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Retrotransposon *OV-RTE-1* from the carcinogenic liver fluke *Opisthorchis viverrini*; potential target for DNA-based diagnosis

Luyen Thi Phung^a, Alex Loukas^b, Paul J. Brindley^c, Banchob Sripa^d, and Thewarach Laha^{a,e,*}

^aDepartment of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand ^bCentre for Biodiscovery and Molecular Development of Therapeutics, Queensland Tropical Health Alliance, James Cook University, Cairns, Queensland 4878, Australia

^cDepartment of Microbiology, Immunology and Tropical Medicine, and Research Center for Neglected Tropical and Infectious Diseases of Poverty, School of Medicine & Health Sciences, George Washington University, Washington, DC 20037, USA ^dTropical Disease Research Laboratory, Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand ^eLiver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

Abstract

Infections by the fish-borne liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* can lead to bile duct cancer. These neglected tropical disease pathogens occur in East Asia, with *O. viverrini* primarily in Thailand and Laos and *C. sinensis* in Cambodia, Vietnam, and China. Genomic information about these pathogens holds the potential to improve disease treatment and control. Transcriptome analysis indicates that mobile genetic elements are active in *O. viverrini*, including a novel non-Long Terminal Repeat (LTR) retrotransposon. A consensus sequence of this element, termed *OV-RTE-1*, was assembled from expressed sequence tags and PCR amplified genomic DNA. *OV-RTE-1* was 3,330 bp in length, encoded 1,101 amino acid residues and exhibited hallmark structures and sequences of non-LTR retrotransposons including a single open reading frame encoding apurinic-apyrimidinic endonuclease (EN) and reverse transcriptase (RT). Phylogenetic analyses confirmed that *OV-RTE-1* was member of the RTE clade of non-LTR retrotransposons. *OV-RTE-1* is the first non-LTR retrotransposon characterized from the trematode family Opisthorchiidae. Sequences of *OV-RTE-1* were targeted to develop a diagnostic tool for detection of infection by *O. viverrini*. PCR specific primers for detection of *O. viverrini* DNA showed 100% specificity and sensitivity for detection of as little as five femtograms of *O. viverrini* DNA whereas the PCR based approach showed 62% sensitivity and 100% specificity with clinical stool samples. The *OV-RTE-1* specific PCR could be developed as a molecular diagnostic for *Opisthorchis* infection targeting parasite eggs in stool samples, especially in regions of mixed infection of *O. viverrini* and/or *C. sinensis* and minute intestinal flukes.

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*Corresponding author: Thewarach Laha, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002 Thailand; phone, +66 43 348387, thewa_la@kku.ac.th.

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Keywords

Opisthorchis viverrini; liver fluke; retrotransposon; diagnosis; feces

1. Introduction

Human liver flukes of the family Opisthorchiidae, including *Clonorchis sinensis*, *Opisthorchis felinus* and *O. viverrini* infect >35 million people in East Asia, Siberia and Eastern Europe, and occasionally in countries of the western Europe including Italy (Mordvinov et al., 2012; Pozio et al., 2013; Sithithaworn et al., 2012; Sripa, 2012; Sripa et al., 2012). *C. sinensis* occurs in Korea, China, Taiwan, northern Vietnam and some regions of the far east of the former USSR, *O. felinus* is endemic in central Europe and Siberia, whereas *O. viverrini* distributes in Thailand, Laos PDR, Cambodia and southern Vietnam (Hong and Fang, 2012; Sithithaworn et al., 2012). Also, migration of *C. sinensis* beyond its more usual endemic range has been reported (Morsy and Al-Mathal, 2011; Traub et al., 2009). The geographical ranges of *O. viverrini* and *C. sinensis* partially overlap; co-endemic areas have been reported in central Vietnam (Le et al., 2006) and in central provinces of Thailand (Sithithaworn et al., 2012; Traub et al., 2009). Given that *C. sinensis* and *O. viverrini* may distribute to other sites beyond their usual geographic ranges, investigation of mixed infections of *O. viverrini* and *C. sinensis* is informative especially from epidemiological and evolutionary perspectives. Furthermore, with the recent establishment of Asean Economic Community (AEC), migration of people infected with these parasites will increase in the AEC. Accordingly, accuracy in the differential diagnosis of clonorchiasis and opisthorchiasis needs to be improved.

