Cryptosporidium parvum causes gastroenteritis epidemics in the Nablus region of Palestine

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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 (Caccio 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 (MacKenzie 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
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 DdeI, SspI and VspI (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...
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 DdeI, SspI, VspI 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
Parasite-free water sources are far from the city by several miles and far away from sources of contamination of slaughterhouses or sewer overflow.

Genotyping of *Cryptosporidium* has revealed that the parasite responsible for the outbreak of gastroenteritis 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; HJJAWI *et al.* 2010). On the other hand, both *C. parvum* and *C. hominis* have been reported from Kuwaiti children.
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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.

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