FULL PAPER *Parasitology*

Genetic Uniformity of *Echinococcus multilocularis* Collected from Different Intermediate Host Species in Hokkaido, Japan

Munehiro OKAMOTO[1)], Yuzaburo OKU[2)], Tsutomu KUROSAWA[3)] and Masao KAMIYA[2)]

[1)]*Department of Laboratory Animal Science, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680–8553,* [2)]*Laboratory of Parasitology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818 and* [3)]*Institute of Experimental Animal Science, Graduate School of Medicine, Osaka University, Suita 565–0871, Japan*

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ABSTRACT. DNA from several isolates of *Taenia taeniaeformis* and *Echinococcus multilocularis* were digested with restriction enzymes and hybridized with digoxigenated oligonucleotide probe $(CAC)_5$. Within the six wild isolates of *Taenia taeniaeformis* from Norway rats in Hokkaido, although several bands were common among isolates, fingerprinting patterns were specific to each isolate. In the case of *E. multilocularis*, regardless of hosts from which each isolate has been isolated, the five isolates collected from Hokkaido, showed the same fingerprinting pattern. These results indicate that there was very little genetic difference among these isolates. Although the fingerprinting pattern of *E. multilocularis* from St. Lawrence Is. was similar to that of the Hokkaido isolates, some bands were different from those in the Hokkaido isolates. *Echinococcus multilocularis* in Hokkaido seems to be closely-related genetically to that from St. Lawrence Is.

KEY WORDS: different intermediate host species, DNA fingerprinting, *Echinococcus multilocularis*, genetic uniformity, Hokkaido.

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Intraspecific variation has been described from different geographic areas or host species in all major groups of parasites by applying a number of differential criteria [25]. Traditionally, the morphology was used as the sole differential criterion. However, more recently several intrinsic or extrinsic characteristics have been used [26]. *Echinococcus granulosus*, which is responsible for cystic hydatid disease in humans and animals, is known to exist as biologically and genetically distinct subspecific variants or strains. However, few intraspecific variations of *E. multilocularis* have been reported [2, 4].

Echinococcus multilocularis, which is responsible for alveolar hydatid disease in humans, is one of the most medically important cestodes in the holarctic region. The natural intermediate hosts of *E. multilocularis* are arvicoline rodents. However, in Hokkaido Japan, natural infections of *E. multilocularis* in swine and horse, which have been scarcely reported in other geographical areas, have been frequently observed [12, 21]. Moreover, natural infection of *E. multilocularis* in a Norway rat, *Rattus norvegicus*, has been found in southern Hokkaido [16]. Although many species of mammals have been reported as possible natural or experimental intermediate hosts of *E. multilocularis*, there are few reports on infection in the Norway rat, swine, or horse outside Hokkaido [15, 20, 23]. Therefore, the possibility that a different strain or population exists in the Hokkaido district from those reported in other geographical areas is considered.

Sequencing of mitochondrial or nuclear genes is a useful tool to infer the phylogenetic relationships of organisms. So we have examined the partial sequences of mitochondrial CO1 gene for Hokkaido isolates of *E. multilocularis*. However, all isolates shared the same sequence of CO1 gene [19].

DNA fingerprinting has proved to be powerful in resolving genetic identity or relationships and is applied in many diverse areas of biological sciences including forensic science, paternity testing, animal breeding and population genetics [6, 8, 9]. One attractive DNA fingerprinting method is the detection of hypervariable simple repetitive DNA by means of oligonucleotide probes, which make it possible to establish highly informative DNA fingerprints for any eucaryotic organisms. Oligonucleotide probe $(CAC)_5$ is multilocus and a very informative probe to identify human individuals [28]. It is also useful for verifying genetic relationships in domestic animals and wild birds [5]. Okamoto *et al.* [18] applied DNA fingerprinting with $(CAC)_5$ to analysis of genetic variation within *Taenia taeniaeformis* and reported that $(CAC)_5$ was a highly resolvable and informative probe for cestodes.