Microscopic examination of the egg of the parasite in feces is the standard method for diagnosis of infection with these opisthorchiid flukes (Sripa et al., 2010). However, the reliability of microscopic diagnosis targeting fecal eggs relies on the expertise of examiner. In addition, stool examination methods offer lower sensitivity than molecular detection approaches in the case of light infection (Carvalho et al., 2012; Duenngai et al., 2008). The eggs of *O. viverrini*, *C. sinensis* and *O. felinus* are very similar to each other, and indeed to eggs of small intestinal flukes such as *Haplorchis taichui*, thus creating diagnostic problems (Sripa et al., 2010). A number of PCR-based techniques using a multiplicity of gene targets including ribosomal DNA and mitochondrial genes can distinguish *O. viverrini* in the cases of mixed infections with *C. sinensis* and small intestinal flukes (Janwan et al., 2011; Le et al., 2006; Sato et al., 2009; Wongsawad et al., 2012). However, improved detection of opisthorchiasis and clonorchiasis in particular with high sensitivity, specificity and simplicity would be welcomed (Johansen et al., 2010; McCarthy et al., 2012).

The genomes of metazoan parasites generally include numerous copies of dispersed repetitive sequences or mobile genetic elements (MGE) (Brindley et al., 2003). MGEs are classified into two classes based on mode of transposition mechanism: Class I has a RNA intermediate; class II has a DNA intermediate. Class I MGEs transpose via transcribed to RNA and reverse transcribed to DNA whereas class II MGEs transpose directly from DNA to DNA. Class I includes the long terminal repeat (LTR) retrotransposons, the non-LTR retrotransposons, the short interspersed nuclear elements (SINEs), and the retroviruses. Class I MGEs occur in diverse taxa from fungi to mammals, are mobilized by replicative processes that generate numerous daughter copies, and thereby expand the size of the host genome (Huang et al., 2012). Retrotransposons have been described from many parasitic species (Laha et al., 2005; Laha et al., 2001; Yadav et al., 2009), and indeed sequences of non-LTR retrotransposons of the RTE family have been targeted for the application of DNA based diagnostics for parasitic infection with high sensitivity (Guo et al., 2012; Wang et al.,

2011; Zhou et al., 2011). Here we report the sequence and predicted structure of a novel non-LTR retrotransposon from *O. viverrini*. Further, we present an analysis of its potential application for PCR based detection of *O. viverrini* and differential diagnosis of infection with *O. viverrini*, *C. sinensis* and/or small intestinal flukes.

2. Materials and methods

2.1. Flukes, parasite eggs

Adult worms of *O. viverrini* were maintained in Syrian hamsters (Flavell et al., 1983; Sripa and Kaewkes, 2002) at animal husbandry facilities of the Faculty of Medicine, Khon Kaen University. Protocols for vertebrate animal studies were approved by the Animal Ethics Committee of Khon Kaen University based on the Ethics of Animal Experimentation of the National Research Council of Thailand. Adult worms of *C. sinensis* were kindly provided by Dr. Nguyen Van De from Department of Parasitology, Ha Noi Medical University, Ha Noi, Vietnam. Adult worms of the small intestinal fluke, *Haplorchis taichui* were kindly provided by Dr. Do Trung Dung from National Institute of Malariology, Parasitology and Entomology, Vietnam (Sripa et al., 2010). Eggs of *O. viverrini* were obtained *in vitro* from adult worms; briefly adult worms were recovered from bile ducts of experimental infected hamster described above. Worms were washed for five times with PBS supplemented with 2X antibiotics (streptomycin/penicillin, 200 µg/ml), and cultured in RPMI 1640 medium supplemented with 1X antibiotics (streptomycin/penicillin, 100 µg/ml) at 37°C under 5% CO₂ atmosphere. Eggs released from worms *in vitro* were collected at intervals of 48 h, washed several times in sterile PBS, and either used immediately or stored at -20°C.

