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A study on PCR-RFLP of *Giardia duodenalis* in Malaysia

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Abstract

Giardia duodenalis causes diarrhoea and malabsorption. The objectives of the study were to detect local isolates of *G. doudenalis* by polymerase chain reaction (PCR) and to determine their restriction fragment length polymorphisms (RFLP). *G. doudenalis* isolated from stools of patients from Hospital Orang Asli Gombak were cultured axenically using TYI-S-33 medium with 10% foetal calf serum. The commercially designed primer-pair 432/433 was used to amplify a 0.52 kb segment known to encode the homologous cysteine-rich trophozoite surface antigen (tsp11 and tsa417). Results showed that the primer-pair 432/433 could amplify the target region of the local isolates. RFLP study on the identical isolates showed that all the restriction enzymes tested (*Hind*III, *Cla*I, *Pst*I and *Kpn*) gave a banding pattern similar to that of the WB strain a reference pathogenic strain from human. The reference pathogenic strain were commercially obtained from the American Type Culture Collection (ATCC).

Key words: Giardia duodenalis, molecular characterization, polymorphism

INTRODUCTION

Giardiasis is a common cause of diarrheal disease in almost all vertebrates, including humans. In industrialized countries, it is referred to as a reemerging infectious disease because of its increasingly recognized role in outbreaks of diarrheal disease in daycare centers and in water and foodborne outbreaks. *Giardia* is also one of the most frequently observed parasites infecting dairy cattle and domestic dogs. In developing countries in Asia, Africa, and Latin America, approximately 200 million people have symptomatic giardiasis .[1]

Molecular techniques such as PCR provide alternative methods for specific detection of pathogens in stool, and in combination with techniques, such as restriction fragment length polymorphism (RFLP) or nested PCR, they have been used to genotype organisms .[2] The sensitivity of detection by PCR is greater than that of microscopy, making it of great use for detection of low numbers of parasites in stool samples.[3]

Two major groups of *G. duodenalis* have been recognized as infecting humans worldwide, but there are some differences in naming of these groups, as evidenced by the following categorizations: Polish and Belgian genotypes;[4] groups 1, 2, and .[5,6] and assemblages A and B.[7]

The existence of fixed genetic differences between isolates belonging to allozyme groups I and II has been demonstrated directly by Southern hybridization using two DNA probes, one specific for the tsp 11 gene and the other detecting an unrelated repetitive sequence.[8] The polymerase chain reaction (PCR) has been used to amplify a 0.52 kb segment of *Giardia doudenalis* DNA, using primer specific for nucleotic sequences conserved within two genes (tsp11 and tsa417) that encode homologous, cystein-rich trophozoite surface proteins. Using products amplified from axenic isolates belonging to genetic group 1 and 11 (defined on the basis of allozyme electrophoresis data), restriction endonuclease analysis revealed both *tsp*11–like and *tsa*417 like fragments within samples. Analysis of genetic Group II showed PCR product corresponding to a previously undetected third gene cleavage while *Pst I* restriction distinguished isolates of genetic group II from genetic group I. The presence of a Pst 1 site I site in the amplified segment of the newly discovered third gene of group 11 prganisms provides a simple diagnostic means of differentiating group 1 and 11 isolates.[9] The objectives of the present study were to detect local isolates of *G. doudenalis* by PCR and to determine the restriction fragment length

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polymorphism (RFLP) pattern using restriction enzyme analyses on the PCR products.

MATERIALS AND METHODS

Origin of G. duodenalis isolates
All local isolates were obtained from six Aborigines with giardiasis admitted to the Hospital for Aborigines at Gombak, Selangor. All the isolates were maintained in *in vitro* culture. Six local isolates used in the study were 110, 7304, 6304, M007, 2002 and 6307. WB was a pathogenic human strain and *G. muris* was of wild mouse origin, which were obtained commercially from American Type Culture Collection (ATCC) and were used as controls.

