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First molecular identification of *Strongyloides vituli* in cattle in Japan and insights into the evolutionary history of *Strongyloides* parasites of ruminants

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ABSTRACT

Traditionally, *Strongyloides* nematode infecting cattle had been thought to be a single species, *S. papillosus*. Surprisingly, Eberhardt et al. in 2008 reported two, rather than one *Strongyloides* species infected cattle, with one being *S. papillosus* and the other *S. vituli*. However, there was no subsequent report to support their findings.

In July 2018, a case of a sudden death of a calf believed to be due to heavy infection with *S. papillosus* at a dairy farm in Miyazaki Prefecture, Japan, was reported. One month after the initiation of a deworming program to prevent further sudden deaths, fecal specimens from 24 cattle housed in the same barn were examined. Eight samples were positive for *Strongyloides* eggs. For species determination, the nucleotide sequences of *18S* rDNA (small subunit ribosomal DNA gene), *rpl-10* (ribosomal protein L10 gene), and mitochondrial (mt) *cox1* (cytochrome *c* oxidase subunit 1 gene) were obtained. Typing data for all three marker genes indicated the presence of both species, *S. papillosus* and *S. vituli,* in the fecal samples. To our knowledge, this study is the first to support the original report by Eberhardt et al. regarding the sympatric existence of *S. papillosus* and *S. vituli* in cattle, and to report the presence of *S. vituli* in Japan.

Interestingly, phylogenetic analyses of both *rpl-10* and mt *cox1* sequences indicated a closer genetic relationship of *S. vituli* with *S. venezuelensis* (*Strongyloides* of rats) than with *S. papillosus*, shedding light on the speciation history of *Strongyloides* nematodes.

1. Introduction

Strongyloides is a genus of intestinal parasites (nematodes) known to infect various terrestrial vertebrates. There are approximately 50 documented *Strongyloides* species [1]. It was generally believed that *Strongyloides* known to infect farm ruminants belonged to a single species: *S. papillosus* (Wedl, 1856) [2]. However, Eberhardt et al. reported that the *Strongyloides* population isolated from sheep and cattle was comprised of at least two different genetically isolated groups with different host preferences [3]. The first group was predominantly found in sheep while the other was found exclusively in cattle. They proposed the name *S. papillosus* for *Strongyloides* found in sheep and *S. vituli* (Brumpt, 1921) [4] for *Strongyloides* preferentially infecting cattle. Prior to the present study, however, there were no subsequent reports to support their findings.

In the 1980s and 1990s, sudden cardiac deaths in calves due to heavy infection with *S. papillosus* drew considerable attention in Japan [5–7]. However, after the introduction of preventive measures such as implementation of deworming programs in many farms, the sudden cattle deaths became rare. However, low grade-infections with *Strongyloides* sp. are still common among cattle in Japan. In July 2018, there was a suspected case of sudden death of a calf due to heavy infection with *S. papillosus* at a farm (farm X) in Miyazaki Prefecture (one of the prefectures where outbreaks of the sudden deaths were reported in the past [8]). After this case, a deworming program was initiated at this farm.

One month after the initiation of the deworming program, fecal examinations were conducted for 24 cattle from farm X. *Strongyloides*

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eggs were identified in eight fecal samples. We obtained *Strongyloides* sp. at the free-living larval and adult stages by culturing these fecal samples. DNA sequence analyses for species identification were conducted on these worm samples, and on worms (parasitic females) isolated from the July 2018 death case. We identified a mixed population of *S. papillosus* and *S. vituli* by these analyses. As per our knowledge, this is the first study to report the presence of *S. vituli* in Japan.

2. Materials and methods

2.1. Ethics statement

Ethical approval was not sought as the fecal samplings were conducted as part of farm animal health management.

2.2. Study on farm animals, and collection of fecal samples

The farm is located in Miyazaki Prefecture, Japan. It raises around 350 dairy calves reared under a loose housing free barn system. Fecal samples were collected manually from the rectum of 24 cattle.