2.2. Stool samples

Human stool samples from endemic sites in Khon Kaen province, Thailand were supplied by the Tropical Disease Research Laboratory, Khon Kaen University. The stools were positive for *O. viverrini* eggs, often included eggs of other helminth parasites, or were microscopically negative for parasite eggs. The stool samples were preserved in 70% alcohol at the time of collection, and were stored at room temperature thereafter until DNA extraction. Fecal egg counts were established using the quantitative formalin-ethyl acetate concentration techniques described (Haswell-Elkins et al., 1991). Additionally, negative human stool samples spiked with *O. viverrini* eggs were used as positive controls. To prepare spiked samples with known numbers of fluke eggs, exact numbers of *O. viverrini* eggs were counted under the microscope in a hemocytometer chamber, and transferred to aliquots of one gram of fresh, parasite-egg negative human feces (feces from one donor). Collection of these samples was approved by the Ethic Committee of Khon Kaen University, approval number HE451132.

2.3. DNA extraction from adult worms and human stool samples

Genomic DNA was extracted from adult developmental stages of *O. viverrini*, *C. sinensis* and *H. taichui* using Bio-Rad's genomic DNA extraction kit (Carlsbad, CA, USA). Genomic DNA was extracted from human feces using QIAgen's DNA extraction kit for stool samples (Qiagen, Germany). Genomic DNA from these sources was stored in kit elution buffers at -20°C.

2.4. Bioinformatics for detection of retrotransposon sequences

The keywords 'reverse transcriptase' and 'retrotransposon' were employed as search query terms of the database of expressed sequence tags of *O. viverrini* (Laha et al., 2007; Young et al., 2010a). Matches were retrieved and employed to search for homologues in the GenBank non-redundant sequence database using Blastn, Blastx and/or tBlastn (Altschul et al., 1997;

Zhang et al., 2000). Consensus sequence of the novel *O. viverrini* retrotransposon was assembled from *O. viverrini* transcriptome sequence with 95% identities and 20 nucleotides overlapped using CAP contig assembly program in BioEdit package software (Hall, 1999).

2.5. *O. viverrini* retrotransposon specific primers design

Retrotransposon-like sequences of *O. viverrini* contig Ov_Contig10163 (Young et al., 2010a) was aligned with cDNA sequence databases of *C. sinensis* (Young et al., 2010a) with pairwise alignment. Non-conserved regions between *O. viverrini* and *C. sinensis* were identified and used to design primers specific for *O. viverrini* and *C. sinensis*. In addition, conserved sequences of *O. viverrini* and *C. sinensis* were identified for universal primer pair for detection of opisthorchiid DNA. Specific primers pairs for the *O. viverrini* retrotransposon were designated Ov_RTE_F1 (5'-GAATCCCTAGATCAGTCCTC-3') and Ov_RTE_R1 (5'-CAGACCTCTATCAACTTGCC-3'). The specific primer for *C. sinensis* were termed CS_RTE_F1 (5'-CCTCAGGTATCTCCAAATCACTC) and CS_RTE_R1 (5'-CAGACATTGATCAACATCCC) and a universal primer pair for opisthorchiid DNA named Universal_OP_F (5'-GTAGCTCTGACACCGTCAAAG) and Universal_OP_R (5'-CGATTGTCCGCACCTTACGCC).