In vitro culture of G. duodenalis trophozoites
Cysts of *G. duodenalis* obtained from fresh faecal specimens were repeatedly washed, sedimented in water and purified by source gradient centrifugation.[10] The trophozoites were inoculated into each of a series of borosilicate glass culture tubes (125mm x 16mm) previously filled with modified TYI-S-33 medium and incubated at 37°C at a 5 to 7 degree angle and examined daily with an inverted microscope.[11] One third of culture liquid in each tube was replaced with fresh medium daily for the first 3 days and every 2 or 3 days thereafter until trophozoites formed a compact monolayer on the inner wall of the tubes. In early stock cultures, multiplication of the organisms was slow. Thereafter, the organisms grew luxuriantly and a monolayer of cells was formed by day 7 to 20 of culture. For passage, the culture tube was cooled on ice followed by vortexing to release the trophozoites from the sides. A sample of the mixture was then used to inoculate a new tube with fresh medium.

Harvesting of parasites
Trophozoites cultivated in the TYI-S-33 medium were harvested in late log phase after incubating on ice. The trophozoites were cooled on ice for 10 minutes, vortexed and spun at 3000 rpm for 10 minutes. After removal of the supernatant, an equal volume of phosphate buffered saline (PBS) was added to the pellet and the contents centrifuged for 10 minutes at 3000 rpm. The supernatant was removed and the parasites transferred to an appropriate container for further work.

Polymerase chain reaction (PCR)
The cells were washed 3 times, by resuspension in cold phosphate buffered saline (pH 7) and followed by centrifugation at 3000 rpm (10 minutes, each time). The cells were processed immediately for DNA extraction. The DNA was extracted using Qiagen DNA Extraction Kit (USA). All six local isolates of *G. duodenalis* were subjected to PCR with primer pair 432/433. The primer pair amplified the 0.52 kb region of *G. doudenalis* DNA specific for nucleotide sequences conserved within two genes (tsp 11 and tsa 417) that encode a homologous, cysteine rich trophozoite surface antigen.[9]

Genomic DNA was amplified by PCR with 2.5 units of the *Taq* DNA polymerase (Fermentas AB, Lithuania) in a reaction mixture (100µl), containing dNTPs (200µM) and 2.5mM $MgCl_2$, subjected to 10 cycles (first PCR program) of 1 minute at 95°C (denaturation), 30 seconds at 58°C (annealing) and 1 minute at 72°C (extension). For the second PCR, the following program was followed: 30 cycles of 45 seconds at 95°C (denaturation), 30 seconds at 60°C (annealing) and 1 minute and 25 seconds at 72°C (extension). The amplification product was purified using High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany). Cross reaction study using *Toxoplasma gondii* and *Giardia muris* was done in order to obtain specificity of the PCR primers.

Restriction endonuclease analysis of amplification product
For restriction analysis, aliquots of PCR reaction mixtures were incubated overnight at 37°C with 2 units of ClaI, HindIII, KpnI and PstI in 20µl of 1X digestion buffer (Boehringer Mannheim, Germany). Half of each reaction mixture was analysed by electrophoresis. For samples that showed no cleavage or where cleavage was incomplete, the digestion was repeated on the residual mixture by the addition of a second aliquot of enzyme. Enzyme digests were subjected to electrophoresis on 1.5% agarose gel in Tri-Borate-EDTA (TBE) and DNA fragments were stained with ethidium bromide, visualized under UV and finally photographed.

RESULTS AND DISCUSSION

Figure 1 shows the PCR amplification result with the primer pair 432/433 using the DNA extracted from *G. doudenalis* WB strain which served as the standard positive control. There

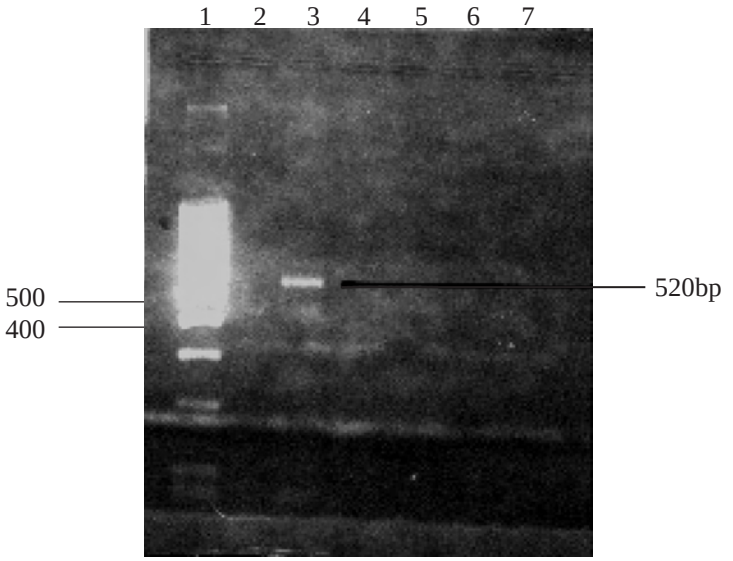

FIG. 1: Agarose gel electrophoresis analysis of PCR amplified DNA using primer pair 432/433. Lane 1: 100bp ladder; Lane 2: *Toxoplasma gondii*; Lane 3: WB strain; Lane 4: *G. muris.*