2.3. Fecal examination and worm isolation

The modified Wisconsin technique [9] was used to examine nematode eggs in fecal samples of 24 calves. In samples where nematode eggs were identified as *Strongyloides* sp., the agar plate culture technique was employed using approximately 1 g feces [10]. The plates were incubated at room temperature (approximately 25 °C) for 4 days. The nematodes that were identified on the plates under a dissection microscope (SZ61, Olympus, Japan) were individually picked up, washed in phosphate-buffered saline containing 0.1% Tween 80 (PBS-T), and transferred to polymerase chain reaction (PCR) tubes containing 10 μL of worm lysis buffer (mixture of 0.5 volume of proteinase K [Qiagen], 0.5 volume of 1 M dithiothreitol [DTT] and 10 volumes of DirectPCR Lysis Reagent [Tail] [catalogue no. 101-T, Viagen Biotech Inc.]), as described before [11].

2.4. Isolation of worms from the sudden death case

Approximately 10 mL of the intestinal (jejunum) contents were collected and preserved at −80 °C until further examination. After thawing, the intestinal contents were observed under the dissection microscope. Twelve worms (parasitic adult stage) were picked up and washed with PBS-T. The worm lysates were prepared as described above.

2.5. PCR and sequencing

The worm lysates were diluted 10-fold with distilled water. Partial sequences of three marker genes, *18S* rDNA (small subunit ribosomal DNA gene), *rpl-10* (ribosomal protein L10 gene), and mt *cox1* (cytochrome *c* oxidase subunit 1 gene) were obtained by PCR and Sanger sequencing. Primer pairs to obtain PCR products for *18S* rDNA, *rpl-10*, and mt *cox1* were ENM173/ENM174, ENM344/ENM345, and ENM351/ENM352, respectively (Table 1). PCR was performed in a 25 μL reaction mixture containing 0.5 μL of KOD FX Neo (Toyobo, Japan), 12.5 μL of 2× PCR buffer for KOD FX Neo, 5 μL of 2 mM dNTPs, 0.75 μL of 10 mM primer solution (forward), 0.75 μL of 10 mM primer solution (reverse), 4.5 μL of distilled water, and 1 μL of diluted worm lysate. The amplification conditions were as follows: 94 °C for 2 min; this was followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s, and 68 °C for 1 min; and finally 68 °C for 7 min. The *rpl-10* gene PCR products for *S. venezuelensis* were obtained using purified *S. venezuelensis* (laboratory strain) genomic DNA (16 ng/μL), instead of crude worm lysate, as a template. DNA PCR products were purified with MinElute 96 UF PCR purification kit (Qiagen, USA). The sequencing reaction was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) with ABI Prism 3110 Genetic Analyzer (Applied Biosystems, USA). For *18S* rDNA sequencing, ENM173 and ENM174 were used; ENM344 and ENM345 were used for the *rpl-10* sequencing, while ENM351 and ENM352 were used for mt *cox1* sequencing. For some samples, ENM354 was also used to obtain the 5′-portion of the mt *cox1* PCR products (ENM351/ENM352). The primer sequences are summarized in Table 1. The sequences obtained in this study were deposited into the DDBJ database under accession numbers of LC459361–LC459362 (*18S* rDNA), LC458855–LC458859 (*rpl-10*), and LC459356–LC459360 (mt *cox1*).

2.6. Phylogenetic analyses

Phylogenetic analyses were conducted using the *rpl-10* and mt *cox1* sequences. The sequences obtained using the forward and reverse primers were assembled into contigs by employing Sequencher software (Gene Codes Corporation, USA). The contigs were aligned together with sequences obtained from the GenBank nucleotide database by Clustal X 2.1 [14]. The aligned sequences were trimmed to have fixed lengths of 340 and 744 bps for *rpl-10* and mt *cox1*, respectively. The MEGA7 program was used to build neighbor-joining or maximum-likelihood trees [15].

3. Results

Among 24 calves, *Strongyloides* eggs were identified in eight animals with eggs per gram (EPG) ranging from 2 to 138 by the modified Wisconsin method. Agar plate culture technique was conducted on these positive specimens. Nematode larvae or adult stages were observed in seven out of eight samples. Up to eight worms were picked up from each plate (44 worms in total) and worm lysates were prepared as described in the Materials and Methods.

