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# Prevalences of *Giardia lamblia* and *Cryptosporidium parvum* infection in adults presenting with chronic diarrhoea

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*Giardia lamblia* and *Cryptosporidium parvum* are both waterborne pathogens associated with diarrhoea in developing countries. In a recent study based at the Aga Khan University in Karachi, 334 adults aged 16–83 years (178 patients with chronic diarrhoea and 156 diarrhoea-free volunteers who acted as controls) were checked for infection with these parasites, using stool microscopy and/or PCR. Overall, 21 (6.3%) and 29 (8.7%) of the subjects were found positive for *G. lamblia* by microscopy and PCR, respectively, while the corresponding values for *C. parvum* were 13 (3.9%) and 14 (4.2%). Although, compared with the diarrhoea-free controls, the patients with diarrhoea were not significantly more likely to be found infected with *Giardia*, either by microscopy [15 (8.4%) *v.* six (3.8%); $P=0.085$] or PCR [19 (10.7%) *v.* 10 (6.4%); $P=0.167$], they were significantly more likely to be found infected with *C. parvum*, both by microscopy [11 (6.2%) *v.* two (1.3%); $P=0.024$] and by PCR [12 (6.7%) *v.* two (1.3%); $P=0.014$].

The 19 patients found PCR-positive for *Giardia* comprised 10 (67%) of the 15 found smear-positive for the same parasite but only nine (5%) of the 163 found smear-negative ($\kappa=0.545$; $P<0.001$). Similarly, the 12 patients found PCR-positive for *Cryptosporidium* comprised all 11 (100%) patients found smear-positive for the same parasite but only one (0.6%) of the 167 found smear-negative ($\kappa=0.954$; $P<0.001$).

Although *C. parvum* was associated with chronic diarrhoea in the present study, the carriage of *G. lamblia* often appeared asymptomatic.

Diarrhoeal diseases are common among humans in the developing world and are major causes of morbidity and mortality (Kosek *et al.*, 2003). There are many causes of diarrhoea, including bacteria (e.g. *Campylobacter jejuni*, *Shigella*, *Salmonella*, enterotoxigenic *Escherichia coli* and cytotoxigenic *Clostridium difficile*), viruses (e.g. Norwalk-like viruses, rotaviruses and enteric adenoviruses) and parasites (e.g. *Giardia lamblia*, *Cryptosporidium* species and *Entamoeba histolytica*) (Guerrant *et al.*, 1990).

*Giardia lamblia* infection is very common throughout the world and considered to be one of the main parasitic causes of infective diarrhoea in developing countries (Mehraj *et al.*, 2008). The laboratory diagnosis of *G. lamblia* infection is usually based on the microscopical examination of stool samples or serological tests such as direct fluorescent antibody assays and antigen-detection ELISA (Garcia and Shimizu, 1997; Mank *et al.*, 1997). Diagnosis based on PCR, however, tends to offer better specificity and sensitivity (Ghosh *et al.*, 2000; Verweij *et al.*, 2003).

In terms of human health, the two most important *Cryptosporidium* species are probably *Cr. hominis* and *Cr. parvum*, which are both associated with diarrhoea and have identical life-cycles and oocyst morphology.

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Although *Cr. hominis* predominantly colonises the human gastro-intestinal tract, *Cr. parvum* has been found in several other mammals. *Cryptosporidium parvum* has been recognised as the cause of waterborne and food-borne outbreaks of gastro-enteritis and is known to cause severe diarrhoea in AIDS patients (Kosek *et al.*, 2003). Modified acid-fast and trichrome stains are commonly used in the microscopical detection of *Cr. parvum* oocysts in faecal smears but the sensitivity and specificity of such microscopy appears to be generally low, varying with the experience of the microscopist (Weber *et al.*, 1991). Although monoclonal antibodies against *Cryptosporidium* antigens have been successfully used for fluorescence microscopy and in antigen-detection ELISA (Garcia and Shimizu, 1997; Schuster and Chiodini, 2001; Chen *et al.*, 2002), cross-reactivity with other microorganisms has limited the usefulness of such immunological techniques (Webster *et al.*, 1996; Fayer *et al.*, 2000; Chappell and Okhuysen, 2002). Molecular methods of detection, based on PCR, have, however, been found to be sensitive and specific in the detection of *Cr. parvum* in faecal samples (Webster *et al.*, 1996; Morgan *et al.*, 1998).