Taenia taeniaeformis is a common parasite of cats in Japan, and its intermediate hosts are Norway rat (*Rattus norvegicus*), small Japanese field mouse (*Apodemus argenteus*) and gray red-backed vole (*Clethrionomys rufocanus bedfordiae*) so far reported. From these intermediate hosts, all isolates of *T. taeniaeformis* from Norway rats shared the same sequences for CO1 gene so far examined [19]. However, DNA fingerprinting patterns of those isolates constructed with the oligonucleotide probe $(CAC)_5$ were different from each other [18]

In this study, we examined the genetic variability of *Echinococcus multilocularis* collected from different intermediate host species using DNA fingerprinting and discussed the genetic features of *E. multilocularis* population in Hokkaido comparing with the case of *T. taeniaeformis*.

Table 1. Hosts and geographical origins of cestodes examined in this study

Species	Isolate	Host	Geographical origin
Echinococcus multilocularis	EmHok	Gray red-backed vole	Higashimokoto, Hokkaido, Japan
	EmTob	Gray red-backed vole	Tobetsu, Hokkaido, Japan
	EmYak	Gray red-backed vole	Yakumo, Hokkaido, Japan
	Empig	Pig	Kitami, Hokkaido, Japan
	Emrat	Norway rat	Kamiiso, Hokkaido, Japan
	EmStL	Tundra vole	St. Lawrence Is., U.S.A.
Taenia taeniaeformis	TtSap1	Norway rat	Sapporo, Hokkaido, Japan
wild isolates	TtSap2	Norway rat	Sapporo, Hokkaido, Japan
	TtSap3	Norway rat	Sapporo, Hokkaido, Japan
	TtTom	Norway rat	Tomikawa, Hokkaido, Japan
	TtKam	Norway rat	Kamiiso, Hokkaido, Japan
	TtEbe	Norway rat	Ebetsu, Hokkaido, Japan
	TtCat	Cat	Sapporo, Hokkaido, Japan

Gray red-backed vole: *Clethrionomys rufocanus bedfordiae*, Norway rat: *Rattus norvegicus*, Tundra vole: *Microtus oeconomus*.

MATERIALS AND METHODS

Parasites: Five Hokkaido isolates and one Alaskan isolate of *E. multilocularis* were examined. Each isolate was passaged by intra-peritoneal injection in Mongolian gerbils (*Meriones unguiculatus*) in Hokkaido University. As for isolates of *Taenia taeniaeformis*, 6 wild isolates were used. These taeniid samples were stored in liquid nitrogen, at –80°C or in 70% ethanol until required for DNA extraction. The sample list and locality map are shown in Table 1 and Fig. 1, respectively.

Preparation of DNA: DNA fingerprinting requires comparably high-molecular weight DNA, so we have prepared the genomic DNA using the extraction with phenol. The details of the method have been given in a previous report [18].

Sequencing for CO1 gene: A partial fragment of CO1 gene was amplified from the total DNA by PCR using the primer pair pr-a and pr-b [19]. Direct sequencing of the PCR amplification product was performed with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) using the pr-a and pr-b.

DNA fingerprinting with oligonucleotide probe $(CAC)_5$: Approximately 1 μg of taeniid DNA was digested with 30–50 units of restriction endonuclease *Pst* I or *Pvu* II (Nippon Gene), using the buffer and reaction conditions as recommended by the manufacturer of the respective enzymes. Reaction was stopped by the addition of 1/10 volume of 50% glycerol, 10 mM $NaHPO_4$, 100 mM EDTA and 0.4% bromophenol blue. Digested DNA was loaded on 0.8% agarose gel and run in TBE buffer (89 mM boric acid, 89 mM Tris, 2 mM EDTA).

After electrophoresis, DNA was denatured by soaking the gel for 30 min in 0.5 M NaOH, 1.5 M NaCl with constant gentle agitation and then neutralized by soaking for 30 min in 0.5 M Tris-HCl (pH 7.8), 1.5 M NaCl. Thereafter, DNA was blotted with 20 × SSC(3 M NaCl, 0.3 M Nacitrate, pH7.0) on to positively charged nylon membranes (Hybond N+, Amersham, U.S.A.) for 6 hr.