Partial *18S* rDNA sequences (436 bp) covering all four nucleotide positions, which had been previously reported to differ between *S. papillosus* and *S. vituli*, were obtained from 42 out of 44 samples. Genotyping of the *18S* rDNA locus was carried out by comparing these sequences with reference sequences (AB923886 for *S. papillosus* and

Table 1
PCR and sequencing primers used in this study.

Target gene	Primers		References
	Name	Sequence (5′–3′)	
18S rDNA	ENM173 (988F)	F: CTCAAAGATTAAGCCATGC	Holterman et al. [12]
	ENM174 (1912R)	R: TTTACGGTCAGAACTAGGG	
rpl-10	ENM344 (AS1267)	F: CCATCCCAAAATCTCGTTTYTG	Eberhardt et al. [13]
	ENM345 (AS1268)	R: AATCCCCATTTCTTTGAAACNGC	
mt cox1	ENM351	F: CTGTATTAACTGCTCATGCA	This study
	ENM352	R: ACATCTGGATAATCWACATATTTACG	
	ENM354	R: CCAAATACTTCCTTCTTACCAGTCA	

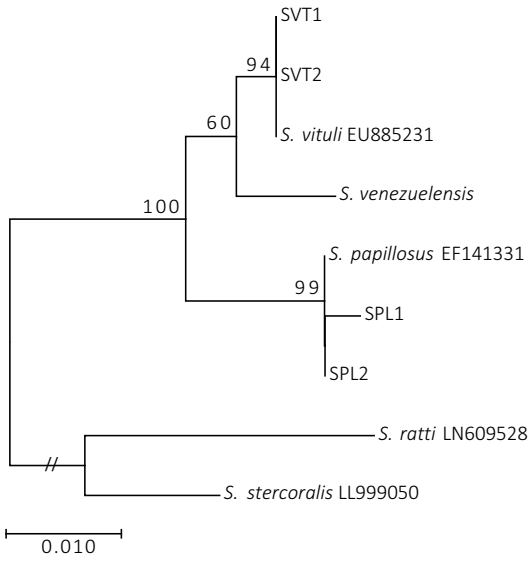

Fig. 1. Neighbor-joining tree for the *rpl-10* haplotypes.
MEGA7 software was used to build a phylogenetic tree using a dataset of four *rpl-10* haplotypes (SVT1, SVT2, SPL1, and SPL2) and *rpl-10* sequences obtained from the GenBank database (*S. papillosus*, *S. ratti*, and *S. stercoralis*) together with a *rpl-10* sequence of *S. venezuelensis* (obtained in this study). Out of the 340 aligned nucleotide positions, 336 informative positions were used for the tree construction, using Tamura 3-parameter method. The numbers at the nodes indicate bootstrap values.

Table 2
rpl-10 genotypes determined for worms identified as either *S. papillosus* or *S. vituli* based on the partial *18S* rDNA sequences.

rpl-10 genotype	Species identification based on *18S* rDNA	
	S. papillosus	*S. vituli*
SPL1/SPL1	6	0
SPL1/SPL2	14	0
SPL2/SPL2	7	0
SVT1/SVT1	0	4
SVT1/SVT2	0	5
SVT2/SVT2	0	1

EU885229 for *S. vituli*). In 32 samples derived from six different cattle, the partial *18S* rDNA sequences showed 100% similarity with AB923886 and were identified as *S. papillosus*. The sequences of the remaining 10 samples, derived from two cattle, showed 100% similarity with EU885229 and were identified as *S. vituli*.

Partial *rpl-10* sequences (340 bp) were obtained from 37 out of the 42 worm samples. A total of four haplotypes were identified. A phylogenetic tree using these four haplotype sequences together with *rpl-10* sequences of *S. venezuelensis*, *S. papillosus*, *S. ratti,* and *S. stercoralis* was constructed (Fig. 1). Two haplotypes were grouped together with the sequence of *S. papillosus* and named haplotypes SPL1 and SPL2, respectively. Whereas, the other two haplotypes were grouped together with the sequence of *S. vituli* and named haplotypes SVT1 and SVT2, respectively. All the worms identified as *S. papillosus* based on the *18S* rDNA sequence showed either haplotype SPL1, SPL2, or both in a homozygous or heterozygous state as shown in Table 2. Whereas all the worms identified as *S. vituli* based on the *18S* rDNA sequence showed either haplotype SVT1, SVT2, or both (Table 2).

