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Isolation and Genotyping of *Cryptosporidium* among HIV and Non-HIV Infected Patients in Jeddah, Saudi Arabia

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Abstract: *Cryptosporidium* is an opportunistic apicomplexan parasite that can cause severe diarrhea in children and immunocompromised persons. In this study, 210 stool samples were collected from adult and children patients, of which 47% are HIV-infected patients in Saudi Arabia. *Cryptosporidium* identification was performed by both modified Kinyoun acid fast staining and molecular analysis using nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Characterization in our analysis was setup on the amplification of a DNA fragment from the *Cryptosporidium* oocyst wall protein (COWP) gene followed by digestion with *RsaI* restriction enzyme. Nested PCR based on SSU-rRNA product after digestion with *Ssp*I and *Vsp*I restriction enzymes was also performed. A total of three samples (1.43%) were positive in both methods. Two samples were identified as *Cryptosporidium parvum* and the third one as *Cryptosporidium hominis*. This is the first study in Saudi Arabia to characterize *Cryptosporidium* in HIV-infected persons. Our finding does not exclude the zoonotic transmission of the disease and further studies are essential for better understanding for the anticipated risk factors and sources of infection in Saudi Arabia.

Key words: *Cryptosporidium* · Parasite · HIV Infected · Saudi Arabia · SSU-rRNA

INTRODUCTION

Cryptosporidium is sporozoan parasite that mainly infects the gastrointestinal tract of a wide range of vertebrate hosts including mammals, rodents, birds, reptiles and fish [1-4]. Life cycle in the host involve both asexual and sexual reproduction [5, 6]. Cryptosporidiosis is a major cause of diarrhea, which is usually self-limiting in healthy host but can be life-threatening in children and immunocompromised individuals [5, 7-9]. Transmission is through the fecal-oral route, following direct or indirect contact with *Cryptosporidium* oocysts via person to person (anthroponosis), animal to person (zoonosis), ingestion of contaminated food/water (foodborne/waterborne), or airborne contact (bioaerosol) [5, 10-12]. Recently, some thirty *Cryptosporidium* species were described, but *C. hominis* and *C. parvum* are the most common species predominantly found in human [13, 14].

Routine diagnostic tests for *Cryptosporidium* in faecal samples rely on detection of oocysts, usually by tinctorial or fluorescence staining of smears. Analytical sensitivity is improved by the use of immunofluorescence microscopy [15]. Since last decade, numerous molecular biological techniques have been developed to detect and differentiate *Cryptosporidium* spp. at species/ genotype and subtype levels. These tools are now increasingly used in epidemiological studies of cryptosporidiosis in endemic and epidemic areas, which has improved significantly the understanding of the transmission of cryptosporidiosis in humans and animals [15-21].

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The aim of this study was to identify the prevalence and genotypes of *Cryptosporidium* parasites among HIV and non-HIV infected patients in Jeddah, Saudi Arabia.

MATERIALS AND METHODS

Stool Samples: Stool samples were obtained from 210 HIV and non-HIV infected patients with or without diarrhea whom referred to pediatric or HIV clinics in three public hospitals in Jeddah. All stool samples were kept unpreserved at 4°C until analyzed.

Kinyouns Modified Cold Staining Microscopy: Very thin layer of feces from the controls and specimens were smeared on microscope slides, then fixed in methanol and allowed to air dry. Smears were flooded with carbol fuchsin stain for 10 minutes. Smears were rinsed with tap water and then decolorized in 1% HCl-Methanol for 10 seconds. After that, all smears were washed with tap water and counter stained with methylene blue for 5 minutes, then washed with tap water and left to air dry. Finally all smears were examined under 100X objectives.

