | C. parvum |
| 14 | Zone de Balan2 | Bore | 153 | 4,902 | + | C. parvum |
| 15 | En Haut Jean | Spring | 170 | 5,640 | + | C. parvum |
| 16 | Mandot | Reservoir | 170 | 3,583 | + | C. parvum |

* Inhabitants [15]; +: positive
was suspended in double-distilled water to a final volume of around 5 ml. Any Cryptosporidium oocysts present were then purified using immunomagnetic beads coated with anti-Cryptosporidium monoclonal antibody (Dynabeads®, Dynal, Oslo, Norway) according to the manufacturer’s instructions. Following the immunomagnetic separation (IMS) procedure [5], about 50 μl of suspension was obtained and used for immunofluorescence assay (IFA) and molecular analysis.

Detection and counting of Cryptosporidium oocysts

Twenty microlitres of suspension derived from the IMS procedure was placed on a glass slide and dried at room temperature. Slides were fixed in cold acetone (−20 °C) for 10 min and were then incubated for 30 min at 37 °C in a humid chamber with a 1:10 final dilution of a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) directed against a Cryptosporidium wall antigen, which was selected because of its lack of cross-reactivity with other microorganisms (FITC-Cow MAb, Monofluokit Cryptosporidium®, Bio-Rad, Marnes la Coquette, France). Slides were rinsed with PBS (pH 7.4) before applying coverslips. The entire smear of each slide was examined using an epifluorescent microscope and oocysts were counted. A positive control slide was used to ensure IFA results. The number of oocysts was expressed per 100 L of filtered water.

Cryptosporidium oocysts genotyping

DNA extraction

Cryptosporidium DNA was extracted using NucliSENS EasyMag kit (Biome™) according to the manufacturer’s instructions. DNA was stored at −80 °C until use.

Nested PCR

The Cryptosporidium 18S rRNA gene was amplified by a nested PCR assay. In order to highlight the presence of multiple species of Cryptosporidium in a single sample, five replicates of nested PCR were performed using different DNA volumes for the first round (10, 2, 1 and 0.5 μl), as reported elsewhere [22]. The first PCR round was performed with primer pair CpXiaoExtF (5′-TTC TAG AGC TAA TAC ATG CG-3′) and CpXiaoExtR (5′-CCC ATT TCC TTC GAA ACA GGA-3′) and the second PCR round was performed with primer pair CpXiaoIntF (5′-GGA AGG GTT GTA TTT ATT AGA TAA AG-3′) and CpXiaoIntR (5′-AAG GAG TAA GGA ACA ACC TCC A-3′). The two PCR rounds were performed under the same conditions. Nested PCR mixtures contained 1x PCR buffer, 5 mM MgCl2, 200 μM each deoxynucleoside triphosphate, 100 nM each primer and 1.25 U HotStart Taq polymerase. Cycling conditions consisting of a hot start at 94 °C for 3 min followed by 35 cycles with denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min, as previously described elsewhere [30, 31].

Restriction fragment length polymorphism

A RFLP assay was performed with two restriction enzymes, AseI and SspI, according to the manufacturer’s instructions (Ozyme, Saint Quentin en Yvelines, France). Part of the second-round PCR products was digested with AseI and another part with SspI, allowing the detection of different species when present [30]. Restriction profiles were visualized by electrophoresis of digested products on a 2% agarose gel with ethidium bromide.

DNA sequencing

Second-round positive PCR products were first purified by ExoSAP-IT® (USB® Products Affymetrix, Inc., Ohio, USA) and bidirectional sequencing analysis was carried out with a 3500xl Dx Genetic Analyser (Applied Biosystems®, Life Technologies) using Big Dye Terminator Cycle Sequencing and Purification Kit, according to the manufacturer’s instructions (Applied Biosystems®, Life Technologies). Electropherograms were analysed and consensus sequences were constructed using DNA Baser Software. BLAST software was used to determine homologies of these consensus sequences with those deposited in GenBank.