was no amplification with DNA from *Toxoplasma gondii* and *G. muris*, showing that the primer was very specific for *G. doudenalis.* Figure 2 shows amplification of DNA from isolates 6307, M007, 2002, 7304, 101, 6304 and WB.

The results of DNA amplification of different isolates treated with restriction enzymes (treated with *Hind*III, *ClaI* , *Kpn* and *Pst*I respectively) are shown in Figs. 3-6. *Hind*III and *Cla*I digestion (Figure 3 and Figure 4) showed that the PCR product from each isolate was in fact heterogenous with respect to restriction sites. For both enzymes, a portion of the PCR was resistant to cleavage at a single site, while the remainder was cleaved at a single site. Fig. 3 showed that all the local isolates and WB strain incubated with *Hind*III, gave fragment product size of 520bp, 350bp and 170bp. Figure 4 showed that all the local isolates incubated with *Cla*I gave product size of 445bp and 75 bp with absence

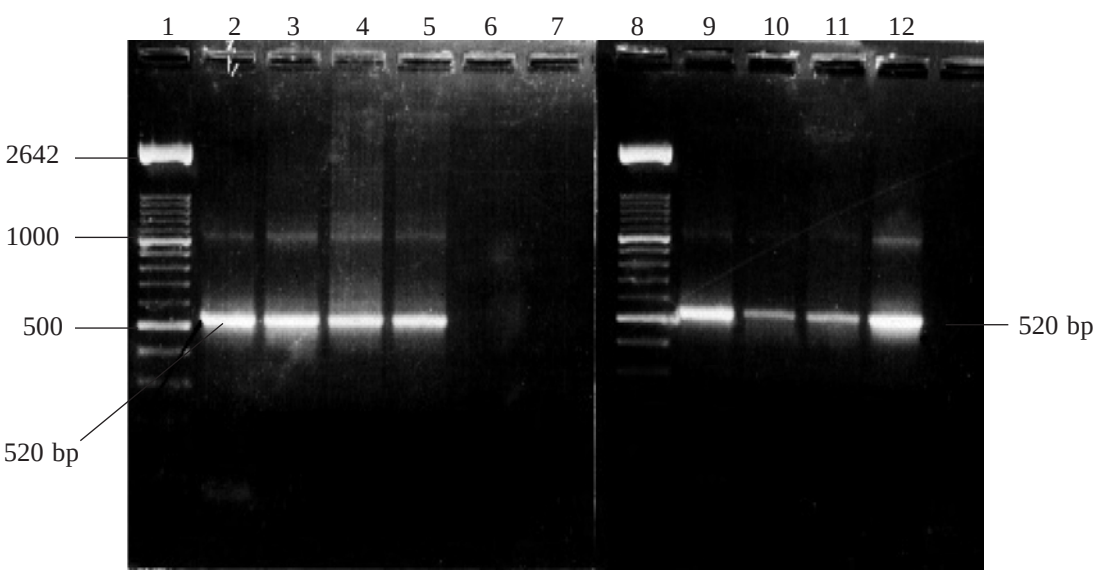

FIG. 2: Agarose gel electrophoresis of PCR amplified DNA using primer 432/433. Lane 1: 100bp ladder; Lane 2: WB strain; Lane 3: 6304; Lane 4: 6307; Lane 5: 2002; Lane 8: 100bp ladder; Lane 9: 101 and Lane 10 : M007; Lane 11: 7304; Lane 12: 2002.

