Short communication

Heterogeneity of the large subunit of ribosomal RNA gene sequences in a *Halicephalobus gingivalis* isolate

Toyoshi YOSHIGA 1,*, Ryusei KUWATA 1, Hikaru TAKAI 2 and Kenichi NISHIMURA 3

*Halicephalobus gingivalis* (Panagrolaimidae) is a nematode parasite of horses and humans. It has been identified as the cause of nasal, maxillary and renal granulomas, as well as meningoencephalitis in horses. More than 50 cases of *Halicephalobus* infection in horses have been reported from Europe, North America, South America, Africa and Asia (Anderson et al., 1998; De Sant’Ana et al., 2012). In addition, four fatal infections of *H. gingivalis* in humans have been reported in Canada and the USA (Gardiner et al., 1981; Ondrejka et al., 2010). In spite of the severe damage, there is no effective method available to prevent or treat infection. Moreover, the ecology and life history of this species is not well elucidated. All the *Halicephalobus* species that have been reported are very small (235-460 μm), reproduce parthenogenetically and are morphologically similar (Andrássy, 1984; Anderson et al., 1998). Because of the poor morphological diagnostic features, identification of *Halicephalobus* species is difficult (Fonderie et al., 2013). DNA analyses are often used to identify the species and to infer phylogenetic relationships. The 5′ end of the DNA sequences of the nuclear large-subunit ribosomal RNA (LSU rDNA) gene from six *H. gingivalis* isolates in humans have been reported in Canada and the USA (Gardiner et al., 1981; Ondrejka et al., 2010). To elucidate the relationship among the *Halicephalobus* isolates, we determined the partial LSU rDNA sequence of another *Halicephalobus* nematode isolated from a horse in Ishikawa Prefecture, Japan, and inferred phylogenetic relationships amongst the *Halicephalobus* isolates.

*Halicephalobus* nematodes were isolated from a frozen renal sample from a horse (horse specimen number: H4677) that died in 2003 at an equestrian club in Ishikawa Prefecture (Takai et al., 2005). DNA was extracted from a single nematode as described by Iwahori et al. (2000). To amplify the partial LSU rDNA, PCR was done using primers 391 and 501 (Nadler et al., 2003) and Takara Ex Taq® (TaKaRa) as described by Akagami et al. (2007). Briefly, the PCR reaction mixture was denatured at 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, followed by a post-amplification extension at 72°C for 8 min. Sequencing was performed using primers 391 and 501 and an ABI PRISM® 310 Genetic Analyzer. Direct sequencing of PCR products was attempted using three different DNA samples from three single nematodes isolated from the renal sample; however, two more cases of infections in horses were detected in 2000 and 2003 at an equestrian club in Ishikawa Prefecture (Shibahara et al., 2002; Takai et al., 2005). In 2006, the first case of infection in a pony in Japan was reported (Akagami et al., 2007). Phylogenetic analysis of the LSU rDNA of the nematode from the pony revealed that it formed a clade with a *H. gingivalis* isolate from Tennessee (Tenn2 strain), USA. In Japan, the first *H. gingivalis* infection in a horse was detected in 1981 in Tokyo (Yoshihara et al., 1985). Two more cases of infections in horses were detected in 2000 and 2003 at an equestrian club in Ishikawa Prefecture (Shibahara et al., 2002; Takai et al., 2005). In 2006, the first case of infection in a pony in Japan was reported (Akagami et al., 2007). Phylogenetic analysis of the LSU rDNA of the nematode from the pony revealed that it formed a clade with a *H. gingivalis* isolate from Tennessee (Tenn2 strain), USA. To elucidate the relationship among the *Halicephalobus* isolates, we determined the partial LSU rDNA sequence of another *Halicephalobus* nematode isolated from a horse in Ishikawa Prefecture, Japan, and inferred phylogenetic relationships amongst the *Halicephalobus* isolates.

In Japan, the first *H. gingivalis* infection in a horse was detected in 1981 in Tokyo (Yoshihara et al., 1985).
complete sequencing could not be successfully achieved because of overlapping peaks beginning from base 432 of the PCR products. To complete the sequencing, the PCR products from a single nematode were directly cloned using the pGEM-T easy vector system (Promega), and plasmid DNA was used for DNA sequencing. Nucleotide sequences data reported in this paper are available in the EMBL, GenBank and DDBJ databases under accession numbers AB289345 and AB289346.

Of eight clones sequenced, four contained the same LSU rDNA sequence (JapanHT4677a) although the rest contained a different sequence (JapanHT4677b). The sequence length of JapanHT4677a was 814 bp, but that of JapanHT4677b was 817 bp. The similarity between the sequences was 96.9%. There were 22 nucleotide substitutions, 18 being transitions (13 G-A and 5 T-C transitions). The presence of a three-nucleotide gap at base 432 (Fig. 1A) appears to account for the overlapping peaks obtained by direct sequencing. Multiple alignments of the two sequences with the H. gingivalis sequences available in the database revealed that there was no sequence gap between bases 1-431; how-

![Fig. 1. A: Variation of partial LSU rDNA sequence of Halicephalobus gingivalis JapanHT4677 relative to other isolates. The alignment was performed using ClustalW (Chenna et al., 2003). The asterisks indicate matched nucleotides, and bars mark gaps between the sequences. The two different sequences (JapanHT4677a, JapanHT4677b) determined in the present study are underlined; B: MP tree showing the phylogenetic relationship of H. gingivalis isolated from a horse in Ishikawa Prefecture, Japan, with Halicephalobus isolates and outgroups based on partial LSU rDNA gene sequences (tree length = 920, consistency index = 0.8565, retention index = 0.7376, rescaled consistency index = 0.6317). Multiple-sequence alignment was created with 15.0 gap-opening penalties and 6.66 gap-extension penalties using ClustalX version 1.81 (Thompson et al., 1997). Phylogenetic analysis was performed using methods in PAUP∗ 4.0 beta Version 10 (Swofford, 1998). The bootstrap values evaluated by bootstrap resampling (1000 replicates) are shown above internal nodes. Four outgroup species were selected according to Nadler et al. (2003). The two different sequences (JapanHT4677a, JapanHT4677b) that were determined in the present study are underlined. JapanMA28826 is the H. gingivalis isolate from Ibaraki, Japan (Akagami et al., 2007). Other H. gingivalis sequences were from Nadler et al. (2003): Tenn2, TN, USA; Tenn1, TN, USA; SAN100, Guelph, ON, Canada; South Pacific, CA, USA; JB128, CA, USA; JB043, Neustadt, Germany.](image-url)