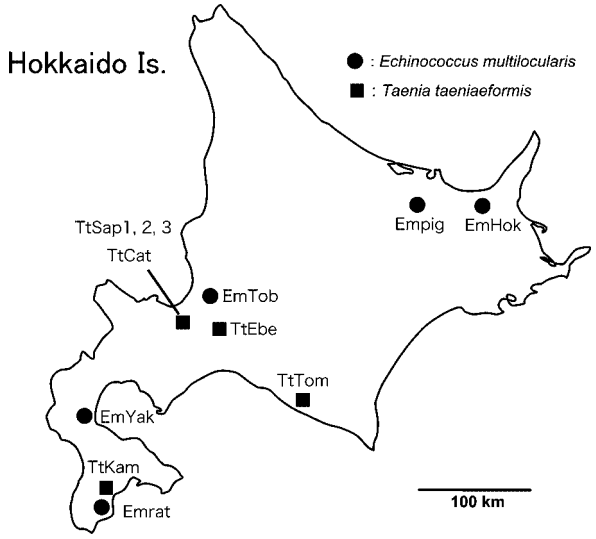

Fig. 1. Collection localities of *Echinococcus multilocularis* and *Taenia taeniaeformis* in Hokkaido Island. For explanations of abbreviations of sample names, see Table 1. Four isolates of *T. taeniaeformis* from Sapporo were collected from distant localities in the same city.

The oligonucleotide $(CAC)_5$ was chemically synthesized and purified by reversed phase HPLC. Labeling with digoxigenated dUTP was done in terminal deoxynucleotidyl transferase using a DNA Tailing Kit (Roche Diagnostics, Germany). Membranes were baked at 80°C for 2 hr. Hybridization with digoxigenated probe and immunological detection were performed by using a DIG Nucleic Acid Detection Kit (Roche Diagnostics). Prehybridization and hybridization were done at 42°C for 1 hr and for at least 10 hr respectively. Filters were then washed twice in 2 × SSC containing 0.1% SDS at room temperature for 5 min and for 15 min, respectively. These were washed twice in 2 × SSC containing 0.1% SDS at 45°C for 30 min. Subsequent immunological detections were performed according to the manufacturer's instructions.

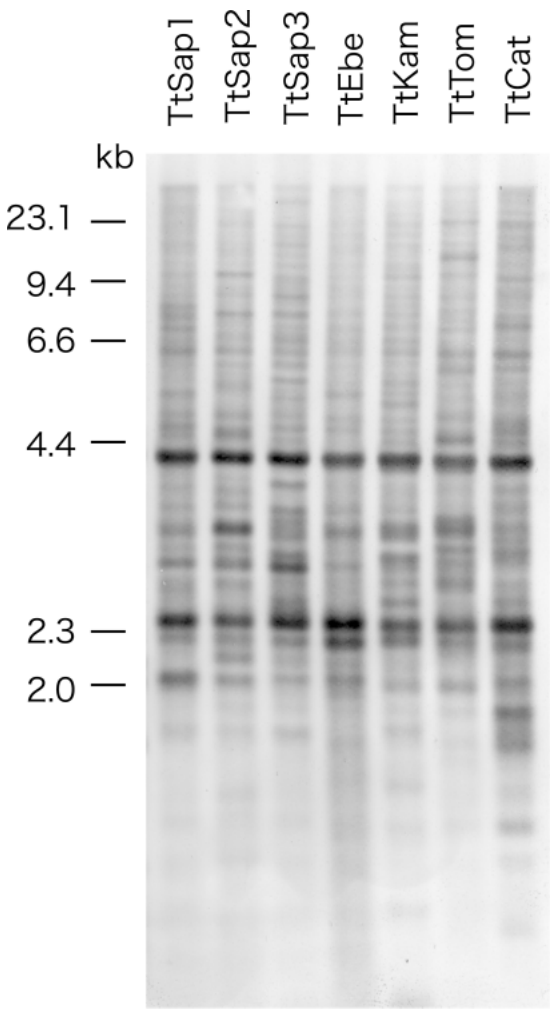

Fig. 2. DNA fingerprinting of wild isolates of *T. taeniaeformis* with digoxigenated oligonucleotide probe $(CAC)_5$. DNA was digested with *Pst* I and electrophoresed in 0.8% agarose gel at 30 V 15 hr. Molecular weight markers are given on the left in kilobases.