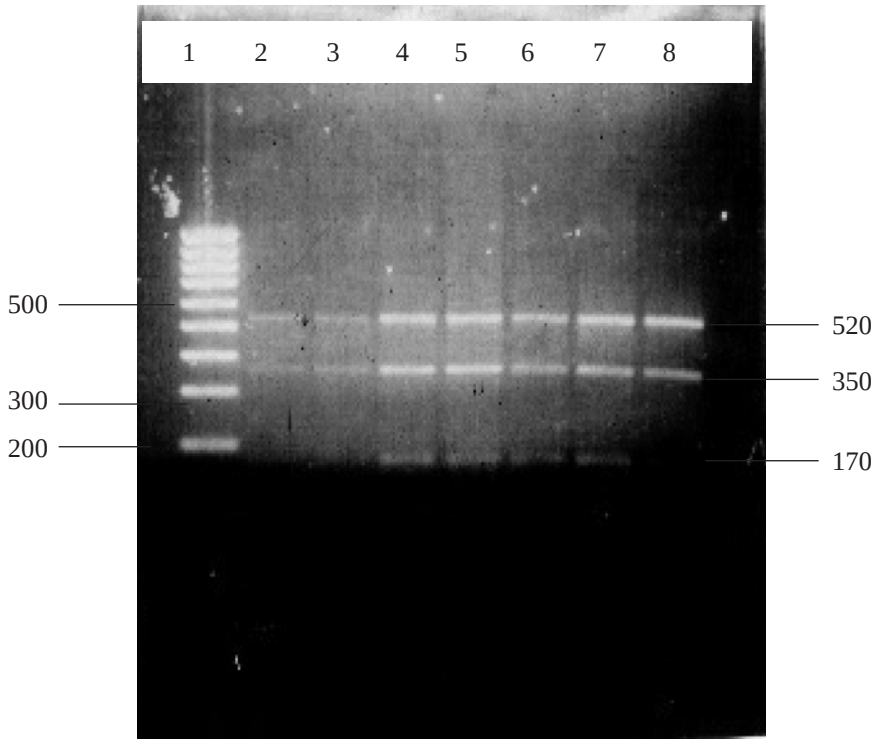

FIG. 3: Analysis of amplified DNA after digestion with *Hind*III from Primer 433/432 of *G. duodenalis* isolates after incubation with *Hind*III. Lane 1: 1kb 'ladder' marker. Lane 2 – 8: WB, 101, 7304, 6304, 6307, M007 and 2002 isolates.

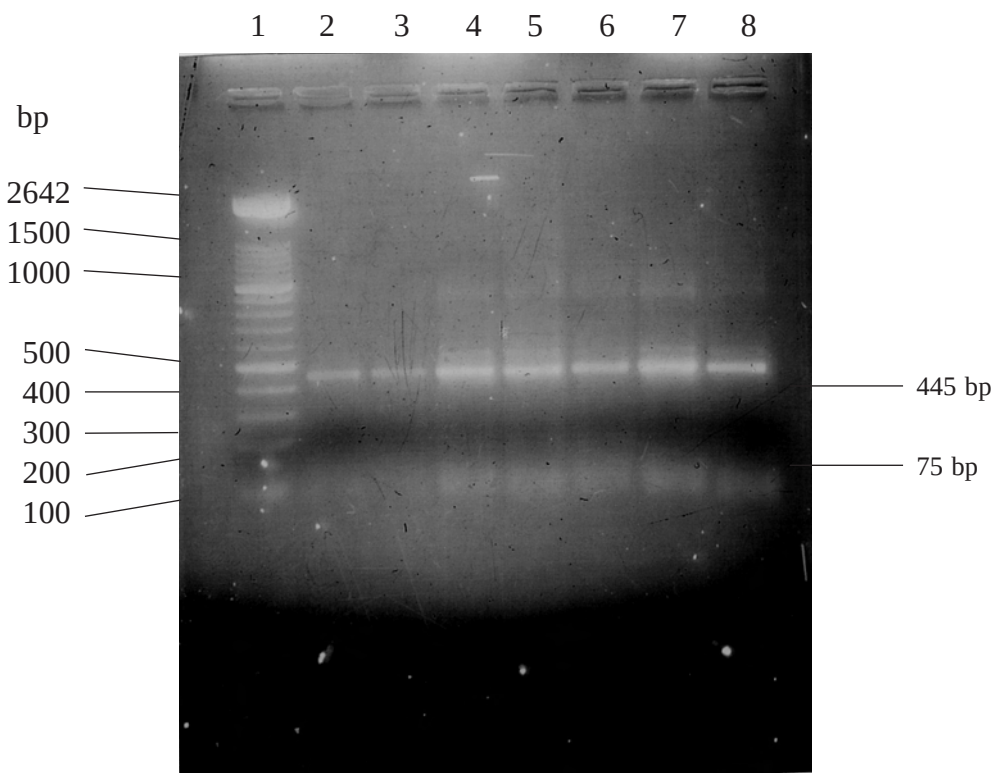

FIG. 4: Analysis of amplified DNA after digestion with *Cla*I . Lane 1: 1kb 'ladder' marker. Lane 2 – 8: WB, 101, 7304, 6304, 6307, M007 and 2002 isolates.

