Tropical Medicine and International Health doi:10.1111/j.1365-3156.2010.02673.x

VOLUME 16 NO 1 PP 12–17 JANUARY 2011

Cryptosporidium parvum causes gastroenteritis epidemics in the Nablus region of Palestine

Ayman S. Hussein

Faculty of Medicine, Genetics Laboratory, An-Najah National University, Nablus, Palestine

Summary A total of 30 faecal samples collected from individuals admitted to a local hospital in Nablus city in Palestine with gastroenteritis symptoms, plus five faecal samples from healthy individuals living in the same area were screened for the presence of *Cryptosporidium* spp. by microscopic analysis using malachite green negative staining. Molecular techniques were used to confirm the microscopic identification. All 30 samples from individuals with gastroenteritis symptoms were positive by both techniques. No other parasites were found in the faecal material of patients or healthy individuals. To explore the source of the outbreak, water was collected from various reservoirs and springs that supply the city with drinking water. Al-Qaryoon water spring was found to be contaminated with *Cryptosporidium* using both microscopic and molecular analysis. No other water resources were found to be contaminated. Genotyping analysis of *Cryptosporidium* oocysts using PCR-RFLP technique identified the parasite as *C. parvum*.

keywords *Cryptosporidium*, PCR-RFLP, Water contamination, Palestine

Introduction

Cryptosporidiosis is a common gastrointestinal disease and has been recognized worldwide as a common cause of diarrhoea in otherwise healthy children. The disease is widespread in many developed and developing countries (Hunter 2003). The risk of infection is associated with drinking water from poorly treated public and private supplies, swimming pools, contact with farm animals and spread within institutions such as day care centres. There are currently 19 spp. of the genus *Cryptosporidium*, but the most important human pathogens are *Cryptosporidium hominis* and *Cryptosporidium parvum* (Fayer *et al.* 2000; Fayer 2009; Xiao 2010). The illness is usually self-limiting; however, it can lead to serious health consequences (Hunter *et al.* 2004). In people with a poor immune system, the disease may be prolonged or even fatal (Cacciò *et al.* 2005). Parasites are usually transmitted from person-to-person or by animals. Person-to-person transmission usually occurs directly by the faecal-oral route. Zoonotic transmission from cattle and sheep has been documented (Hunter & Thompson 2005).

Drinking water contaminated with *Cryptosporidium* oocysts is a recognized risk factor for human illness (McAnulty *et al.* 2000; Goh *et al.* 2004 Goh *et al.* 2005; Almeida *et al.* 2010; Smith *et al.* 2010). Water contamination can arise from a variety of sources including oocysts from infected humans and livestock (Smith *et al.* 1995). Oocysts are resistant to most disinfectants used to treat drinking water, and thus infectious oocysts can be transmitted to susceptible consumers of that water (Mac Kenzie *et al.* 1994; Smith & Nichols 2009).

In October 2008, an outbreak took place in the city of Nablus in which several people were admitted to hospital showing symptoms such as diarrhoea, strong abdominal pain and periodic vomiting. The present study describes an outbreak of *C. parvum* using microscopy and molecular techniques.

Materials and methods

Study location and study population

The study was approved by the Ethics Committee at An-Najah National University and carried out in the Genetics Laboratory/Faculty of Medicine. The present study reports the identification of *C. parvum* in both stool samples collected from patients admitted to a local hospital in Nablus city in October 2008 and from Al-Qaryoon water spring that supplies Nablus city (Figures 1 and 2). Nablus is a city in Palestine located about 80 km north of Jerusalem (Figure 1). The total population of Nablus city is about 200 000 (Palestinian Central Bureau of Statistics 2009). The city depends on eight reservoirs and springs for

Figure 1 The regional setting of the West Bank and the location of Nablus.

drinking water (Figure 2) where water is treated only with chlorine dioxide. Al-Qaryoon spring supplies the old city with water.

Stool samples were collected from 30 patients admitted to Alwatni hospital with abdominal pain, vomiting and diarrhoea and from five healthy individuals (controls) who did not show any symptoms of gastroenteritis. Stool samples were transferred to the laboratory and used for parasite staining and DNA extraction.

For the identification of parasites in water 1-l samples from wells, springs and reservoirs used by Nablus city (Al-Qaryoon, Ain Defna water springs, Der Sharaf, Al-Bithan and Al-fara'a wells (Figure 2)) or 1-l samples of sterilized water (control) were used for the isolation of *Cryptosporidium* spp. using the ferric sulphate flocculation method as described by Karanis and Kimura (2002). Oocysts in the final pellets (0.5 ml) were counted and used for microscopic examination or DNA extraction. For further use, oocysts were stored in 2.5% potassium dichromate solution at 4 °C.

