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Summary

Our recent work regarding pathogenic parasites of domestic and wild animals and microbial communities in the composting processes has been summarized. Cryptosporidiosis is one of the important protozoan zoonotic diseases that causes diarrhea and occasionally death of humans, domestic animals, and wild vertebrates. We isolated a novel type of *C. andersoni* from cattle that grazed on the Kawatabi farm in Tohoku University, and we refer to this strain as the *C. andersoni* Kawatabi strain. We also isolated cryptosporidian oocysts from a dog and large Japanese field mice, *Apodemus speciosus*. Gene analysis suggested possibilities to be a novel type of *C. canis* (*C. parvum* dog genotype) and a new species of *Cryptosporidium*, respectively.

A commercial microbiological additive (MA) was effective in the composting process, e.g., quicker elevation of temperature, lower emission of ammonia gas, and lower production of nitrate. Functional microbes obtained by the cultivation method with the MA did not coincide with the dominant species in the microbial community detected by DNA analyses. Various species of microbes in the MA grew at 55 and 72°C incubation; however, they did not coincide with the dominant species detected in chicken manure composting processes. It was clarified that the MA contained a variety of microbes, including thermophilic microbes and that these microbes did not become dominant during the composting processes. However, microbes in the MA that are smaller in number than the dominant species may act functionally in the composting process.

The control of pathogens and the appropriate management of waste and wastewater are essential for healthy and safe livestock production. The research work in our laboratory is focused in these directions. In the present paper, two topics from our recent work in parasitology and environmental microbiology are reviewed.

1. Cryptosporidiosis of Domestic and Wild Animals

Cryptosporidium species are protozoan parasites that infect humans, domestic

animals, and wild vertebrates. In farm animals, *Cryptosporidium* spp. cause disorders of the gastrointestinal tract, which lead to poor health of the infected animals and significant economic losses.

C. muris-like oocysts, which parasitized the bovine abomasums, were isolated in the U.S. (Anderson, 1987) and Japan (Kaneta and Nakai, 1998; Nakai *et al.*, 2004). Recently, this species was proposed to be a new species, *C. andersoni*, which was distinct from *C. muris* that infected the stomach of rodents (Lindsay *et al.*, 2000). However, it is still unclear whether *C. andersoni* is the representative species of the *C. muris*-like oocysts isolated from cattle and if subpopulations of *C. andersoni* actually exist. Subsequently, we surveyed bovine *Cryptosporidium* and analyzed the characteristics of the isolated oocysts. We also surveyed cryptosporidian oocysts from domestic and wild animals in and around the farm contaminated with *C. andersoni*.

We detected ovoid *Cryptosporidium* oocysts from 4 of 118 and 6 of 113 grazing cattle in 1999 and 2000, respectively, on the Kawatabi farm of Tohoku University in the Miyagi prefecture. The average size of the oocyst isolates ranged from 7.4 to 7.5×5.1 to 5.8 μm in size, which was significantly smaller than that of *C. muris* and significantly larger than that of *C. parvum*. Various developmental stages of *Cryptosporidium* were detected in the epithelial cells of the abomasum of a 3-year-old cow.

We detected oocysts from six adult and two newborn cattle in 2000 and two adults and two newborns in 2001. The two adults were positive in both the years. In 2002, seven newborns were positive; one adult had been positive since 2000. These results indicated that this parasite persistently infected some adult cattle but did not infect other adult cattle. It was also observed that this parasite infected newborn calves. The newly infected individuals were not detected in barns but on the pasture.

We observed the oocyst discharging patterns. The number of oocysts discharged from the positive cattle was approximately 1 million/g of feces, and the number was almost similar to that in the pasturing season. This data also indicated a persistent infection of the parasite.

We investigated the body weights of two positive calves that continuously discharged oocysts. Although the growth rate of one calf was slower than that of the negative group, that of the other was better than or the same as that of the negative group. No oocysts were detected in the mothers of any of the positive calves.

We investigated the calf-to-calf transmission of *Cryptosporidium*. We kept the positive calves that were continuously discharging oocysts and the negative calves in the same pen. Although the total number of oocysts discharged from one positive calf was estimated to be more than 1 trillion, the negative calves did not discharge any oocysts during the 2-month observation period.

We inoculated two calves with 10 million oocysts each on day 4 after birth. No oocysts were detected in the 1 month fecal observation period.

PCR amplification products for genes of 18S ribosomal RNA (rRNA), heat shock protein 70 (HSP70), and *Cryptosporidium* oocyst wall protein (COWP) of the isolate were sequenced. Similarity of the sequences indicated that there was no difference between the isolates from the farm. They were identical to the data of published bovine strains of *C. muris* and *C. andersoni*; however, they were different from those of *C. muris* isolated from rats, hylaxes, and camels.

