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Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam

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Abstract

The two species common of liver fluke, *Fasciola hepatica* and *Fasciola gigantica*, cause human fascioliasis. Hybrids between these species, and introgressed forms of *Fasciola*, are known from temperate and subtropical regions of eastern Asia. Here, we report the presence of hybrid and/or introgressed liver flukes in Vietnam where it has recently been recognised that human fascioliasis is an important zoonotic disease. Specimens examined came from domestic stock (cattle and buffalo) at slaughter and also from human patients. DNA sequences were obtained from the nuclear ribosomal second internal transcribed spacer (ITS-2) and from portions of two mitochondrial protein-coding genes. Mitochondrial sequences in every case were similar to those of *Fasciola gigantica*. Nuclear ITS-2 sequences belonged to one or other of the *Fasciola* species, or, sequences from both were found in the same individual worm. This study extends the known range of hybrids or introgressed forms of *Fasciola* into tropical regions of Asia.

Keywords: *Fasciola gigantica*; *Fasciola hepatica*; Hybridisation; Internal transcribed spacer region; Introgression; Vietnam

1. Introduction

The commonest and most widespread liver flukes of the genus *Fasciola* are *Fasciola hepatica* Linnaeus, 1758 (mostly in temperate regions) and *Fasciola gigantica* Cobbold, 1856 (mostly tropical in distribution). Adults of both species occur in many domestic ruminants and in humans (Mas-Coma et al., 2005; Le et al., 2007) and can cause serious disease. The two liver fluke species appear to be sympatric in many subtropical and warm temperate areas, especially in Africa and Asia (Le et al., 2007). They can generally be distinguished on the basis of their morphology (e.g. Ashrafi et al., 2006), but the existence of individuals with intermediate morphological characteristics can cause confusion (e.g. Terasaki et al., 1982; Itagaki et al., 2005a) and has led to the increasing use of molecular methods (e.g. Marcilla et al., 2002) or morphometric methods (e.g. Ashrafi et al., 2006) to distinguish between the species. It is desirable to know which species is the agent of human or animal disease in a given area. The two species differ in pathological and epidemiological characteristics (Mas-Coma et al., 2005).

Difficulties in specific identification have been most intensively studied in Japan and adjacent areas. Research there has revealed not only a confusing range of morphological forms but also the presence of worms of different ploidies (diploid, triploid and "mixoploid"), all of which are parthenogenetic and do not produce normal sperm (Terasaki et al., 1982, 2000). Genetic studies on Japanese

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and Korean worm populations have detected individuals that have both nuclear and mitochondrial sequences typical of *F. hepatica* and others that appear on the same grounds to be *F. gigantica*. Individuals also occur that resemble one species in their nuclear DNA (usually assayed using ribosomal RNA gene or spacer sequences) but have a mitochondrial genotype typical of the other species (Agatsuma et al., 2000; Itagaki et al., 2005a,b). Individuals have also been found that have, in their tandem array of nuclear ribosomal genes, copies of genes apparently derived from both liver fluke species (Agatsuma et al., 2000 in Korea; Huang et al., 2004 in north eastern China; Itagaki et al., 2005a,b in Japan and Korea; Lin et al., 2007 in China). An obvious conclusion is that hybridisation and introgression (definitions in Dowling and Secor, 1997) are common in *Fasciola* populations in this part of Asia, processes that can lead to production of polyploid and parthenogenetic individuals (Dowling and Secor, 1997). Such individuals are often aspermic. There have been no convincing demonstrations of hybrid/introgressed liver fluke populations outside Asia, although aspermic triploid individuals of "pure" *F. hepatica* are now known from Britain (Fletcher et al., 2004). Examples of triploid *Fasciola* sp. are also known from Assam and Hawaii (reviewed in Terasaki et al., 2000) and Vietnam (Itagaki et al., 2005a). In addition to Japan and Korea, aspermic *Fasciola* spp. of unknown ploidy are present (but generally uncommon) in the Philippines, Vietnam, Thailand, Taiwan, India, Nepal and Hawaii (Terasaki et al., 1982).

