Detection of *Halicephalobus gingivalis* in soil nematode samples using PCR

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*Halicephalobus gingivalis* is a panagrolaimid nematode that causes fatal infections in horses. Once the nematode infects and propagates within horses, it causes nasal, maxillary, and renal granulomas as well as meningoencephalitis. More than 50 cases of infection in horses have been reported from all over the world (Anderson et al., 1998; Takai et al., 2005a). In addition to infecting horses, 3 cases of fatal infection of *Halicephalobus* in humans have also been reported in Canada and the USA. (Takai et al., 2005a).

In Japan, the first *Halicephalobus* infection in horses was reported in 1981 in Tokyo (Yoshihara et al., 1985). In 2000 and 2003, 2 cases of infections in horses were reported in an equestrian club in Ishikawa Prefecture (Shibahara et al., 2002; Takai et al., 2005b). In 2006, an infected pony was reported in Ibaraki Prefecture (A kagami et al., 2007). Although infections have been reported from different places in recent years, there is no information regarding the natural reservoir of the nematode or its mode of infection.

*H. gingivalis* is a small nematode with a maximum body length of approximately 400 µm (Andrássy, 1984). Due to the poor morphological characterization and lack of information regarding the distribution of the nematode, its detection in the mixed soil nematode population is laborious and time consuming. The establishment of a simple, sensitive, and reliable method for the identification of the nematode from the soil nematode population is the key to determining the natural reservoir of the nematode and preventing the *Halicephalobus* infection. In this paper, I describe the method for identifying *H. gingivalis* from the nematodes isolated from soil.

**MATERIALS AND METHODS**

Nematodes:

Nematodes were isolated from soil in a compost pile obtained from the equestrian club in Ibaraki Prefecture where the *Halicephalobus* infection was reported in 2007 (A kagami et al., 2007) using the Baermann funnel method at 27°C for 48 hr. Because *H. gingivalis* has been isolated from compost pile in the USA (Nadler et al., 2003) the soil from the equestrian club was chosen.

*H. gingivalis* juveniles were obtained from a frozen renal sample of a horse (A kagami et al., 2007). A single juvenile was picked up from the thawed renal sample by a dental file and was used to confirm the efficiency of the primers used. In order to evaluate the DNA extraction method and perform the polymerase chain reaction (PCR) analysis, the single or 5 juveniles were added to about 3,000 nematodes isolated from the soil in the compost pile.

Isolation of genomic DNA from the nematodes:

DNA extraction from a single nematode was performed as described by Iwahori et al. (2000). DNA from nematode mixtures was extracted using the QIA amp DNA mini kit (Qiagen). The nematodes were lysed in 200 µl of the supplied cell lysis buffer containing proteinase K solution at 65°C for 1 hr with occasional vortexing. After the nematode lysis was confirmed microscopically, DNA was purified by using the spin column provided, according to the manufacturer's
instructions. DNA was eluted from the spin column into 400 µl of the supplied buffer and stored at -30°C until further use. The eluent (20–30 ng DNA/µl) was diluted with sterile water (1/1, 1/10 and 1/100 dilution) and the diluted DNA solution was used as a template for PCR.

PCR analysis:

To amplify partial large-subunit ribosomal DNA (LSU rDNA), PCR was carried out according to the procedure described by Nadler et al. (2003) using the Halicephalobus-specific primers #632 (5'-GTAGCGTATAGGAATATATTATGG-3') and #634 (5'-CTTCATCCTGCCTAGCAAGCATAGA-3'). PCR was performed in a 10-µl reaction mixture containing 1 µl of 10-fold PCR buffer, 0.8 µl of dNTP mixture, 0.05 µl of TaKaRa Ex Taq® (TaKaRa), 1 µM of each primer, and 0.8 µl of template DNA solution (1/1, 1/10, or 1/100-diluted DNA solution). The mixture was incubated at 95°C for 3 min followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min, followed by post-amplification extension at 72°C for 8 min. The PCR products were separated on 1.5% agarose gel, and DNA was visualized using 1 µg/ml ethidium bromide solution. For DNA sequencing, a DNA band measuring approximately 700 bp in length was excised from the gel, and DNA was purified using the Gel-M Gel Extraction System (Viogene).

RESULTS AND DISCUSSION

A DNA fragment—expected to be approximately 700 bp long—was successfully amplified by using a primer set (#632 and #634) and a DNA template that was extracted from a single H. gingivalis obtained from an equine sample from Ibaraki Prefecture. Using this primer set, the amplification of the partial LSU rDNA of H. gingivalis from nematode mixtures was per-
formed. Following nematode isolation by Baermann funnel method, the nematodes were examined under a microscope, but Halicephalobus nematodes were not detected. The nematode sample was thus considered as a Halicephalobus-free nematode sample. When the DNA extracted from approximately 3,000 soil nematodes except H. gingivalis was used for PCR, no amplicon was detected (data not shown). On the other hand, in the DNA extracted from approximately 3,000 nematodes containing a single juvenile or 5 juveniles of H. gingivalis, an amplicon of approximately 700 bp was detected (Fig. 1). This 700-bp amplicon was purified and was used for direct sequencing. The DNA sequence was identical to the partial LSU rDNA of H. gingivalis from a frozen renal sample from Ibaraki prefecture (data not shown). There was no significant difference between the DNA samples isolated from 1 or 5 H. gingivalis juveniles in 3,000 nematodes. These results demonstrate that this method is sufficiently sensitive to detect a single juvenile of H. gingivalis among 3,000 soil nematodes.

For the detection of nematodes, the method used for nematode extraction from the soil is critical. However, no information is available on the efficiency of extraction of H. gingivalis; moreover, live H. gingivalis specimens are not available in Japan. In the present study, the Baermann funnel method was used to extract nematodes because no information regarding the distribution of H. gingivalis is available, and the processing of many soil samples is required to increase the possibility of detection. Unlike plant parasitic nematodes, H. gingivalis is a bacterivorous free-living nematode that generally exhibits high motility. The Baermann funnel method that is based on nematode mobility is simple and reproducible and appears to be suitable for the present purpose of processing many samples. Further study is necessary to improve the extraction efficiency of this method.

The nematode cuticle is a three-layered structure covered with a trilaminar epicuticle (Bird and Bird, 1991), which protects the nematodes from physical and chemical stress. In addition, nematodes are difficult to homogenize using mechanical homogenizers such as a glass homogenizer and Polytron® because of their small size. To increase the reproducibility and sensitivity along with the ease of handling, the QIAamp DNA mini kit (Qiagen) was selected for DNA extraction. After incubation in the lysis buffer containing proteinase K solution, most nematodes were observed to be lysed in 1 hr. In the present study, the yield of DNA from the 3,000 soil nematodes by this method was 6–12 µg which appears to be within the maximum capacity of this kit. This type of kit with an efficient lysis buffer may be suitable for the isolation of genomic DNA from nematodes.

By using the simple and reliable method described in the present report, the detection of H. gingivalis from soil nematodes samples can be simplified. Moreover, information regarding its natural reservoir will also help in preventing further infection.

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