2.6. Evaluation of sensitivity of the PCR

Genomic DNA extracted from parasites and human feces were used as PCR templates. The retrotransposon gene of *O. viverrini* and *C. sinensis* were amplified using retrotransposon specific primers, as above. PCR conditions were optimized for the specific amplification of the *OV-RTE-1* fragment. PCRs were performed in a volume of 25 µl, including 50 ng of DNA template, PCR buffer (20 mM Tris- HCl pH 8.4, 50mM KCl, 1.5 mM MgCl₂), 200µM of each deoxynucleotide triphosphate, 1 µM Taq DNA polymerase, and each primer at 0.2 µM. The PCR was accomplished with a thermal cycler with 35 cycles of denaturation at 94°C for 30 sec, 60°C for 1 min and 72°C for 1 min, and final extension for 5 min at 72°C. Amplicons were sized by electrophoresis through 0.8% agarose in 1X Tris-acetate-EDTA buffer, stained with ethidium bromide, and photographed under UV illumination.

Sensitivity of PCR detection of the *O. viverrini* DNA was calculated from the least quantity of genomic DNA detectable by the PCR. Genomic DNA of adult *O. viverrini* was 10-fold serial diluted, starting at 0.5ng/µl, with one µl of each dilution employed as template for amplification, as above. Sensitivity for detection of isolated *O. viverrini* eggs was determined using increasing numbers of *O. viverrini* egg, ranging from one to seven eggs. In brief, eggs were added to 20 µl sterile distilled water and the preparation freeze-thawed three times in liquid nitrogen/ hot water, then incubated for 15 min at 100°C. Two µl of egg lysate was employed as the PCR template. The diagnostic sensitivity and specificity were calculated and expressed using the Galen's method: Sensitivity = [no. of true positives/(no. of true positives + no. of false negatives)] × 100; Specificity = [no. of true negatives/(no. of true negatives + no. of false positives)] × 100 (Galen, 1980).

2.7. Long range PCR amplification

Long range PCR method was used to amplify the full length *OV-RTE-1*. Primers used were OV_RTEfull_F1 (5'- ATGCCACATCGGCCGTCGCG-3') and OV_RTEfull_R1 (5'- GTTCCCCATCCTGGAAGGCG-3') as forward and reverse primers, respectively. The conditions for long range PCR were recommended in the manufacturer's protocol (Qiagen, Germany). PCR amplification was carried out in a final volume of 50 µl, including 5 µl of 10× long range PCR buffer with Mg²⁺, 2.5 µl of dNTP mix (10mM each), 0.4 µM of each primer, 2 units of long-range PCR enzyme, 200ng *O. viverrini* genomic DNA, 30 µl RNase-free water. The PCR was carried out in a thermal cycler with initial denaturation at 93°C for 3 min, then 35 cycles including denaturation at 93°C for 15sec, annealing at 60°C for 30 sec,

extension at 68°C for 4 min and final extension for 10 min at 68°C. Amplification products were analyzed as above.

2.8. The OV-RTE-1 gene

PCR products of the desired size, resolved through agarose by electrophoresis, were recovered after excision of bands from gels and purified from agarose using the GeneJET Gel Extraction Kit (Fermentas, EU). Amplicons were cloned into plasmid T-vector for propagation using a cloning kit (pGEM-T easy, Promega, USA). *E. coli* strain JM109 cells were transformed with the cloned products. Plasmid DNA was isolated from bacteria using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, USA). DNA sequencing of PCR products and recombinant plasmids was undertaken on an automated fluorescent DNA sequencing platform using the Big Dye terminator method (1st Base, Malaysia). Sequences were conceptually translated into amino acids and functional domains and motifs compared and contrasted with those of other retrotransposons. Alignments of amino acid sequences of functional domains were accomplished with ClustalW (Thompson et al., 1994), edited and annotated with BioEdit v5.0.9 software, and also manually edited to highlight conserved endonuclease (EN) and reverse transcriptase (RT) domains (Malik et al., 1999).

2.9. Phylogenetic analysis

Alignments of the RT and EN domains were utilized to construct phylograms of the relationships of the novel retrotransposon using MEGA version 5.2.2 (Tamura et al., 2011). Phylograms were assessed for bootstrap values of 1,000 replicates using the neighbor-joining method. The substitution model of Jones-Taylor-Thornton (JTT) was used. Alignment gaps were treated with complete deletion method. Sequences for phylogenetic analyses were accessed from the GenBank, EMBL and PIR databases, and included representatives from 11 major clades of the non-LTR retrotransposons (Malik et al., 1999). RT sequences of Group II introns and EN sequences from bacteria were used as outgroups for the RT and EN phylograms, respectively. The names and accession numbers of the aligned sequences included in the phylogenies are provided in the supporting information, Supplementary Table S1.