Sequencing data for only one fifth the length of the 18S rRNA gene of *C. andersoni* has been published. The length is too short to compare the sequence of the Kawatabi isolate and *C. andersoni*, because the sequence of this portion of *C. andersoni* was the same as that of *C. muris*. The sequence of a longer length of the COWP gene of *C. andersoni* was published, and its sequence showed 100% similarity to that of the Kawatabi isolate.

On day 6 after inoculation of SCID mice with *C. muris* oocysts, all the four mice discharged oocysts. All the eight mice inoculated with the Kawatabi isolate discharged oocysts on day 14.

During the histological examination, round shaped parasites were detected from the lumen and in the stomach epithelium of the SCID mice infected with the Kawatabi isolate.

The results are summarized in Table 1. The oocyst size of Kawatabi isolate was similar to that of *C. andersoni*. Both the Kawatabi isolate and *C. andersoni* parasitized the bovine abomasum. DNA sequences of the three genes were the same as those of the *C. muris* bovine strain and *C. andersoni*. The Kawatabi isolate and *C. muris* were infective to SCID mice; however, *C. andersoni* did not infect SCID mice. Therefore, we concluded that this isolate was a novel type of *C. andersoni*. We refer to this strain as "*C. andersoni* Kawatabi strain" (Satoh *et al.*, 2003).

We tried to isolate *C. andersoni* from domestic animals and wild animals in and around the Kawatabi farm; however, we failed to detect the oocysts. Nevertheless, we detected cryptosporidian oocysts from a dog and large Japanese field mice, *Apodemus speciosus*. Genes of these isolates were analyzed, and there were possibilities to be a novel type of *C. canis* (*C. parvum* dog genotype) and a new species of *Cryptosporidium* (unpublished data), respectively.

2. Microbial Community in a Microbiological Additive and Composting Process

We have investigated microbial communities in composting and wastewater treatment processes (Nakai *et al.*, 1997, 1999b, 1999d; Nakai, 2001; Sasaki, 2002, 2004a, 2004b) and functional microbes involved in such processes (Kohda, 1997a,

Table 1. Characteristics of Kawatabi Isolate and Large Type Oocysts Isolated from Cattle

	Kawatabi isolate	*C. muris* RN66	*C. muris* bovine strain (Xiao *et al.*, 1999)	*C. andersoni* (Lindsay *et al.*, 2000)
Oocyst size (μm)	7.5×5.4	8.1×5.2	N.C	7.4×5.5
Similarity to Kawatabi strain (%)				
18s-rDNA		98	100	100
HSP70		98	100	N.C
COWP		98	N.C	100
Infectivity to mice	+ (SCID)	+ (SCID)	− (Young mice)	− (ICR, BALB/c, SCID, γ-INF•KO)
Bovine Abomasal infection	+	?	N.C	+

N.C. indicates not compared.

1997b ; Nakai *et al.*, 1999a, 1999c). We isolated several ammonia-assimilating microbes from a lagoon for animal wastewater treatment, and some of them showed high ability of ammonia assimilation even in mixed cultures with living microbes in the wastewater in the laggon. The results indicated that these isolated microbes could survive in the lagoon and assimilate ammonia in the wastewater. There is a possibility that we can use such ammonia-assimilating microbes for wastewater treatment.

Some farms use microbiological additives for the treatment of animal wastes and wastewater in Japan. More than 90 types of commercial microbiological additives are available in Japan for animal manure treatment. However, only a limited number of these additives disclose the microorganisms present. Moreover, the fate and function of the microorganisms in most of these commercial additives in manure treatment processes have not been known. When microbiological additives are precisely used to improve the animal manure treatment, it is essential to know the function and mechanisms of the additives. Therefore, we have observed the effects of a commercial microbiological additive (MA) on beef manure composting and investigated microbial communities in the MA and those in the chicken manure composting process by both the conventional cultivation method and advanced DNA sequencing analysis.

Five hundred kilograms of beef manure with or without 10 kg of MA was composted in piles, which were turned every 10 days. The manure that was inoculated with the MA showed quicker elevation of temperature at the start of composting and the first turning, lower emission of ammonia gas, lower production of nitrate, larger number of mesophilic aerobes and anaerobes and thermophilic anaerobes, and smaller number of thermophilic aerobes, compared with the composting process without the MA. These results suggested that mesophilic aerobes increased in number and metabolized ammonia assimilatively rather than nitrificatively to accelerate the temperature elevation of the compost by the inoculation of the MA.

