Zoonotic onchocerciasis in Hiroshima, Japan, and molecular analysis of a paraffin section of the agent for a reliable identification


Summary:
Japan is a country of high specific diversity of Onchocerca with eight species, the adults of two not yet known. *Onchocerca dewittei japonica*, a common filarial parasite of wild boar, had been proved to be the agent of five zoonotic onchocerciasis in Kyushu island with morphological and molecular studies. The sixth case, at Hiroshima, was identified to the same *Onchocerca* species, based on adult characters observed on histological sections. To consolidate the identification, mitochondrial cytochrome c oxidase subunit I (CO1) gene analysis was attempted with the formalin-fixed, paraffin-embedded parasite specimen. The sequence (196 bp) of a CO1 gene fragment of the parasite successfully PCR-amplified agreed well with those of *O. dewittei japonica* registered in GenBank, confirming the morphological identification. Moreover a comparison with the CO1 gene sequences of six other *Onchocerca* species in GenBank excluded the possibility that *Onchocerca* sp. from wild boar and *Onchocerca* sp. type A from cattle in Japan, were the causative agents in this case. Mitochondrial DNA analysis proved to be a valuable tool to support the morphological method for the discrimination of zoonotic *Onchocerca* species in a histological specimen.

KEY WORDS: zoonotic onchocerciasis, *Onchocerca dewittei japonica*, mitochondrial DNA analysis, Japan.

Résumé : *Onchocercose zoonotique à Hiroshima, Japon, et analyse moléculaire d’une coupe histologique de l’agent pour conforter l’identification*

Le Japon est une région de grande diversité du genre *Onchocerca* avec huit espèces, dont deux identifiées par les microfilaries, les adultes étant encore inconnus. Il a été démontré par les analyses morphologiques et moléculaires qu’*Onchocerca dewittei japonica*, filaire fréquente du cochon sauvage, est l’agent des cinq cas d’onchocercose zoonotique dans l’île de Kyushu. Le sixième cas, à Hiroshima, a été identifié à la même espèce d’onchocercose d’après les caractères de l’adulte observés sur coupes histologiques. Afin de vérifier cette identification, l’analyse du gène mitochondrial de la sous-unité 1 de la cytochrome c oxydase (CO1) a été effectuée avec une coupe du specimen fixé au formol et inclus en paraffine. La sequence (196 bp) d’un fragment du gène CO1 du parasite, amplifié par PCR, est semblable à celles d’*O. dewittei japonica* enregistrées dans GenBank, confirmant l’identification morphologique. En outre, la comparaison avec les séquences du gène CO1 de six autres espèces d’*Onchocerca* déposées dans cette base de données exclut comme agents possibles *Onchocerca* sp. du sanglier et *Onchocerca* sp. type A du bétail au Japon. L’analyse de l’ADN mitochondrial est donc un outil valable qui complète la méthode morphologique de discrimination des espèces zoonotiques d’onchocercses sur coupes histologiques.


Human zoonotic onchocerciasis is rare. Only 16 cases have so far been reported in the world, including six cases in Japan, five from Oita, Kyushu, and the most recent from Hiroshima (Uni et al., 2010). The causative agents of all the Japanese cases were identified as *Onchocerca dewittei japonica* Uni, Bain & Takaoka, 2001, a common filarial parasite of wild boar (*Sus scrofa* Linnaeus) in Japan (Uni et al., 2001), based on the morphological characteristics of the adult worms (Beaver et al., 1989; Hashimoto et al., 1990; Takaoka et al., 1996, 2001, 2004, 2005; Uni et al., 2010). As for the first two cases, the species was confirmed retrospectively because at that time *O. dewittei japonica* had not yet been discovered (Takaoka et al., 2001; Uni et al., 2010).

In Japan, six other *Onchocerca* species are known (Takaoka et al., 2005; Uni et al., 2007): three cosmopolitan parasites of domestic animals, *O. cervicalis* Railliet and Henry, 1910 from horses, *O. gutturosa* Neumann, 1910, and *O. lienalis* (Stiles, 1982) from cattle; three of wild animals, *O. eberhardi* Uni & Bain, 2007 from sika deer (*Cervus nippon* Temminck), *O. skrjabini* Rukhiyadev, 1964 from sika deer and serows (*Capricornis crispus* Temminck), and *O. suzukii* Yagi, Bain & Shoho, 1994 from serows.
Recently another *Onchocerca* species was found from wild boars in Japan (Fukuda *et al*., 2008, 2010a). This unnamed species, the adult of which is unknown, is distinguishable from *O. dewittei japonica* by the body size of the microfilaria (Fukuda *et al*., 2008). In addition, there is another unnamed *Onchocerca* species (its adults unknown) found from cattle in Japan (Takaoka & Bain, 1990). This, designated as type A, is also distinguishable from other *Onchocerca* species by the morphology of the microfilaria and the infective larva (Takaoka & Bain, 1990; Fukuda *et al*., 2010b). Thus, there remains the possibility that either of these two unnamed species was involved as the causative agent of all or some of six Japanese cases so far reported. On the other hand, we have already shown that both of these two unnamed species are distinguishable from *O. dewittei japonica* by the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene analysis (Fukuda *et al*., 2010a, 2010b).