Partial mt *cox1* sequences (744 bp) could be obtained from 33 out of 42 samples. The 33 sequences were grouped into five haplotypes. A phylogenetic tree was constructed using these five haplotypes together with mt *cox1* sequences of *S. venezuelensis*, *S. fuelleborni*. *S. papillosus*, *S. stercoralis*, *S. ratti*, and *Parastrongyloides trichosuri*, retrieved from the GenBank database (note that no mt *cox1* sequence of *S. vituli* had been deposited before the present study). All the worms that were identified as *S. papillosus* based on the *18S* rDNA sequence showed a single haplotype, SPL1, which was clustered together with a *S. papillosus* sequence from the database (LC050210). All worms that were identified as *S. vituli* by *18S* rDNA sequences had one of four haplotypes (SVT1–4) that showed a high degree of similarity to each other with pair-wise p-distances ranging from 0.003 to 0.007. Haplotypes SVT1–4 formed a clade (*S. vituli* clade) that was clearly different from *S. papillosus* clade (Fig. 2). The number of individual nematodes having respective mt *cox1* haplotypes are summarized in Table 3.

Twelve parasitic adults were collected from the intestinal content of the calf from the July 2018 sudden death case. *18S* rDNA, *rpl-10*, and mt *cox1* sequences were obtained for these worms. All worms were identified as *S. papillosus* based on the sequencing results for these three genes. No additional haplotype for *rpl-10* or mt *cox1* was found.

4. Discussion

Using three marker genes, *18S* rDNA, *rpl-10*, and mt *cox1*, it was clear that *Strongyloides* nematodes isolated from cattle reared on farm X were comprised of two genetically isolated populations. Based on the *18S* rDNA sequences, we predicted one population to be *S. papillosus* and the other to be *S. vituli*. Our results support the findings of Eberhardt et al. regarding the sympatric presence of two different species of *Strongyloides* nematodes in cattle [3]. The present study represents the first case of detection of *S. vituli* in cattle in Japan. The sympatric presence of *S. papillosus* and *S. vituli* in cattle population might be a cosmopolitan condition, due to the artificial distribution of domestic animals around the world. What differed in our study from the original report [3] was the dominant species. While *S. papillosus* was isolated from six animals, *S. vituli* was found only in two animals (there was one case of mixed infection). *S. vituli* accounted for only 23.8% (10/42) of all the isolated worms, and the rest were identified as *S. papillosus*. Nevertheless, the *S. vituli* population appeared to be more diverse at least in this farm, given that only one mt *cox1* haplotype was identified for *S. papillosus*, while four haplotypes were identified for *S. vituli*. We expect large-scale sampling will clarify whether these findings are generally true for the cattle population in Japan.

Thus far, there has been no formal description of morphology of *S. vituli*. Eberhardt et al. failed to identify reliable morphological differences that would enable distinction between *S. papillosus* and *S. vituli* [3]. In the present study, we did not attempt to conduct any morphological analysis. Therefore, it remains unknow whether these species can be distinguishable by morphological features at this moment, requiring thorough investigations.

Interestingly, *S. papillosus* and *S. vituli* do not appear to be in a sister-taxon relationship - although they are both parasites of ruminants and were once considered to be the same species. Rather, the phylogenetic analysis based on the mt *cox1* gene suggests common ancestors of *S. papillosus* and *S. fuelleborni* (*Strongyloides* of the Old World primates and human), and common ancestors of *S. vituli* and *S. venezuelensis* (*Strongyloides* of rats). Presence of multiple *Strongyloides* species with wide phylogenetic separation, capable of infecting a single vertebrate host species appears to be rather common (*i.e. S. ratti* and *S. venezuelensis* in rats, *S. stercoralis* and *S. fuelleborni* in human, and *S. papillosus* and *S. vituli* in cattle). This may indicate a general capability and tendency of this genus to switch hosts that are phylogenetically distant from each other. Potential host-switching of *S. stercoralis* from dog to human was hypothesized, recently [11].