3. Results

3.1. OV-RTE-1, a non-LTR retrotransposon from the genome of *O. viverrini*

The entire open reading frame of a non-LTR retrotransposon gene designated *OV-RTE-1* was amplified by long range PCR with the primers OV_RTEfull_R1 and OV_RTEfull_F1. The amplicon was cloned into pGEM-T and the construct used to transform bacteria from which the plasmid we have termed pOV-RTE-1 was recovered. The consensus nucleotide sequence of the partial *OV-RTE-1* retrotransposon was 3,330 bp in length and encoded a single read through open reading frame (ORF) (Fig.1). The ORF of *OV-RTE-1* encoded 1,101 amino acids including endonuclease (EN) and reverse transcriptase (RT) domain, in that order. The EN domain of *OV-RTE-1* was 261 amino acids in length and the RT domain was 319 amino acids. The consensus sequence of *OV-RTE-1* has been assigned GenBank accession KF598866.

The conserved domain architecture of the non-LTR retrotransposons encoded by the *pol* gene, both the reverse transcriptase (RT) and endonuclease (EN) domains, was used for phylogenetic investigation of the relationships of *OV-RTE-1* to other retrotransposons (Malik et al., 1999). Alignments of RT and EN of *OV-RTE-1* with orthologous motifs from other non-LTR retrotransposons from other informative families and taxa revealed high similarity and conservation of functional motifs. The seven conserved blocks of the RT domain (Xiong and Eickbush, 1990) were identified, as shown in Fig.2A. The RT domain of

OV-RTE-1 contains an active site YXDD motif for reverse transcriptase in the sequence (Flavell, 1995). The nine conserved boxes of the EN domain of *OV-RTE-1* were also identified (Fig.2B). The conserved sequences in non-LTR retrotransposon sequences were indicated in black colored font (Fig. 2, panel A and B), as described (Laha et al., 2005; Malik et al., 1999).

3.2. *OV-RTE-1* is a member of the RTE clade of non-LTR retrotransposons

By Blast, the entire sequence of the novel *O. viverrini* retrotransposon shared highest similarity to the *SR2* non-LTR retrotransposon from *Schistosoma mansoni* (Drew et al., 1999; Ziegler et al., 2011) and to the closely related *SjR2* element of *Schistosoma japonicum* (Laha et al., 2002a). *OV-RTE-1* shared 54% identity to *SR2* and 53% identity to *SjR2* at the amino acid level when compared with 1,101 amino acids. Phylograms were constructed to compare and contrast the reverse transcriptase (RT) and endonuclease (EN) domains of *OV-RTE-1* with those of other retrotransposons including representatives from 11 clades of non-long terminal repeat retrotransposons. Comparison of the RT domains of these diverse elements revealed that the closest relatives of *OV-RTE-1* were uncharacterized retrotransposon-like sequence from *Clonorchis sinensis* (Cs_Contig4981) and *O. viverrini* (Ov_Contig7125) retrieved from cDNA sequence databases of Young et al. (2010), *SR2* from *S. mansoni* and *SjR2* from *S. japonicum*, *SR3* and *Perere3* from *S. mansoni*, and *ShR3* from *S. haematobium* (Fig. 3), firmly locating *OV-RTE-1* within the RTE clade of non-LTR retrotransposons. *Ov-RTE-1* groups with *SR3*, *Perere3* and *ShR3* with bootstrap value less than 50%. In addition, a phylogenetic tree was constructed based on the EN domain of eight clades of non-LTR retrotransposons. The topography of the EN tree, as well as the position of *OV-RTE-1* within the RTE clade, was similar to the topography represented on the RT-based tree, thereby providing additional support to the assignment of *OV-RTE-1* as a member of the RTE clade of RTE-like retrotransposons (Supplementary Fig. S1).