of fragment at 520bp. Ey *et. al.* (1993), reported that using primers 432 and 433 in the PCR of giardiasis belonging to the allozyme defined genetic group I or II, gave rise to a single product of 520 bp with high yield.

Figure 5 shows the pattern obtained with local isolates incubated with *Kpn*. Residues of fragment size 520bp, 350bp and 170bp were observed. Incubation of the PCR amplification products with *Kpn*, yielded fragments that would be expected from cleavage of the tsa 417 gene product, according to its published segments.[12] (The published segments are 520bp, 350bp and 170bp).

When a similar analysis was carried out with *Pst*I, a different and more complex results was obtained (Figure 6). Once again, the DNA amplified from all isolates comprised sequences that corresponded to both tsa 417 gene sequences and all tsa 417 band. This can be seen by the presence of some resistant PCR product (tsp II-like band) plus two cleavage fragments (330 bp and 190 bp respectively), which correspond to those expected from tsa 417 sequence.[12] There is absence of fragment size 370bp and 150bp. The findings of this study showed that they are similar to that of an earlier study.[9] Restriction enzyme *Pst*I was used to differentiate *G. doudenalis* isolates between genetic group I and II. Group II showed extra fragments of size 370bp and 150bp. The presence of a *Pst*I site on the PCR product will differentiate the *G. doudenalis* isolate belonging to groups I and II easily. The presence of a *Pst*I site in the amplified segment of the newly-discovered third gene of group II organisms provides a simple diagnostic means of differentiating group I and II isolates. All the six local isolates from aborigines can be detected specifically using primer 432/433. There is no amplification of PCR product from *Toxoplasma gondii* DNA and *G. agilis* DNA. The local isolates belong to group I. All the six local isolates gave a similar banding pattern as the pathogenic strain WB which was used as a standard. Table 1 showed the restriction fragments of *Pst*I, *Cla*I, *Kpn* and *Hind*III of PCR product of *G. duodenalis* isolates amplified using primer 432/433.Table 1 showed the action of restriction enzymes on PCR product (520 bp) using primer 432/433).