Isolation of *Cryptosporidium* Oocysts: Oocysts were separated from stool samples by flotation technique using saturated sodium chloride (brine) solution. For each specimen, two 15ml centrifuge tubes were used. The first tube was marked at 8, 10 and 13ml. The second tube was marked at the 10ml only. Using first tube, 8ml of brine solution and 2ml of stool were vortexed for 5 seconds. Then 3ml of reverse osmosis water was trickled drop by drop until reached the mark 13ml. This tube was centrifuged at 1100 g for 8 minutes, while break is off. This resulted in formation of four layers. Using the tip of a narrow plastic pasture pipette, the water layer was swirled to form a vortex moving oocysts from the fecal suspension into the water. The water layer was transferred into the second tube and mixed well with 10ml reverse osmosis water. This tube was centrifuged at 1100 g for 5 minutes and then whole supernatant was discarded. Finally the sediment was re-suspended in 1ml reverse osmosis water and stored in fridge until needed.

DNA Extraction: DNA was extracted using QIAGEN DNA mini kit (QIAGEN, Hilden, Germany, Cat # 51306) according to manufacturer's instructions with slight modification as follow; for each sample, 200µl of the oocysts suspension was boiled for one hour then centrifuged at 6000 rcf for 5 seconds. 20µl of proteinase K and 200µl Buffer AL were added and vortexed for 15-20 seconds then centrifuged at 6000 rcf for 5 seconds. Tube was incubated at 56°C for 10 minutes and left at room temperature for 5 minutes to cool down. 200µl of ethanol (96–100%) was added to the lysate, vortexed for 15-20 seconds then centrifuged at 6000 rcf for 5 seconds. The complete lysate was carefully transferred to the QIAamp spin column without moistening the rim into a 2ml collection tube and then centrifuged at 6000 rcf for 1 minute. The QIAamp spin column was placed in a new 2ml collection tube, 500µl Buffer AW1 was added and then centrifuged at 6000 rcf for 1 minute. The QIAamp spin column was placed in a new 2ml collection tube, 500µl Buffer AW2 was added and then centrifuged at 20000 rcf for 4 minutes. The collection tube containing the filtrate was discarded and the QIAamp spin column was transferred into a new labeled 1.5ml flip top microcentrifuge tube. 100µl Buffer AE was directly pipetted onto the center of QIAamp membrane, tube left for 5 minutes then centrifuged at 6000 rcf for 5 minutes to elute DNA. Finally, DNA tube was kept at -20°C until needed.

***Cryptosporidium* Oocyst wall (COWP) Nested-PCR Amplification:** A nested PCR approach using oligonucleotide primers specific for the *Cryptosporidium* oocyst wall (COWP) 5-GTAGATAATGGAAGAGATTGTG-3 and 5-GGACTGAAATACAGGCATTATCTTG-3 was performed. Each PCR mixture contained 5µl of 10x Low Mg buffer, 5µl of each deoxynucleoside triphosphate at a concentration of 2 mM, 3µl of 25 mM $MgCl_2$, 3µl of each primer at a concentration of 10 µM, 0.5µl of 5 U/µl of *Taq* polymerase, 20.5µl of nuclease free water, 10µl of DNA template. The amplification reactions of 40 cycles were initiated by denaturation of the DNA at 95°C for 15 minutes, denaturation at 94°C for 50 seconds, annealing of the primer at 60°C for 30 seconds and extension at 72°C for 50 seconds, with an additional 10 minutes extension at 72°C. Positive (DNA of *C. parvum* and *C. hominis*) and negative controls were included in each amplification run. The PCR product was analyzed by electrophoresis in a 3% agarose gel and visualized after Sybrgreen staining and recorded by UV illumination.

***Cryptosporidium* Oocyst wall Protein (COWP) RFLP Analysis and Visualization:** For the restriction-fragment analysis, 10µl of the PCR products was digested in a 10µl reaction mixture containing 1µl of *Rsa*I restriction enzyme, 2µl of the appropriate 10X restriction buffer and 7µl of water at 37°C for 1 hour. The digest products were fractioned by 3% agarose gel, visualized after Sybrgreen staining under UV illumination.