Parasite staining

Isolates either from faecal materials of healthy individuals or patients and from water purified by the ferric sulphate flocculation were analysed microscopically. For identification of *Cryptosporidium* spp., malachite green negative staining was used as described by Elliot *et al.* (1999). Microscopy for all samples either from faecal material of healthy individuals and patients or from water sources was also performed for other parasites such as *Amoeba*, *Giardia* and parasitic worms. Positive samples for *Cryptosporidium* spp. were further analysed by PCR amplification after DNA extraction. Faecal materials from healthy individuals were used for DNA extraction and DNA amplification as control.

Isolation of DNA from human stool and water and amplification of Cryptosporidium 18S rDNA

Genomic DNA extraction was performed from approximately 150 mg of faecal samples and from purified oocysts of Al-Qaryoon water (200 oocysts/ml), which were microscopically positive for *Cryptosporidium* using the QIAamp DNA stool mini test kit (Qiagen, Hilden, Germany), applied according to the manufacturer's instructions.

For the identification of *Cryptosporidium* spp., a two-step nested PCR protocol was used to amplify the 18S rDNA gene using a previously described method (Xiao *et al.* 2001). PCR products were subjected to electrophoresis in 1.5% (w/v) Agarose-TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) gels stained with ethidium bromide. Sequencing of the primary PCR product was performed using an ABI Prism Dye Terminator cycle sequencing kit (Life Sciences, Amersham, UK). Nucleotide sequences were analysed using Chromas Lite version 2.0.

PCR-RFLP analysis

Isolates were further genotyped using a two-step nested PCR using the CDC 18S rDNA primers and restriction enzyme analysis using *Dde*I, *Ssp*I and *Vsp*I (Promega, Madison, WI, USA) according to the method described by Xiao *et al.* (1999, 2001) where 20 μl of secondary PCR product was used with each restriction enzyme reaction.

Results

All 30 faecal samples from individuals with diarrhoea showed *Cryptosporidium* oocysts when stained with malachite green (Figure 3). Faecal material from healthy individuals did not show any parasites. *Cryptosporidium* oocysts were shown in Al-Qaryoon spring water only and not in the other water sources. No other parasites such as *Amoeba*, *Giardia* or parasitic worms were detected in faecal material from patients, healthy individuals or other water sources in the city.

PCR using DNA extracted from the 30 stool samples that were microscopically positive amplified the expected DNA fragments of approximately 760 and 580 bp at the 18S rDNA locus (Figure 4, lanes 2 & 3). No amplification was shown using DNA extracted from stool samples of five healthy individuals living in the same area (Figure 4, lane 1). Only DNA extracted from Al-Qaryoon water spring

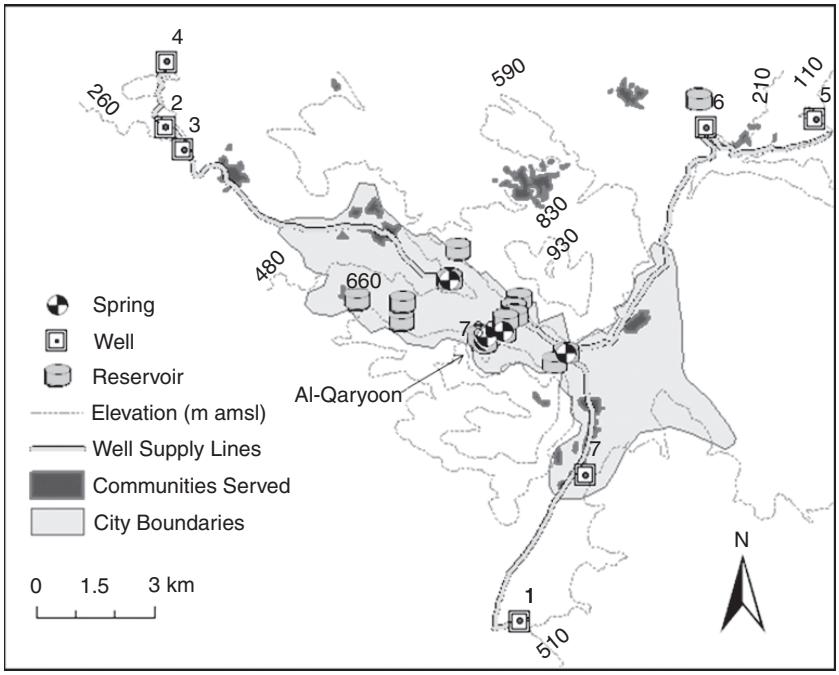

Figure 2 Water collection sites in Nablus. The spatial distribution of the wells, springs and reservoirs utilized by the city along with the communities supplied with water by the municipality is shown. Selected elevation contour lines are shown in the figure. Al-Qaryoon spring is shown by an arrow.