Humans become infected when they ingest *G. lamblia* cysts or *Cr. parvum* oocysts in contaminated food or water or — in disadvantaged community settings and day-care centres where hygiene levels may be compromised — in faeces. The main aims of the present study were to determine the prevalences of *G. lamblia* and *Cr. parvum* infection among adults with chronic diarrhoea (who presented at a gastro-enterology clinic in Pakistan) and among the apparently healthy subjects used as controls, and to determine if either parasite was associated with the diarrhoea.

## PATIENTS AND METHODS

### Patients

The 334 stool samples that were examined were all collected between January 2008 and January 2010, either from 178 patients (121 men and 57 women) aged 16–83 years attending the gastro-enterology outpatient clinic at the Aga Khan University (AKU) in Karachi, Pakistan, or from 156 apparently healthy volunteers (102 men and 54 women, all residents of Karachi) who served as controls. At the time his or her stool sample was collected, each patient had suffered from intermittent diarrhoea for at least the previous 6 months. The mean (S.D.) ages of the patients and controls were similar, at 40 (15) and 42 (14) years, respectively.

The study protocol was approved by the AKU's Ethics Review Committee.

### Parasitology

Each stool specimen was checked for *G. lamblia* cysts and *Cr. parvum* oocysts by microscopy and for *G. lamblia* and *Cr. parvum* DNA by PCR (see below). Other parasites detected during the microscopy (such as *Blastocystis hominis* and *Entamoeba* species) were recorded.

Although each stool sample was also checked, using standard microbiological methods, for *Salmonella* spp., *Shigella sonnei*, *Campylobacter jejuni*, *Clostridium difficile* and *Vibrio cholerae*, the funding for a viral screen was not available.

#### MICROSCOPY OF FAECAL SMEARS

Stool samples were examined for *G. lamblia* cysts as unfixed wet smears and for *Cr. parvum* oocysts as dry, fixed and stained smears (Zaman and Khan, 1994; Ignatius *et al.*, 1997). For each wet smear, approximately 2 mg of faeces were emulsified on a glass slide in a drop of physiological saline or Lugol's iodine and covered with a coverslip. Dry smears were fixed in sodium-acetate–acetic acid–formalin (SAF) and then stained with acid-fast trichrome stain (Ignatius *et al.*, 1997). Each smear was carefully examined, at both $\times 100$ and $\times 400$, for at least 10 min.

EXTRACTION OF GENOMIC DNA

DNA was extracted from unfixed stool samples, using a commercial kit (Stool DNA Extraction kit; QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and then stored at −20°C until used as template in each of two types of PCR (one for *G. lamblia* and one for *Cr. parvum*).

PCR

The primers used in the PCR have been described previously (see Table 1). One PCR used a primer pair that amplifies a 485-bp *G. lamblia* glutamate dehydrogenase (*gdh*) locus and the other a primer pair that amplifies a 369-bp region of the thrombospondin-related adhesive protein (TRAP-C2) of *Cr. parvum* (Peng *et al.*, 1997; Elwin *et al.*, 2001; Read *et al.*, 2004).

Each 25-μl reaction mixture contained 2.5 μl 10× PCR buffer (Promega, Fitchburg, WI), 2.0 μl 25 mM $MgCl_2$ (Promega), 0.4 μl of a mix containing each deoxyribonucleotide triphosphate at 10 mM (Promega), 2.5 U *Taq* polymerase (Promega), 0.25 μmol of each primer, and 2.0 μl template DNA. The thermocycler used was set to give 5 min at 94°C, then 35 cycles, each of 60 s (*Giardia*) or 70 s (*Cryptosporidium*) at 94°C, 60 s at 59°C and 90 s at 72°C, followed by 5 min at 72°C. The PCR products and molecular markers producing a 100-bp ladder (Promega) were subjected to electrophoresis in 2%-agarose gel, with Tris–acetate–EDTA buffer. The PCR amplification for each primer pair was repeated at least three times for each stool sample. Bands were visualized using the Gel DocJ 2000 imaging system (Bio-Rad, Hemel Hempstead, U.K.) after being stained with ethidium bromide.