Results

During the surveys, a total of 16 water samples (12 in Port-au-Prince and 4 in Cap Haïtien) were collected from different sites supplying the majority of the population via various modalities of distribution (reservoirs, bores, springs and surface water). Detailed characteristics of sampling sites are presented in Table 1. As shown in Table 1, the IMS-IFA method demonstrated contamination of all water samples (100%) collected in Port-au-Prince and Cap Haïtien by Cryptosporidium oocysts with concentrations ranging from 6/100 L (site No. 10 in Port-au-Prince) to 6088/100 L (site No. 13 in Cap Haïtien). PCR detection of Cryptosporidium oocyst DNA was successful in 15 of the 16 samples analysed and confirmed 94% of the IMS-IFA results.

Under our working conditions, repeated nested PCR-RFLP using 10 μl of DNA extracts for species identification demonstrated the presence of Cryptosporidium parvum in all 15 water samples (Table 1). However, positive PCR results were obtained for four samples (No. 13, 14, 15, 16) when using 2, 1 and 0.5 μl samples of DNA extracts. Species identification was confirmed by direct DNA sequencing, as analysis of electropherograms failed to demonstrate any species diversity in the samples studied. BLAST results showed a 100% identity of our sequences with C. parvum 18S ribosomal RNA gene sequences available in GenBank.

Discussion

Cryptosporidium is a strict intracellular parasite and its infectious forms are oocysts eliminated in the stools of various hosts, including humans. Domestic and wild animals are important reservoirs in the transmission of this zoonosis to humans,
and water plays an important role in the spread of contaminating oocysts, which are excreted in a variety of environments [6, 10]. Oocysts can survive for long periods in fresh water and are resistant to water chlorination processes. Correct identification of this pathogen is extremely important, not only because of the clinical implications, but also for epidemiological studies. In developing countries such as Haiti, Cryptosporidium infection represents a major public health threat. Several factors facilitate transmission of Cryptosporidium and account for the propensity to cause large-scale outbreaks of diarrhoea such as those observed in developed countries: (i) Cryptosporidium can infect many mammalian species and is frequently identified in farm animals, particularly calves, and in domestic animals; (ii) oocysts are very resistant and can conserve their infectivity in moist environments for a long time; (iii) the genus Cryptosporidium is composed of a large number of species, several of which can infect humans; (iv) the infectious dose is very low, and infected individuals excrete large numbers of oocysts, up to 10^8 in a single day [7].

This study demonstrated the very poor biological quality of drinking water in the two cities. Although only a limited number of water samples were collected in Cap Haitien, these samples were the most heavily contaminated, probably due to the large number of stock farms located around sampling sites. Exposure to such concentrations of Cryptosporidium oocysts in drinking water could generate major biological risks for human health, particularly for children under the age of 5 years, undernourished individuals and immunocompromised patients. Greater commitment to environmental improvement in these cities is therefore required to improve the quality of drinking water in order to limit human transmission of cryptosporidiosis. This is particularly important for the safety of young children and HIV/AIDS individuals. The results concerning the high level of Cryptosporidium oocyst contamination were not surprising, as previous studies conducted in Port-au-Prince led to similar results [4]. However, a possible impact of the January 2010 earthquake on these results cannot be excluded, as the study was conducted after the earthquake and the subsequent breakdown in infrastructures damaged a large part of the water reservoirs and water distribution network. This damage may have worsened pre-existing water pollution by Cryptosporidium oocysts. Moreover, the presence of Cryptosporidium oocysts in water samples from bores ranging in depth from 60 to 106 m raises a number of questions. How were oocysts transferred from the surface to such depths? Damaged pipelines used for water distribution would not explain oocysts contamination, as water samples were collected from bores. The geological characteristics of the ground may explain transfer of Cryptosporidium oocysts to groundwater, as Haiti is mostly mountainous and these mountains are mainly limestone, although some volcanic formations can be found, particularly in the Massif du Nord. Karstic features, such as limestone caves, grottoes, and subterranean rivers, are therefore present in many parts of the country, including the Cul-de-Sac Plain aquifer that constitutes the main source of drinking water for the urban population of Port-au-Prince. Karst aquifers are known to be highly susceptible to microbiological pollution [11, 17, 18].

