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

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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 3330 bp in length, encoded 1101 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 5 fg 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.

1. Introduction

Human liver flukes of the family Opisthorchiidae, including *Clonorchis sinensis*, *Opisthorchis felineus* and *Opisthorchis 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. felineus* 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

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O. viverrini, *C. sinensis* and *O. felineus* 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 (MGEs) (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., 2001, 2005; 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, *H. 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% CO_2 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 1 g 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′-CGATTTGTCCGCACCTTACGC).

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, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of each deoxynucleotide triphosphate, 1 μM Taq DNA polymerase,