TABLE 1. *Sequences of the primers used in the PCR for* Giardia lamblia *and* Cryptosporidium parvum

| Primer | Sequence (5′–3′) | Amplicon size (bp) | Reference |
|---|---|---|---|
| *Giardia lamblia* | | 485 | Read *et al.* (2004) |
| Forward | TCA ACG TCA ACC GCG GCT TCC GT | | |
| Reverse | GTT GTC CTTGCA CAT CTCC | | |
| *Cryptosporidium parvum* | | 369 | Peng *et al.* (1997) |
| Forward | CAT ATT CCC TGT CCC TTG AGT TGT | | |
| Reverse | TGG ACA ACC CAA ATG CAG AC | | |

## Data Analysis

All data were analysed using version 16.0 of the SPSS software package (SPSS Inc, Chicago, IL). Results are expressed as means (S.D.) for the continuous variables (such as age) and as numbers and percentages, of the subjects or two groups of subjects, for the categorical data (such as gender, positivity in one of the investigations, presence of diarrhoea etc). Univariate analysis was performed using independent-sample *t*-test, Pearson $\chi^2$ test or Fisher's exact tests, as appropriate. The level of agreement between the results of the two methods of testing was evaluated using kappa (κ) tests. A *P*-value of $<0.05$ was considered indicative of a statistically significant difference or association.

## RESULTS

The main results are summarized in Table 2. Overall, 21 (6.3%) of the 334 subjects were found smear-positive for *Giardia* cysts, 29 (8.7%) PCR-positive for *Giardia* DNA, 13 (3.9%) smear-positive for *Cryptosporidium* oocysts, and 14 (4.2%) PCR-positive for *Cryptosporidium* DNA. *Endolimax nana* cysts, *Blastocystis hominis*

and *Entamoeba* sp. were seen in the faecal smears of two (0.6%), 19 (5.7%) and eight (2.4%) of the subjects but bacteria that are known to cause diarrhoea were not detected in the stools of any of the subjects.

### Prevalences of *Giardia* and *Cryptosporidium* Infection

Although the prevalence of *Giardia* infection — whether detected by microscopy or PCR — was not significantly higher among the patients than among the controls (Table 2), the patients were significantly more likely to be found smear-positive for *Cryptosporidium* oocysts ($P=0.024$) and PCR-positive for *Cryptosporidium* DNA ($P=0.014$) than the controls (Table 2).

#### COMPARISON OF THE RESULTS OF STOOL MICROSCOPY AND PCR

The 19 patients found PCR-positive for *Giardia* comprised 10 (67%) of the 15 found smear-positive for the same parasite but only nine (5%) of the 163 found smear-negative ($\kappa=0.545$; $P<0.001$). Similarly, the 12 patients found PCR-positive for *Cryptosporidium* comprised all 11 (100%) patients found smear-positive for the same parasite but only one (0.6%) of the 167 found smear-negative ($\kappa=0.954$; $P<0.001$).

#### *Giardia–Cryptosporidium* CO-INFECTION

The prevalence of *Giardia–Cryptosporidium* co-infection in the patients, whether detected by microscopy alone ($P=0.251$) or PCR alone ($P=0.126$), was not significantly different to that in the controls (Table 2).

## DISCUSSION

*Giardia lamblia* and *Cr. parvum* are both considered important causes of diarrhoea but infections with either parasite often cause non-specific symptoms, making it difficult to identify clinically which, if either, species is involved. Of the diagnostic tests currently available, those based on PCR appear to offer the highest sensitivity and specificity, as well as reasonable cost-effectiveness and speed, whether designed to detect *Giardia* (Nikaeen *et al.*, 2003; Savioli *et al.*, 2006) or *Cryptosporidium* DNA (Webster *et al.*, 1996; Morgan *et al.*, 1998).