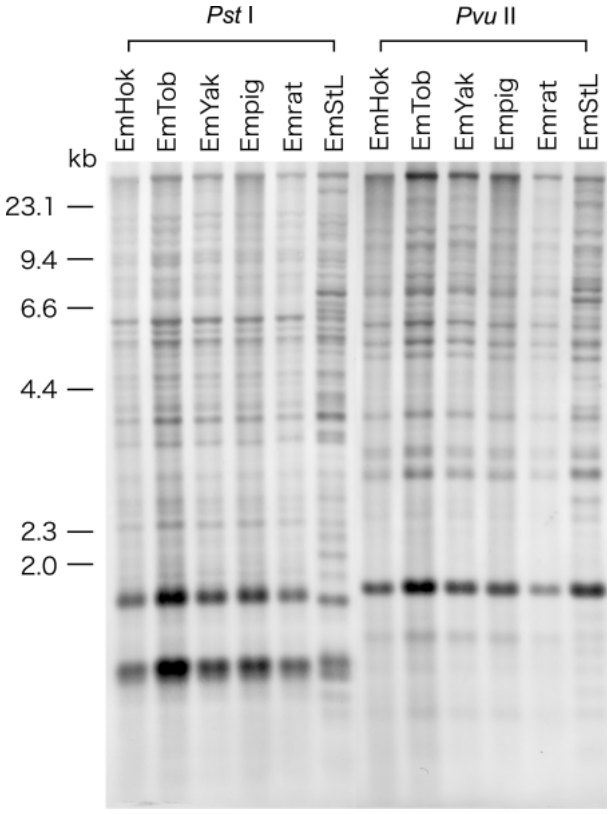

Fig. 3. DNA fingerprinting of six isolates of *Echinococcus multilocularis* with digoxigenated oligonucleotide probe $(CAC)_5$. DNA was digested with *Pst* I or *Pvu* II and electrophoresed in 0.8% agarose gel at 20 V 24 hr. Molecular weight markers are given on the left in kilobases.

RESULTS

Sequencing of the CO1 gene: Partial sequences of the mitochondrial CO1 gene from some samples had been examined [19]. In this study, therefore, all wild isolates of *T. taeniaeformis* except TtTom were examined. All wild isolates of *T. taeniaeformis*, including TtCat, shared the same sequence as that from TtSRN (GenBank/EMBL/DDBJ accession no. AB221484) [19]. No sequence variation was observed in *E. multilocularis*, regardless of the hosts or geographical areas from which each metacestode had been isolated [19]. Phylogenetic relationships of taeniid cestodes, including some isolates examined in this study, inferred from the CO1 gene have been reported [19].

DNA fingerprinting: DNA from *T. taeniaeformis* were digested with *Pst* I and hybridized with digoxigenated oligonucleotide probe $(CAC)_5$. All isolates showed clear multibanding patterns, which were characteristic of multilocus DNA fingerprinting (Fig. 2). Fingerprinting patterns of wild isolates of *T. taeniaeformis* resembled each other. However, several bands were specific to some isolates, so isolate was easily distinguishable each other by fingerprinting patterns.

Fingerprinting patterns of 6 isolates of *E. multilocularis* when digested with *Pst* I and *Pvu* II are shown in Fig. 3. Regardless of hosts from which each isolate had been isolated, the five isolates from Hokkaido showed completely the same fingerprinting pattern. Fingerprinting pattern of EmStL was similar to that of the Hokkaido isolates. However, some bands seen in EmStL were either different in molecular size, or missing in Hokkaido isolates. Moreover additional bands were seen in EmStL.

DISCUSSION

The DNA fingerprinting pattern often is specific to an individual, except in extreme case of inbreeding or in monozygotic twins, or clones. It was reported that $(CAC)_5$ represented the informative fingerprints for genetic analysis

of *T. taeniaeformis*, when digested with *Hinf* I [18]. In the case of *E. multilocularis*, digestion with either *Pst* I or *Pvu* II was effective in preliminary study (data not shown). In this study, therefore, the digestion with *Pst* I was applied.

One of the major problems with applying multilocus DNA fingerprinting to population analysis is that it is impossible to identify which bands are derived from the same locus, especially between distant organisms. Although wild populations usually are highly polymorphic, with many alleles at a single locus, bands that appear to be shared by individuals are not always identical alleles at the same locus [3]. On the contrary, among closely-related, especially blood-related organisms, many identical alleles are derived from the same locus, and the fingerprinting patterns resemble each other. In the present study, it seemed that six wild isolates of *T. taeniaeformis* were genetically related to each other. However, no isolate had a fingerprinting pattern that was same as that of any other isolate.