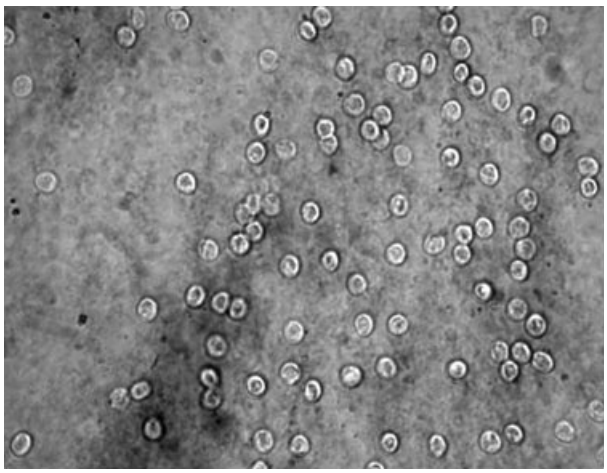

Figure 3 Microscopic detection of *Cryptosporidium*. Oocysts collected from the stool of Palestinian patients with gastroenteritis symptoms were stained with malachite green (100 × magnification).

showed *Cryptosporidium*-specific amplification using 18S rDNA primers (Figure 5, lanes 2 & 3). PCR-Restriction Fragment Length Polymorphism (RFLP) analysis using the CDC 18SF2/CDC 18SR2 nested primers and D*del*, S*sp*I, V*spI* restriction enzymes revealed *C. parvum*-specific patterns for all human samples and for the Al-Qaryoon water spring (Figure 6).

Discussion

The results of the present study suggest that *Cryptosporidium* was the causative agent of the diarrhoea and vomiting outbreak that occurred in the Nablus region of Palestine. In tropical countries, *Cryptosporidium* transmission is usually associated with the rainy season, and water-borne transmission is considered a major route in the epidemiology of cryptosporidiosis in these areas (Bhattacharya *et al.* 1997; Bern *et al.* 2000). The water in Al-Qaryoon spring is considered as shallow surface water that could have been contaminated from dirt washed out by rainfall which usually falls for the first time in October, the start of the rainy season. There is no filtration of water in Nablus that would trap the parasite oocysts. The results of this study thus indicate that the source of infection is because of contamination of Al-Qaryoon water with *Cryptosporidium* oocysts. No other water sources were found contaminated with the parasite. These

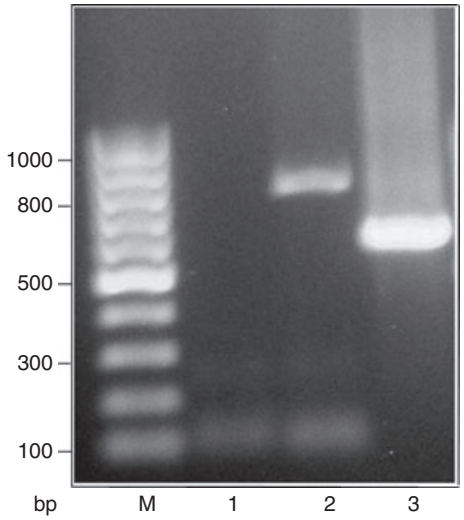

Figure 4 Detection of *Cryptosporidium* by polymerase chain reaction amplification of DNA. Lane M, Low 100-bp molecular ladder. Lane 1, DNA template extracted from stool of healthy person and amplified with outer primers; lane 2, DNA template extracted from stool of patient amplified with outer primers; lane 3, DNA template extracted from stool of healthy person and amplified with inner primers. Outer primers: (18SiCF2/18SiCR2); inner primers: (18SiCF1/18SiCR1.