***Cryptosporidium* SSU rRNA gene (18 s) Nested-PCR Amplification:** For the primary PCR, a PCR product was amplified by using primers 5 -TTC TAG AGC TAA TAC ATG CG-3 and 5 -CCC ATT TCC TTC GAA ACA GGA-3. Each PCR mixture (total volume, 50µl) contained 5µl of 10x Low Mg buffer, each deoxynucleoside triphosphate at a concentration of 2 mM, 5mg/ml of BSA (bovine serum albumin), 1µl of each primer at a concentration of 10µM, 0.5µl of 5U/µl of *Taq* polymerase, 27.5µl of nuclease free water, 5µl of DNA template. The amplification reactions of 35 cycles were initiated by denaturation of the DNA at 95°C for 15 minutes, denaturation at 94°C for 45 seconds, annealing of the primer at 62°C for 45 seconds and extension at 72°C for 1 minutes, with an additional 7 minutes extension at 72°C.

For the secondary PCR, a PCR product was amplified by using 5µl of the primary PCR product and primers 5 –GGA AGG GTT GTA TTT ATT AGA TAA AG-3 and 5 - CTCATAAGGTGCTGAAGGAGTA -3. The PCR mixture and cycling conditions were identical to primary PCR, except that 3µl of 25 mM $MgCl_2$ and 27.5µl of nuclease free water were used. Positive (DNA of *C. parvum* and *C. hominis*) and negative controls, were included in each amplification run. The PCR product was analyzed by electrophoresis in a 2% agarose gel and visualized after Sybrgreen staining and recorded by UV illumination.

***Cryptosporidium* SSU rRNA Gene (18 s) RFLP Analysis and Visualization:** For the restriction-fragment analysis, 10µl of the secondary PCR products was digested in a 10µl reaction mixture containing 1µl (10 U/µl) of *Ssp*I or *Vsp*I, 7µl of nuclease free water and 3µl of the appropriate 10X restriction buffer at 37 °C for The digest products were fractioned by 2% agarose gel, visualized after Sybrgreen staining under UV illumination.

Statistical Analysis: Data analysis was performed using SPSS V17.0 statistic Package.

The study was approved by the Research Ethical Committee of the Saudi Ministry of Health in addition to the Ethics and Research Committee in the Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia.

RESULTS

Patients: this study included 99 (47.1%) HIV infected patients of which 44 suffering from diarrhea and 111 (52.9%) non-HIV infected patients of which 54 having diarrhea (Figure 1). There was no significance association between HIV infection and diarrhea (p>0.5).

Nationality: the majority of patients (90.5%) were Saudis; including the ninety nine HIV infected patients.

Table 1: Summary of patients' age group in relation to diarrhea, HIV infection and *Cryptosporidium* infection.

Age Group (Years)	Total NO. (%)	With Diarrhea (%)	HIV infected (%)	*Cryptosporidium* infected
1-5	107 (51.0)	51 (52.1)	9 (9.1)	2
6-10	26 (12.4)	11 (11.2)	17 (17.2)	0
11-15	16 (7.6)	7 (7.1)	12 (12.1)	0
16-20	2 (1.0)	0 (0.0)	2 (2.0)	0
21 and above	59 (28.1)	29 (29.6)	59 (59.6)	1
Total	*210 (100)*	*98 (100)*	*99 (100)*	*3*

Table 2: Structural analysis of the COWP gene after digestion with the restriction enzymes *RsaI*. Also the structural analysis of the 18S rRNA gene after digestion with the restriction enzymes *SspI* and *VspI*.

Species	*RsaI* digestion	*SspI* digestion	*VspI* digestion
C. parvum	413, 107, 34	449, 254, 108/119	625/628, 104
C. hominis	284,129,107,34	449, 254, 111	561, 104, 70

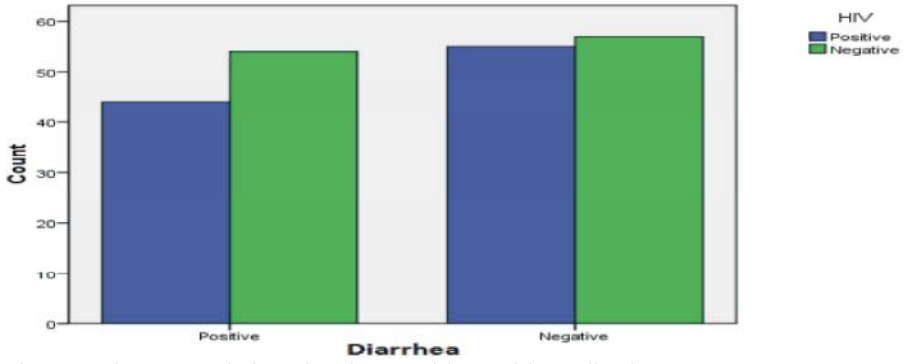

Fig 1: Comparison of HIV and non-HIV infected patients' with or without diarrhea.