From the MA, 14 isolates having a high ability of fiber degradation and 16 isolates having a high ability of protein degradation were cultivated. They were identified as members of *Bacillus*, *Paenibacillus*, and *Clostridium* by the analysis of partial DNA sequences of 16S rRNA genes. The PCR-DGGE method (Sasaki *et al.*, 2004a, 2004b), directly applied to the extract from the MA, indicated that dominant microbes were groups of Bacteroidetes and *Lactobacillus*. The clone library method indicated that groups of Actinobacteria and alpha-Proteobacteria were also dominant microbes. These dominant microbes did not coincide with those among the cultured isolates. This indicated that the microbes obtained by the cultivation method were not dominant in the microbial community in the MA.

Band patterns observed during PCR-DGGE differed for each treatment stage of the composting process. This indicated a change in the dominant species

Table 2. Dominant Microbes in Composting Process

band	identified species	simirality (%)	order
S-1	*Porphyromonas levii* ATCC 29147	94	Bacteroidales
S-2	Uncultured eubacterium WJGRT-161	91	Clostridiales
S-3	Uncultured eubacterium WJGRT-161	91	Clostridiales
S-4	Uncultured bacterium 24-45 : 3	93	Clostridiales
S-5	*C. ultunae*	100	Clostridiales
A-1	Uncultured bacterium SP31-1	98	Bacillales
A-2	Uncultured bacterium HZ_05J	99	Bacillales
A-3	Uncultured bacterium SP23-11	95	Bacillales
A-4	Uncultured bacterium SP23-11	95	Bacillales
A-5	*B. thermocloacae*	100	Bacillales
A-6	*B. thermocloacae*	99	Bacillales
A-7	Bacillaceae bacterium NS1-3 1	100	Bacillales
E-1	Uncultured Bacilli bacterium	93	Bacillales
E-2	Uncultured bacterium SP31-5	95	Bacillales
E-3	*B. oleronius*	98	Bacillales
E-4	*B. thermocloacae*	100	Bacillales
E-5	*Corynebacterium* sp.	99	Actinomycetales
E-6	*Brevibacterium linens* WS2906	100	Actinomycetales

during the composting process.

DNA sequences of major bands observed in PCR-DGGE were determined (Table 2). The dominant species were members of *Clostridium* at the start of composting (S), members of uncultured Bacterium and *Bacillus* at the end of the first treatment (A), and members of *Bacillus* and *Corynebacterium* at the end of the second treatment (E). The clone library method revealed additional groups of dominant microbes during the treatment processes. None of them coincided with microbes demonstrated in the MA by the cultivation method and DNA analysis.

There was a possibility that microbes were killed by the heat during the composting process. Subsequently, we placed the MA in a liquid medium at 55°C and 72°C and investigated the changes in the microbial community by the PCR-DGGE method (Table 3). During the incubation at 55°C, the band patterns changed as the incubation proceeded, and it was observed that the dominant microbes were members of *Bacillus* and Clostridiales. During incubation at 72°C, the dominant microbe was an uncultured bacterium of Rhizobiales. These dominant microbes detected from cultures at 55°C and 72°C incubation did not coincide with those detected during the composting processes.

It was determined that the MA contained a variety of microbes including thermophilic microbes and that these microbes did not become dominant during

Table 3. Dominant Microbes in the MA after Incubation at 55 or 72°C

band	identified species	similarity (%)	Order
L0-1	Uncultured bacterium	99	Rhizobiale
L24-1	*B. smithii*	100	Bacillales
L24-2	*B. smithii*	100	Bacillales
L48-1	*B. thermoalkalophilus*	99	Bacillales
L48-2	*B. thermoalkalophilus*	99	Bacillales
L48-3	*Soehngenia saccharolytica*	97	Clostridiales
L48-4	*Soehngenia saccharolytica*	97	Clostridiales
L72-1	*B. thermoalkalophilus*	99	Bacillales
L72-2	*Bacillus* sp.	99	Bacillales
L72-3	*Bacillus* sp.	99	Bacillales
L72-4	Uncultured bacterium	99	Clostridiales
H0-1	Uncultured bacterium	100	Rhizobiales
H24-1	Uncultured bacterium	100	Rhizobiales
H48-1	Uncultured bacterium	100	Rhizobiales
H72-1	Uncultured bacterium	100	Rhizobiales

L0-1 : 55°C 0 hr incubation-band number 1
H0-1 : 72°C 0 hr incubation-band number 1

the composting process. The microbes in the MA that were smaller in number than the dominant species may act as functional microbes during the composting process.

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