In order to investigate the possibility of one of the two unnamed *Onchocerca* species being the causative agent of the sixth case of zoonotic onchocerciasis in Japan (Uni *et al*., 2010), we performed the mitochondrial DNA analysis for a formalin-fixed, paraffin-embedded parasite specimen.

**MATERIALS AND METHODS**

**SPECIMEN EXAMINED**

A tissue sample stored as a paraffin block of the sixth case of zoonotic onchocerciasis in Japan was examined, where the worm found in the histological sections had been already identified as female *O. dewittei japonica* based on its morphology (Fig. 1) (Uni *et al*., 2010). In short, in July 2009, a subcutaneous nodule (2 cm in diameter) was surgically removed from the left knee of the patient, a 70-year-old man living in Hiroshima Prefecture, Japan. The tissue excised (1 × 2 cm) was fixed in 4 % paraformaldehyde for 24 hr and embedded in paraffin by a routine procedure (Uni *et al*., 2010). For molecular analysis the section was cut at thickness of 20 μm.

**DNA EXTRACTION**

The tissue of the worm (ca. 2.8 mm²) was scraped from the section on a glass slide with a disposable sterilized scalpel blade and transferred into a 1.5 ml microcentrifuge tube. The tissue was incubated with 0.5 ml of DEXPAT (Takara Bio Inc., Otsu, Japan) for 10 min at 100 °C and then centrifuged for 10 min at 12,000 rpm at 4 °C. Ten microliters of the supernatant was used as template DNA for PCR.

**PCR AND SEQUENCING OF THE PARTIAL MITOCHONDRIAL CO1 GENE REGION**

Two primer sets, general filarial primers CO1intF-CO1intR (Casiraghi *et al*., 2001) and newly designed CO1fF (5’-TTGTCTGTTCCTGTTTTGG-3’)–CO1fR (5’-GCAAAAGTTATTCTAGTTTGACCA-3’) respectively, were used to amplify a fragment of the mitochondrial CO1 gene (coding sequence). CO1fF-CO1fR was constructed inside CO1intF-CO1intR on the basis of the known sequences of *Onchocerca* species in Japan. The positions of the primers on the complete mitochondrial genome of *O. volvulus* (GenBank accession
number: AF015193) are: CO1intF, 2519-2538; CO1intR, 3207-3186; CO1ffF, 2884-2902; CO1ffR, 3199-3122.  

Amplifications were performed in 50 µl containing 1× PCR buffer for KOD -Plus- Ver.2 (Toyobo, Osaka, Japan), 1.5 mM MgSO₄, 200 µM each of dNTPs, 0.1 µM each of primers, 0.5 units of KOD -Plus- (Toyobo), and 10 µl of template DNA. The thermal conditions were as follows: larger fragments (689 bp), an initial denaturation at 94 °C for 2 min, followed by five cycles of 98 °C for 45 s; smaller fragments (239 bp), an initial denaturation at 94 °C for 2 min, followed by five cycles of 98 °C for 10 s, 48 °C for 30 s, and 68 °C for 45 s; and 37 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 45 s and 37 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 30 s. 

PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and directly sequenced using the primers for PCR, a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). PCRs were conducted twice and each of the amplification products were sequenced. The sequence determined was deposited in DDBJ/EMBL/GenBank databases under the accession number AB604943 (Table I).

**DATA ANALYSIS**

The sequence obtained was aligned with published sequences of seven *Onchocerca* species in Japan. Using this alignment, sequences were compared by MEGA 4.0.2 based on 196 bp available for comparison (Tamura et al., 2007). Used GenBank database accession numbers were as follows: *O. dewittei japonica* (AM749266, AB518874, AB518875), *O. eberhardi* (AM749268), *O. gutturosa* (AJ271617), *O. skrijabinii* (AM749269), *O. sp. type A sensu* Fukuda et al., 2010 (AB518876), *O. sp. wild boar sensu* Fukuda et al., 2010 (AB518693), and *O. suzukii* (AM749275).

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 Values above the diagonal are the numbers of nucleotide differences, and those below the diagonal are the percentages of nucleotide differences. * Diagnosis by morphological observation.

Table I. – Nucleotide differences over 196 sites of the CO1 gene sequences among *Onchocerca* species in Japan.
**Research note**

**species** is uninvestigated. The causative agent of zoonotic onchocerciasis in view of morphological method for species identification of the causative agent, species identification from a section with a usual thickness of 4 µm may be possible, but is yet to be confirmed. This type of molecular analysis will be a useful tool for the definitive diagnosis in similar cases of zoonotic onchocerciasis in future.

In conclusion, our study suggests that mitochondrial DNA analysis is a useful tool to support the traditional morphological method for species identification of the causative agent of zoonotic onchocerciasis in view of the fact that few specimens, especially histological sections, are available and the life cycle of the causative species is uninvestigated.

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**REFERENCES**


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