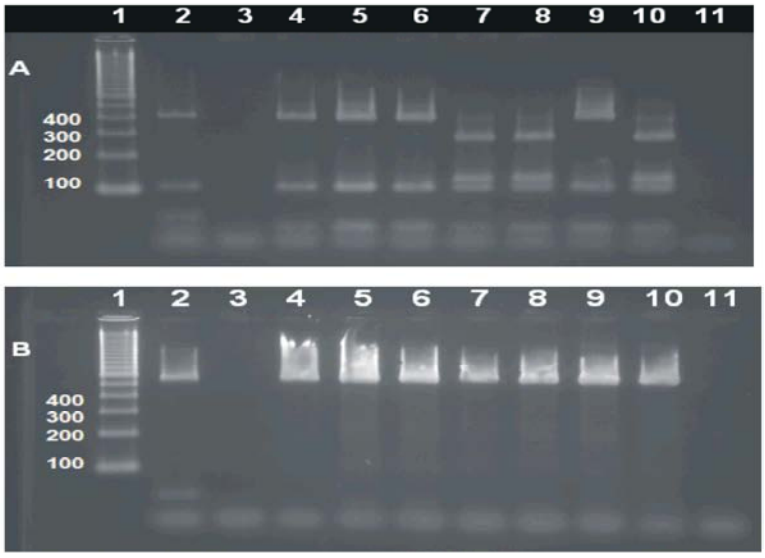

Fig 2: Molecular diagnosis of *Cryptosporidium* parasites by a nested PCR based on COWP before digestion (B) and after digestion with *Rsa*I (A). Lane 1: molecular weight marker; Lane 2, 3, 4, 5, 6, 7 and 8: patient isolates; Lane 9: positive control (*C. hominis*); Lane 10: positive control (*C. parvum*); Lane 11: negative control.

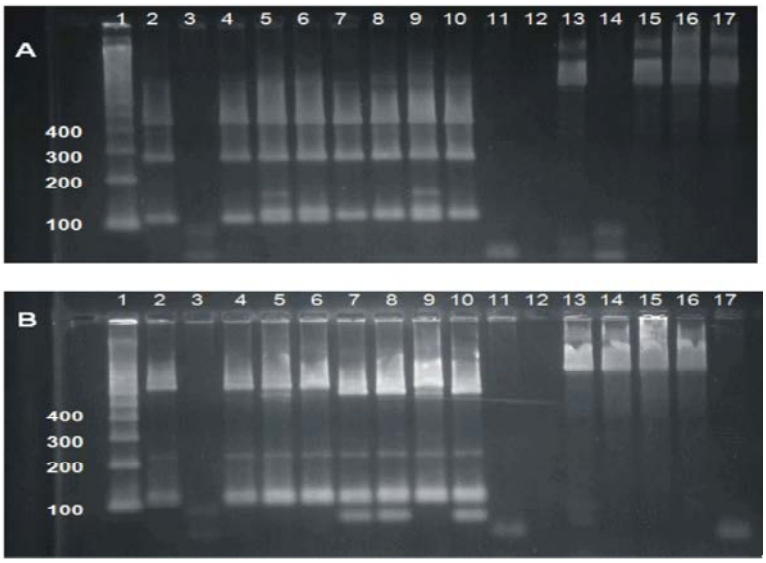

Fig 3: Molecular diagnosis of *Cryptosporidium* parasites by a nested PCR based on SSU-rRNA product, *Ssp*I digestion (A) and *Vsp*I digestion (B). Lane 1: molecular weight marker; Lane 2, 3, 4, 5, 6, 7 and 8: patient isolates after digestion of the nested PCR product; Lane 9: positive control (*C. hominis*); Lane 10: positive control (*C. parvum*); Lane 11: negative control, after digestion of the nested PCR product. Lane 12: no sample, Lane 13, 14, 15, 16 and 17: patient isolates of undigested nested PCR products for the same samples used in Lane 2, 3, 4, 5 and 6 respectively. Some samples were repeated in more than one lane