S. papillosus was generally considered to be a helminth of minor importance, infrequently associated with clinical symptoms in calves. In Japan however, *S. papillosus* drew considerable attention in the 1980s and 1990s due to its ability to cause “sudden death syndrome” in calves characterized by cardiac arrest without any premonitory signs [16]. Miyazaki is located in southern Kyushu, where outbreaks of such

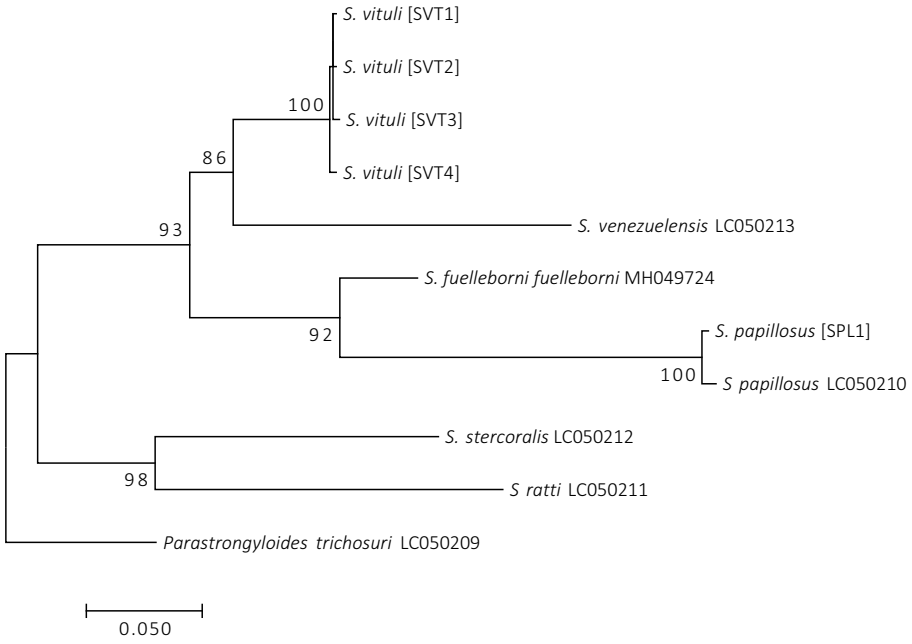

Fig. 2. Maximum-likelihood tree based on the mt *cox1* haplotype data.
MEGA7 software was used to build a phylogenetic tree using a dataset of five mt *cox1* haplotypes (SVT1, SVT2, SVT3, SVT4 and SPL1) together with mt *cox1* sequence of *S. venezuelensis*, *S. fuelleborni*, *S. papillosus*, *S. stercoralis*, *S. ratti*, and *Parastrongyloides trichosuri* (obtained from the GenBank database). A total of 744 aligned nucleotide positions were used for tree construction based on the general reversible model. The numbers at the nodes indicate bootstrap values.

Table 3
Mitochondrial *cox1* haplotypes for worms identified as *S. papillosus* or *S. vituli* by *18S* rDNA sequences.

mt *cox1* haplotype	Species determination based on *18S* rDNA	
	S. papillosus	*S. vituli*
SPL1	25	0
SVT1	0	3
SVT2	0	3
SVT3	0	1
SVT4	0	1

sudden death syndrome were observed mostly in the 1980s. At the time, all the sudden death cases were believed to be caused by a single species, *S. papillosus*, because the presence of *S. vituli* was not known. In the present study, all the *Strongyloides* worms identified from the sudden death case were also identified as *S. papillosus*. It is of interest to know whether *S. vituli* can also cause "sudden death syndrome" in calves; if not, a new research opportunity may present itself to determine why this might be the case.

Declaration of competing interests

The authors declare that they have no conflict of interest.

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