In Vietnam, fascioliasis (caused by morphologically identified *F. gigantica*) is very common in cattle and water buffaloes, with prevalences of more than 50% in the Red River and Mekong deltas, as well as in other coastal regions (Bui et al., 2003). In recent years, an extraordinary number of human cases of fascioliasis has been reported, to such an extent that this zoonotic infection has become a major public health concern in Vietnam. More than 500 human cases were recorded in the three years from 1997 to 2000 based on serological tests (Tran et al., 2001). Prior to 1991, fascioliasis had been regarded as rare in Vietnam. For example, only two cases were reported in 1978 (Tran et al., 2001). Unusual cases of cutaneous fascioliasis have also been described (e.g. Xuan et al., 2005; Le et al., 2007). Morphologically, the adult flukes found in animals and human patients in Vietnam fall into two categories, one typical of *F. gigantica* and the other closely resembling *F. hepatica*. Here we report data indicating that hybrid/introgressed populations of *Fasciola* occur in Vietnam and that these are implicated in human infection.

2. Materials and methods

2.1. Sources of Fasciola specimens

Parasite material and nucleotide sequences of *Fasciola* species, their host and geographical origin used in this study are listed in Table 1.

Adult worms of *Fasciola* sp. of Vietnamese origin, collected during 2001–2005 from human patients and animals (cattle and buffaloes), were preserved in 70% ethanol and kept at −20 °C until used for extraction of DNA. Of 21 Vietnamese samples, 12 were of human origin, of which two (FspN-VN and FspQB-VN) were from the cases involving unusual cutaneous migration of worms reported in Le et al. (2007). Specimens from eight human cases were obtained surgically. In two further cases, eggs morphologically identified as being of a *Fasciola* sp. were recovered from faeces of human patients serologically positive for fascioliasis. These eggs were allowed to develop and hatch in water (1–2 weeks) and 10–20 miracidia from each patient were collected for subsequent DNA extraction.

2.2. Genetic markers

Genetic markers including mitochondrial genes (*cox1, nad1*) and the nuclear second internal ribosomal spacer (ITS-2) sequences were obtained. ITS-2 is a useful marker for distinguishing between *F. gigantica* and *F. hepatica*. It is rather conserved, especially in *F. hepatica*. There are seven sites at which the two species typically differ. One of these is a deletion in *F. gigantica* relative to *F. hepatica* that appears to be a diagnostic difference between the species (e.g. Adlard et al., 1993; Semyenova et al., 2005). There is some variability, especially in *F. gigantica*, that can be confusing (Le et al., 2007). Sequence data from mitochondrial genes are more variable than is the case for ITS-2, but also provide unambiguous recognition of the two species (Le et al., 2007).

2.3. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from adult worms and miracidia using the commercial QIAamp DNA extraction kit (QIAGEN Inc.) according to the manufacturer's instructions. In the case of adult worms, only a single specimen was used in each DNA extraction. Genomic DNA was diluted to a working concentration of 50 ng/μL and 2 μL of this was used as template in a PCR reaction of 50 μL.

PCR was used to amplify the entire nuclear ITS-2 and two mitochondrial genetic markers (a portion of each of *cox1* and *nad1*). Primers 3SF (forward) (5′GGTACCG GTGGATCACTCGGCTCGTG3′) and BD2R (reverse) (5′TATGCTTAAATTCAGCGGGT3′) were used for amplification of ITS-2; JB3F (forward) (5′TTTTTTGG GCATCCTGAGGTTTAT3′) and JB4.5R (reverse) (5′TAAAGAAAGAACATAATGAAAATG3′) for *cox1* as previously published (Bowles and McManus, 1993; Bowles et al., 1995). Primers FND1F (forward) (5′TGG GGTCTGTTGCAGAGATTTGC3′) and FND1R (reverse) (5′ATCCAATGGAGTACGGTTACA3′) for *nad1* were designed for use in this study.

PCR amplification was carried out in a final volume of 50 μL, including 100 ng template, 10 pmol of each primer