As contrasted with *T. taeniaeformis*, five isolates of *E. multilocularis* from Hokkaido showed the identical fingerprinting pattern. The main intermediate host of *E. multilocularis* in Hokkaido is a gray red-backed vole. However, several reports have been published on natural infections of *E. multilocularis* in uncommon intermediate hosts in Hokkaido Island, namely, swine [21], horses [12] and Norway rats [16]. There have been few reports of natural infection of *E. multilocularis* in these intermediate hosts in other endemic areas. In the present study, although we compared isolates derived from gray red-backed voles, swine and Norway rats, no difference was detected in their fingerprints. These results indicate that there was very little genetic difference among these isolates. It seemed that infections with *E. multilocularis* to the unusual animals in Hokkaido were not responsible for the variation in *E. multilocularis*.

Generally, wild populations are highly polymorphic, with many alleles at a single locus. It is very rare that the same fingerprinting pattern is obtained from two individuals, except in the case of monozygotic twins. Nevertheless, why were fingerprints from all Hokkaido isolates of *E. multilocularis* identical?

Although all isolates from Hokkaido Island showed an identical fingerprinting pattern, this did not mean that all isolates were as uniform as clones. Actually, Nakao *et al.* [13] reported that polymorphism of microsatellite DNA was detected in Hokkaido's population of *E. multilocularis*. Because the multilocus fingerprint is the technique used to detect RFLPs of genome DNA, sometimes it cannot detect a slight difference in DNA such as several base indels in microsatellites. Although it is known that self-insemination occurs in *Echinococcus* [10, 24], the heterozygosity of microsatellite alleles indicates that cross-fertilization also occurs in *E. multilocularis*. However, the heterozygosity observed was low in Hokkaido's population of *E. multilocularis* [13].

Since asexual proliferation occurs in the larval stage of *Echinococcus*, a large number of clonal protoscoleces are produced in intermediate hosts. When foxes prey on the rodent infected, the majority of adult worms, therefore, are clonal. An increase in homozygosity within a population of *Echinococcus* is well explained by self-fertilization [13], which can be achieved by a sperm of the same individual (autogamy) or of another clonal individual (geitonogamy) [11].

Even though the population of *Echinococcus* has such properties, if *E. multilocularis* is native to Hokkaido and has inhabited that area for a long time, geographical variation in fingerprinting should be detected, because the mutation rate of fingerprinting is very high. In humans, the spontaneous mutation rate of fingerprinting with $(CAC)_5$ has been estimated to be approximately 0.001 per DNA fragment and gamete [14]. *Echinococcus multilocularis* in Hokkaido seems to be a recent population that invaded from another endemic area. Actually, endemic areas of *E. multilocularis* on Hokkaido Island were restricted to its eastern part before 1975 [7]. In addition, if the *E. multilocularis* that invaded Hokkaido had been polymorphic genetically, variation in its fingerprinting should still have remained. The origin of *E. multilocularis* endemic in Hokkaido seems to be a single or very uniform population.

Yamashita [27] assumed that the *E. multilocularis* prevalent in Hokkaido Island was introduced from St. Lawrence Island via Komandorskie and Kuril Islands. It was reported that a sequence of CO1 gene of *E. multilocularis* from Kunashiri Island, which was the southern Island of Kuril Islands, was same as that from Hokkaido [22]. The fingerprinting pattern of EmStL resembled that from Hokkaido isolates with slight differences in several bands. Comparing with the case of *Taenia taeniaeformis*, it appears that Hokkaido's *E. multilocularis* is not identical to EmStL, but is closely-related genetically.

A partial sequence of the mitochondrial CO1 gene of EmStL examined is shared with that from Hokkaido's isolates. However, it was recently revealed that *E. multilocularis*, which had a different type of the mitochondrial CO1 gene, also inhabits St. Lawrence Island (data not shown). Thus, *E. multilocularis* endemic to St. Lawrence Island may be polymorphic. In the present study, only one isolate from St. Lawrence Island was examined. Therefore, it is possible that population, which is identical to *E. multilocularis* in Hokkaido, may inhabit St. Lawrence Island. In order to determine the origin of *E. multilocularis* in Hokkaido, additional investigations are needed, including isolates from St. Lawrence Island, Komandorskie and Kuril Islands.

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