3.3. Retrotransposon PCR detection for *O. viverrini* infection

Three sets of primers were designed based on the sequence of the novel *O. viverrini* non-LTR retrotransposon. PCR conditions were optimized for detection of *O. viverrini* DNA using these primers. Primer pairs *OV_RTE_F1* and *OV_RTE_R1* exhibited 100% specificity to distinguish *O. viverrini* from *C. sinensis* and *H. taichui*. In addition, primer pairs *CS_RTE_F1* and *CS_RTE_R1* showed 100% specificity to distinguish *O. viverrini* from *C. sinensis* DNA. The universal_OP_F and Universal_OP_R primer pairs successfully amplified genomic DNA of both *O. viverrini* and *C. sinensis*, providing a positive control. The *OV_RTE_F1* and *OV_RTE_R1* primers amplified a product of 388 bp solely from *O. viverrini*, whereas products were not amplified from *C. sinensis* or *H.taichui* DNA (Fig. 4A). The *O.viverrini*-specific primers *OV_RTE_F1* and *OV_RTE_R1* were also evaluated for specificity using stool DNA samples (17 samples) containing eggs of other helminths: minute intestinal flukes, echinostomes, *Strongyloides stercoralis*, *Ascaris lumbricoides* and *Taenia* spp. PCR products were not amplified from these samples.

In evaluating the sensitivity of the PCR assay using the *O. viverrini*-specific *OV_RTE_F1* and *OV_RTE_R1* primers, we observed that the minimal amount of *O. viverrini* genomic DNA detectable by the specific PCR assay was 5 fg (Fig.4B). For detection of *O. viverrini* eggs by specific PCR, primers *OV_RTE_F1* and *OV_RTE_R1* detected a single egg of *O. viverrini* egg (Fig. 4C).

A total of 47 stool samples (confirmed to be infected with *O. viverrini* by examination for eggs by light microscopy using the FECT method) were examined. (These feces had been preserved in 70% ethanol and stored at room temperature). Of these, 11 fecal samples included 5-50 eggs per gram (EPG), 19 included 200-500 EPG and 17 included 500-1,000

EPG. In addition, 71 fresh, parasite-negative stool samples were spiked with increasing numbers of *O. viverrini* eggs: 5-50, 50-200, and 200-1,000 eggs in one gram of stool. Of these latter positive controls, 24 samples had 5-50 eggs, 35 had 50-200 eggs and 12 included 200-1,000 eggs. Furthermore, 17 stool samples that were positive for other helminth parasites were investigated; the other parasites were minute intestinal flukes (MIF) (n=2 stool samples), *S. stercoralis* (n=8), echinostomes (n=3), *Taenia* (n=1). Three additional samples were co-infected; one sample was co-infected with MIF and *Ascaris lumbricoides* whereas two samples were co-infected with MIF and *S. stercoralis*. In addition, five negative-by-microscopy stool samples were included as negative controls.

For the 47 samples preserved in ethanol, the specific PCR detected 18 samples (38.3%). Of these, four of 11 (36.4 %) samples with 5-50 EPG, five of 19 (26.3%) samples with 50-200 EPG and nine of 17 (52.9%) samples with 200-1,000 EPG were detected. To compare the efficiency of retrotransposon PCR versus FECT, 71 samples of fresh negative human stool samples into which different numbers of *O. viverrini* eggs were spiked were screened by PCR. The PCR detected 44 samples of 71 samples (62%); more specifically, the assay detected 14 of 24 (58.3 %) samples with 5-50 EPG, 23 of 35 (65.7%) samples with 50-200 EPG and 7 of 12 (58.33%) samples with 200-1,000 EPG. To examine the sensitivity of FECT, 40 fresh human stool samples spiked with increasing numbers of *O. viverrini* eggs (5-50, 50-200, 200-1,000) were screened. The assay failed to detect *O. viverrini* eggs in 15 samples to which 5-50 eggs had been added. However, the assay detected eight of 15 (53.33%) samples spiked with 50-200 eggs and 10 of 10 (100%) samples spiked with 200-1,000 eggs. In total, 18 of 40 (45%) samples were detected by FECT. PCR targeting DNA from five negative fecal samples failed to yield amplicons.