Gender: out of the 210 patients, 117 (55.7%) were males and 93 (44.3%) were females. There was no significance association between gender and diarrhea (p=0.5), but there was significant correlation with HIV infection (p<0.05).

Age: most of the examined samples (71%) were from the children age groups as illustrated in Table 1. There was no association between age and diarrhea, but there was highly significant correlation with HIV infection (p>0.5 and <0.001 respectively).

Detection of Parasite: Out of the 210 tested samples, *Cryptosporidium* parasite was detected in 3 Saudi male patients (1.43%) by using both microscopic examination and molecular techniques; 2 non-HIV infected children and one HIV infected adult (see Table 1). Genotyping of the detected cases revealed that 2 isolates were *C. parvum* while the third one was *C. hominis* (Table.2, Figure 2 and 3).

DISCUSSION AND CONCLUSION

Cryptosporidiosis in human is cosmopolitan in developed or underdeveloped countries, the most common species associated with more than 90% of human infection are *C. hominis* and *C. parvum* [22-31].

Cryptosporidiosis of human is self-limited in healthy persons, but it can cause chronic watery diarrhea and lead to deadly life threatening complications in immunocompromised patients –mainly HIV patients- and children, accompanied by dehydration, weight loss, abdominal pain, fever, nausea and vomiting [5, 8, 23, 32, 33].

In our study, oocysts were isolated from stool samples by flotation technique using saturated brine solution.

Neither permanent nor fluorescence-based stains can determine the species of *Cryptosporidium*. PCR is more sensitive than conventional and immunological assays for detecting oocysts in faeces. In these instances and particularly when clinical suspicion is high, oocyst negative stool samples should be subjected to molecular methods, which offer both improved sensitivity and species/genotype/subtype identity. Most of these informations about *Cryptosporidium* species and genotypes were determined by nested PCR of small-subunit (SSU) ribosomal RNA gene fragment (18S SSU rRNA) and RFLP analysis.

The present study investigated *Cryptosporidium* molecular characterization for the first time in Saudi Arabia using stool samples from HIV and non-HIV infected patients. The prevalence of *Cryptosporidium* was 1.43%. Modified cold staining revealed oocysts stained red on a blue background with their size ranging between 4-6 µm. Some oocysts appeared amorphous while others contained the characteristic crescent forms sporozoites. We were not able to observe any oocysts' morphological differences in the three infected cases.

Previous worldwide studies revealed different prevalence's rates of *Cryptosporidium* in children and/or HIV infected patients' from Asian [7, 16, 24, 35-48], American [26, 28, 31, 34, 49-59], African [31, 60-65] and European [29, 31, 58] countries or sometimes from different countries in the same study [28, 31]. Our result is consistent with the results for most of these studies.

Our data and finding are not enough to recognize the possible ways of transmission of *Crypstosporidium* in Jeddah. However, we confirmed interestingly that there was no significant association between *Cryptosporidium* infection and diarrhea ($p>0.5$) and among the 98 patients having diarrhea, 54 (55.1%) were non-HIV infected patients. The three Saudi positive cases were: 1 adult, HIV-infected male with diarrhea; 1 child, non-HIV infected female with diarrhea; 1 child, non-HIV infected female with no diarrhea.

In conclusion, further work on more sample sizes is needed for better understanding of the molecular epidemiology, the role of man, animals, water or other environmental sources on *Cryptosporidium* transmission and risk factors. According to that, the needed measures can be taken to control cryptosporidiosis in Jeddah and Saudi Arabia.

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