The other objective of this study was to identify Cryptosporidium species from oocysts collected in water samples. Although PCR is extremely sensitive and efficient when a pure DNA suspension is used, amplification can be reduced by inhibitory factors frequently present in environmental samples, which could be a possible explanation for the unsuccessful PCR assay for sample No. 7 despite the positive IMS-IFA result. Contrary to the experimental results of Ruecker’s study in 2011 [26], PCR assays performed with DNA extract volumes < 10 µl were less successful for our environmental samples, as these assays were positive for only four samples (No. 13, 14, 15, 16) containing the highest loads of Cryptosporidium oocysts (Table 1). The success of this method therefore depends on the presence of a sufficient quantity of Cryptosporidium DNA before dilution. In Haiti, Cryptosporidium species identification has been previously performed on the stools of patients with cryptosporidiosis, but never on environmental samples. Under our working conditions, using both genus-specific nested PCR-RFLP analysis and direct DNA sequencing, only Cryptosporidium parvum was identified in the water samples studied (Table 1). This result was somewhat surprising, as a diversity of Cryptosporidium species was expected in view of the insalubrious environment in the two cities. Practically no tests are performed in Haiti to ensure the quality of the water distributed by public services. In urban areas, basic services for collection and treatment of wastewater and solid wastes including sewerage are not available. Latrines and septic tanks result in faecal contamination of alluvial and karstic aquifers [9]. Moreover, animals are allowed to roam freely in the cities and scatter their stools everywhere. Although animals have not yet been evaluated as a possible reservoir for Cryptosporidium in Haiti, the close proximity between Haitians and both domestic and wild animals may affect the rate of Cryptosporidium infection, as a study conducted in Port-au-Prince between 2000 and 2001 based on 1,529 stool sample examinations revealed the presence of Cryptosporidium oocysts in 158 (10.3%) stool samples [24]. In this same study, genotyping of 69 Cryptosporidium oocysts demonstrated the presence of three Cryptosporidium species: C. hominis (41), C. parvum (26) and C. felis (2). Poor resolution of possible mixed species could therefore be due to the methodology used. However, the performance of nested PCR-RFLP analysis for Cryptosporidium genotyping has been clearly established [8]. More recently, the performance of repeated nested PCR-RFLP, as used in our study, was assessed for its ability to detect mixtures of Cryptosporidium species in water samples [26]. Furthermore, it had been reported that direct sequencing of PCR products is more accurate than cloning to characterize Cryptosporidium species in water [27]. Despite the use of these two methods, only C. parvum was found in the water samples analysed in the present study. Either these samples effectively contained a single species, or they contained a mixture of species that was not detected by our methods. A possible explanation could be that C. parvum was the predominant species present, preventing identification of the less abundant species by molecular methods. The results of this study therefore cannot exclude the presence of other less abundant Cryptosporidium species in the water samples analysed, as most of the roaming animals in the cities of the study
were pigs, oxen, goats and donkeys, which could be the main sources of \textit{C. parvum} oocysts in the environment. However, this explanation would appear to be in disagreement with the results of a previous study showing that \textit{C. hominis} was the leading cause of human cryptosporidiosis in Haiti [24]. Human-to-human transmission of \textit{C. hominis}, the species most commonly responsible for human infection, would therefore appear to be frequent. However, a study conducted in the same area in 2006 revealed that human-to-human transmission appeared to be an unlikely route of infection, as among 123 persons living in close contact with 56 HIV-positive subjects contaminated by \textit{Cryptosporidium}, only 1 was infected [25].

**Conclusion**

This study confirmed \textit{Cryptosporidium} oocysts pollution of drinking water in the study sites, probably worsened by the January 2010 earthquake, but it failed to reveal the expected species diversity of \textit{Cryptosporidium}. Nevertheless, the study constitutes the first molecular characterization of \textit{Cryptosporidium} oocysts conducted on environmental samples in Haiti and it also highlights the difficulty of detecting mixed populations of \textit{Cryptosporidium} species on environmental samples.

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