4. Discussion

Mobile sequences are key drivers of genome evolution (Kazazian, 2004). A number of families of retrotransposons have been characterized from trematode genomes including *CsRn1*, *PwRn1*, *Gulliver*, the LTR retrotransposons from *C. sinensis* (Bae et al., 2001), *Paragonimus westermani* (Bae et al., 2008) and *S. japonicum* (Laha et al., 2001), respectively. Moreover, numerous non-LTR retrotransposons have been characterized from schistosome genomes (Brindley et al., 2003; Drew et al., 1999; Laha et al., 2005). These reports generally employed approaches pairing bioinformatics and screening of genomic DNA libraries to locate novel non-LTR retrotransposons (Laha et al., 2002a; Laha et al., 2002b; Laha et al., 2005, 2006). The approach presented here used bioinformatics and long range PCR approaches, both of which are straightforward methods, to isolate a long fragment of retrotransposon sequence from genomic DNA template.

The retrotransposon *OV-RTE-1* is the first mobile genetic element to be described in the genome of *O. viverrini*, and the first non-LTR retrotransposon characterized in depth from the family Opisthorchiidae. The sequence, structure, and phylogenetic relationships of *OV-RTE-1* are presented here. Transcriptome-based estimates indicate that the genome of *O. viverrini* includes ~20,000 protein encoding genes along with numerous repetitive sequences (Young et al., 2010a; Young et al., 2010b). Abundant retrotransposon sequences occur in the transcriptome of *O. viverrini* which, along with the presence of putatively functional domains of *OV-RTE-1*, indicates that *OV-RTE-1* and other retrotransposons are actively mobilized in the genome of *O. viverrini*. Indeed, it is feasible that active or autonomous *OV-RTE-1* copies may be of practical use as a transgenesis vector in future functional genomics analysis of these fish born flukes (Izsvak et al., 1997; Kopera et al., 2011; Laha et al., 2001).

Multiple sequence alignments of both the reverse transcriptase (RT) and endonucleases (EN) domains of the deduced polyprotein of the retrotransposon and phylograms demonstrated that *OV-RTE-1* belonged to the RTE clade of non-LTR retrotransposons. The closest relatives of *OV-RTE-1* were *SR2* from *S. mansoni* and *SjR2* from *S. japonicum*. *OV-RTE-1* encoded a single open reading frame containing a domain with homology to the apurinic-aprimidic endonucleases and reverse transcriptase domain. The RT and EN domains of *OV-RTE-1* represented the most highly conserved regions that characterization of RTE clade of non-LTR retrotransposon (Malik et al., 1999; Malik and Eickbush, 1998). Deeper analysis of *O. viverrini* retrotransposons can be expected to enhance understanding on the evolutionary relationships and history of *O. viverrini* and platyhelminths at large and will facilitate the annotation of the liver fluke genome (which is ongoing; unpublished).

The potential for deployment of *OV-RTE-1* as a gene target for opisthorchiasis detection was supported by the findings presented here. Retrotransposon *OV-RTE-1* specific primers, *OV_RTE_F1* and *OV-RTE_R1*, showed 100% specificity to detect *O. viverrini* DNA and could detect as little as five femtograms of genomic DNA. The outcome showed, further, that *OV_RTE_F1* and *OV-RTE_R1* primers were specific for *O. viverrini* and do not interact with the DNA of other known trematode and nematode species. The findings also indicated that *OV_RTE_F1* and *OV-RTE_R1* primers can differentiate *O. viverrini* from other parasites especially the closely related species, *C. sinensis*.