TABLE 2. *Prevalences of* Giardia lamblia *and* Cryptosporidium parvum *infection detected, using microscopy or PCR, among the 178 cases of chronic diarrhoea and the 156 asymptomatic controls*

| Parasite(s) and detection method | Result | No. and (%) of: Patients with diarrhoea | Controls | *P* |
|---|---|---|---|---|
| *Giardia lamblia* | | | | |
| Microscopy | Positive | 15 (8.4) | 6 (3.8) | 0.085 |
| | Negative | 163 (91.6) | 150 (96.1) | |
| PCR | Positive | 19 (10.7) | 10 (6.4) | 0.167 |
| | Negative | 159 (89.3) | 146 (93.6) | |
| *Cryptosporidium parvum* | | | | |
| Microscopy | Positive | 11 (6.2) | 2 (1.3) | 0.024 |
| | Negative | 167 (93.8) | 154 (98.7) | |
| PCR | Positive | 12 (6.7) | 2 (1.3) | 0.014 |
| | Negative | 166 (93.2) | 154 (98.7) | |
| *Giardia lamblia–Cryptosporidium parvum* CO-INFECTION | | | | |
| Microscopy | Positive | 3 (1.7) | 0 (0) | 0.251 |
| | Negative | 175 (98.3) | 156 (100) | |
| PCR | Positive | 4 (2.2) | 0 (0) | 0.126 |
| | Negative | 174 (97.8) | 156 (100) | |

In the present study, curiously, *Cr. parvum* appeared to be associated with chronic diarrhoea but *G. lamblia* did not. The numbers of subjects found infected with either parasite were, however, quite low, and statistically significant associations between chronic diarrhoea and both *G. lamblia* infection and *Giardia–Cryptosporidium* co-infection may have been detected if the sample sizes had been much larger.

Although more subjects were found *Giardia*-positive by PCR than by stool microscopy, the two detection methods gave very similar results for *Cr. parvum*. Encouragingly, there was a high level of agreement between the results of the microscopy and the results of the PCR, both for *G. lamblia* and *Cr. parvum*. It would have been interesting if the present study had included antigen-detection ELISA (for both *Giardia* and *Cryptosporidium*) and *Cryptosporidium* genotyping (the results of which can indicate the probable source of infection) but the necessary resources were not available.

In the present study, of the 21 subjects found stool-positive for *Giardia*, 15 had chronic diarrhoea but six (29%) appeared diarrhoea-free and asymptomatic. Similarly, of the 29 found PCR-positive for *Giardia*, 10 (34%) were asymptomatic. Asymptomatic *Giardia* infection has previously been reported to be quite common in Pakistan (Ensink *et al.*, 2006). In a cross-sectional survey of a random sample of 350 children aged 1–5 years from an urban slum in Karachi, faecal samples from 29% of the subjects were found smear-positive for *G. lamblia*, which was the most frequently detected intestinal parasite (Mehraj *et al.*, 2008).

In their study of children from the Pakistani city of Rawalpindi, as in the present study, Iqbal *et al.* (1999) found a positive association between *Cryptosporidium* infection and diarrhoea, such infection being detected in 10.3% of the children with diarrhoea and 3.3% of the other children. Of the immunosuppressed patients from Karachi investigated by Baqai *et al.* (2005), almost half (40%) were found faecal-smear-positive for *Cryptosporidium*.

The results of other case–control studies have demonstrated the importance of person-to-person spread, travel, and potable and recreational water as risk factors for sporadic disease associated with *Giardia–Cryptosporidium* co-infection (Hunter and Thomson, 2005). Although conventional sewage treatment considerably reduces the load of *Cryptosporidium* oocysts and *Giardia* cysts, large numbers may remain in the treated water (Smith and Grimason, 2003). Fortunately, although *Giardia* cysts and *Cryptosporidium* oocysts in surface and ground water have been linked to disease outbreaks (Nichols and Smith, 2002; Smith and Grimason, 2003; Karanis *et al.*, 2007), the small numbers of *Cryptosporidium* oocysts sometimes found in drinking water generally appear to have little epidemiological significance (Smith and Grimason, 2003). In fact, host immunity, kept intact by low-grade exposure, may help to prevent severe outbreaks of cryptosporidiosis and giardiasis.

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