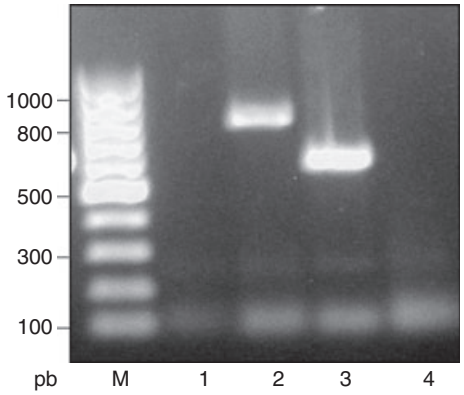

Figure 5 Detection of *Cryptosporidium* by polymerase chain reaction amplification of DNA. Lane M, Low 100-bp molecular ladder. Lane 1, template from sterilized water using outer primers; lane 2, DNA template extracted from Al-Qaryoon water spring amplified with outer primers; lane 3, DNA template extracted from Al-Qaryoon water spring amplified with inner primers. Lane 4, template from other water sources amplified with outer primers. Outer primers: (18SiCF2/18SiCR2); inner primers:18SiCF1/18SiCR1.

parasite-free water sources are far from the city by several miles and far away from sources of contamination of slaughterhouses or sewer overflow.

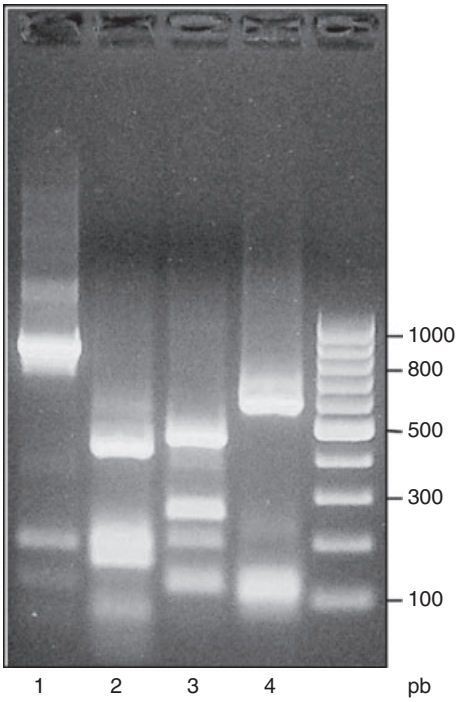

Figure 6 Genotyping of *Cryptosporidium* DNA extracted from stool of patient with SSU rRNA-based PCR-RFLP technique. Secondary PCR product was digested with *Dde*I (lane 2), *Ssp*I (lane 3) and *Vsp*I (lane 4). As a control, secondary PCR product was used using all PCR-RFLP ingredients except of digestion enzyme (lane 1). Lane M, Low 100-bp molecular ladder.

Genotyping of *Cryptosporidium* has revealed that the parasite responsible for the outbreak of gastroenterities in Nablus city is *C. parvum*. Abu-Alrub *et al.* (2008) studied the prevalence of *Cryptosporidium* spp. in West Bank, Palestine by acid fast staining technology. However, the present study is the first to use the molecular technology in diagnosis of cryptosporidiosis in Palestine. In neighbouring countries, *C. parvum* has been reported in a number of studies to be the causative agent of cryptosporidiosis (Areeshi *et al.* 2007, Mahgoub *et al.* 2004; Tanriverdi *et al.* 2006). In Israel for example, *C. parvum* has been reported to be the causative agent of cryptosporidiosis in infants (Robin *et al.* 2001) or in cattle (Tanriverdi *et al.* 2006) using either enzyme-linked immunosorbent assay (Robin *et al.* 2001) or multiple polymorphic genetic markers (Tanriverdi *et al.* 2006). In other countries, such as Jordan and Saudi Arabia, *C. parvum* in addition to other species have been reported (Mahgoub *et al.* 2004; Areeshi *et al.* 2007; Hijjawi *et al.* 2010). On the other hand, both *C. parvum* and *C. hominis* have been reported from Kuwaiti children

with gastrointestinal symptoms using genetic tools (Sulaiman *et al.* 2005).

Our study shows the importance of routine surveillance of water reservoirs in the country. Further studies using more differentiating targets such as GP60 are recommended.

Acknowledgements

I am grateful to Mrs Alaa Bashir and Mr Methqal Ahmad for their assistance in the microscopic analysis. My thanks are to professor Murray Selkirk (Imperial College London, UK) for revising the manuscript. My thanks go also to Dr Nawal Hijjawi (The Hashmite University, Jordan) for valuable discussion regarding the molecular analysis throughout this study. Funding of this research is obtained from An-Najah National University.

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Corresponding Author **Ayman S. Hussein,** Genetics Laboratory, Faculty of Medicine, An-Najah National University, Nablus, Palestine. Tel.: 972 9 23 45113/7; Fax: 972 9 23 45982; E-mail: ashussein@najah.edu