The sensitivity of PCR using *OV-RTE-1* specific primers for detection of *O. viverrini* infection with DNA extracted from human stool that had been preserved in ethanol was lower than that for detection of *O. viverrini* by microscopic examination of stool specimens using the FECT technique. However, the sensitivity of PCR using DNA extracted from human stool samples spiked with *O. viverrini* eggs was higher than FECT. These results revealed that *OV-RTE-1* specific primers exhibit potential for development as molecular diagnostic tools for *Opisthorchis* infection especially in the areas of mixed infection with *O. viverrini*, *C. sinensis* and/or *H. taichui* such as in northeastern Thailand and adjacent countries (Pitaksakulrat et al., 2013; Rim et al., 2013; Wongsawad et al., 2012).

Because the sensitivity of PCR detect of *O. viverrini* was lower than might be expected, in future trials we will consider inclusion of a spike of *O. viverrini* genomic DNA and additional sets of primers against human gene(s) that would be expected in feces as positive controls. This should improve interpretation of negatives as being truly negative and address whether potential inhibitors for the PCR in the stool samples influenced the outcome. Furthermore, the variation of the number of stool samples used in each group might have affected the sensitivity but availability of these kinds of samples can be problematic. To improve efficiency, sufficiently more samples can be included in future investigations. For ethanol preservation, the conditions imposed by long term preservation may have led to inhibitory factors, and we will investigate alternative methods for preservation of samples. To conclude, however, The *OV-RTE-1* specific PCR can likely be developed as a molecular diagnostic for *Opisthorchis* infection targeting parasite eggs in stool samples, especially in regions of mixed infection of *O. viverrini* and/or *C. sinensis* and minute intestinal flukes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The genome of *Opisthorchis viverrini* has a multi-copy retrotransposon
- This novel non-long terminal repeat retrotransposon has been termed Ov-RTE-1
- PCR based analysis targeting Ov-RTE-1 detected *O. viverrini* eggs in human feces
- PCR targeting Ov-RTE-1 discriminated *O. viverrini* from *Clonorchis sinensis*

Figure 1.

Schematic representation of the consensus structure of *Ov-RTE-1* and the *Ov_Contigs* used for its construction. EN and RT refer to the endonuclease and reverse transcriptase domains of the *pol* open reading frame. Individual *Ov_Contigs* are shown as black lines, contigs names are listed on the right. Numbers beside each contig refer to regions sequenced. The sequence of *Ov-RTE-1* obtained from long range PCR is shown at the bottom. The region of PCR product from specific primers *Ov_RTE_F1* and *Ov_RTE_R1* is indicated by the red line.

Figure 3.

Phylogram constructed using the neighbor-joining method to compare the relationships among reverse transcriptase encoding regions of *OV-RTE-1* and of representative elements belonging to the major clades of non-long terminal repeat retrotransposons from a range of host genomes. Bootstrap values, where 500 or greater from a maximum of 1,000 replicates, are presented at the nodes.

A. **B.** **C.** **Figure 4.**

Panel A: PCR detection *O. viverrini* DNA with retrotransposon specific primers. Lanes 1-3 represent PCR product amplified from genomic DNA extracted from *O. viverrini*, *C. sinensis* and *H. taichui*, respectively. Lane 4 represents no DNA control. Lane M represents a DNA size marker. Panel B: Evaluation of the sensitivity of the specific PCR assay with *O. viverrini* genomic DNA by agarose gel electrophoresis. Lane 1 represents PCR product amplified from *O. viverrini* DNA with amount 0.5ng/ μ l. Lanes 2-7 represent PCR products amplified from *O. viverrini* DNA diluted for 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 times, respectively. Lane M shows DNA size standards. Panel C: Agarose gel electrophoresis showing PCR products of the *OV-RTE-1* PCR assay using *O. viverrini* egg as the template. Lane 1

represents PCR products amplified from DNA of one *O. viverrini* egg. Lanes 2-7 represent PCR products amplified from 2, 3, 4, 5, 6, 7 eggs of *O. viverrini*, respectively. Lane M shows DNA size standards.