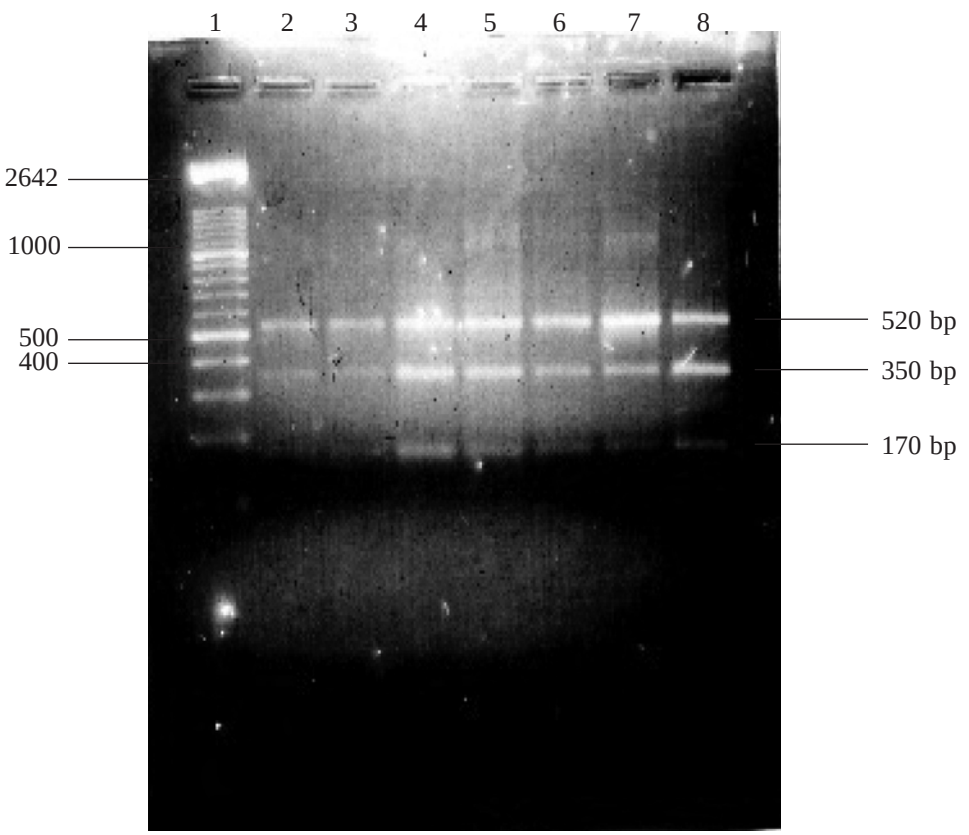

FIG. 5: Analysis of PCR DNA after incubation with *Kpn*. Lane 1: kb 'ladder' marker. Lane 2 – 8: WB, 101, 7304, 6304, 6307, M007 and 2002 isolates.

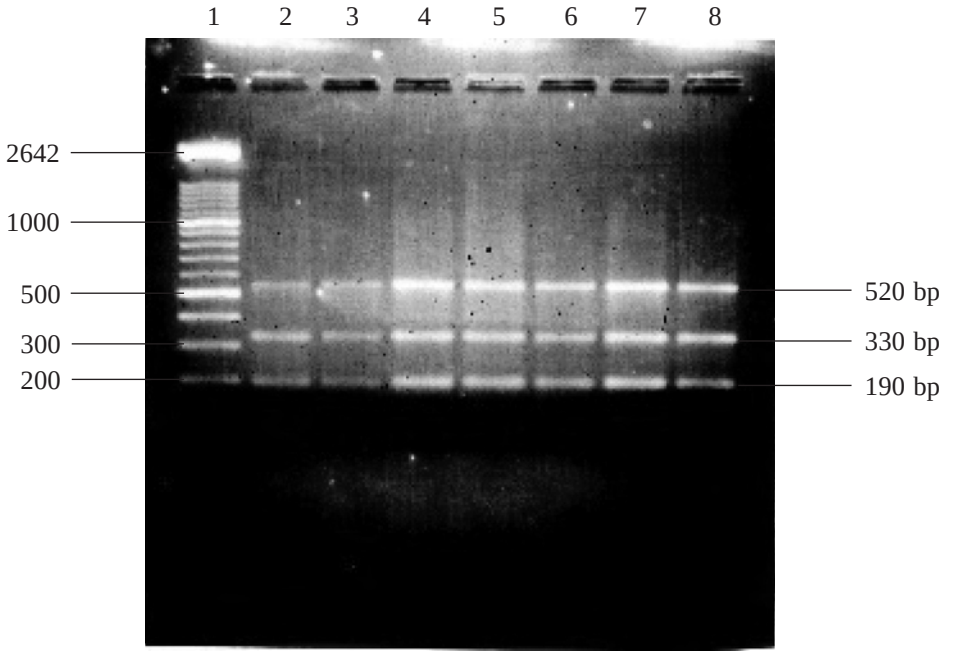

FIG. 6: Analysis of PCR product after digestion with *Pst*1. Lane 1: 1kb 'ladder' marker. Lane 2 – 8: WB, 101, 7304, 6304, 6307, M007 and 2002 isolates.

TABLE 1: The restriction fragments of *Pst*I, *Cla*I, *Kpn* and *Hind*III of PCR product of *G. duodenalis* isolates amplified using primer 432/433.

***G. duodenalis* isolates**	***Pst*I**	***Cla*I**	***Kpn***	***Hind*III**
WB (standard)	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp
110	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp
7304	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp
6307	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp
6304	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp
2002	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp
M007	330bp 190bp	445bp 75bp	350bp 170bp	350bp 170bp

No polymorphism occurred in all the six local isolates. It is concluded that the PCR finger printing method described in this paper provides a reliable characterization method for *Giardia* isolates and has the potential to be used as a direct method of typing *G. duodenalis* cyst from feces.

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