Original Paper

Monitoring of pathogenic microorganisms originating from nomadic livestock feces in the Tuul River basin of Mongolia

Chika Tada, Tomoe Kotogaoka, Moe Takada, Yasuhiro Fukuda, Yutaka Nakai

Graduate school of Agricultural Science, Tohoku University, 232-3, Yomogida, Naruko-onsen, Oosaki, Miyagi, 989-6711

Abstract In Mongolia, livestock concentration is occurring in places from which drinking water is obtained along with overgrazing, and there is concern over contamination of river water by nomadic livestock feces. In this study, we investigated the pathogenic microorganisms (*E. coli*, *Cryptosporidium, Giardia*) originating from the feces of nomadic livestock (horse, sheep, goat, cattle) and river water on the Tuul River from 2012 to 2014. A comparison with past reports suggests that nomads in Mongolia tended to test positive at a higher rate for pathogenic microorganisms than in domestic livestock in other countries. These results indicate the need to consider new nomadic ways that can prevent river water pollution due to the feces derived from nomadic livestock in order to preserve a healthy water environment.

Keywords: nomadic livestock, feces, river water, *E.coli*, *Cryptosporidium*, *Giardia*

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INTRODUCTION

In Mongolia, precipitation has decreased by 7% over the past 68 years. In Mongolia, which has been supplying water to rivers and lakes, glaciers and year-round snow of Mongolia was 659 km^2 in 1983, but it is said that it decreased by 30% in the main area by 2002, and there is concern about depletion of water resources.

With respect to its social structure, Mongolia has maintained a nomadic society that balances forest and grassland ecosystems, water circulation, and the like under a socialist regime. However, due to the introduction of democratization policy in 1989 following the fall of the Berlin Wall, the discretion of pastoral farming is entrusted to individuals as part of a change to a market economy. Domestic animals such as horses, cattle, goats, sheep and camels are increasing in number (National Statistical office of Mongolia, 2007). Also, democratization has liberalized the use of land, and many nomads now tend to highly convenient areas, such as near urban areas and abundant water resources[1]. The increase in the quantity of livestock grazing in the suburbs of the city and overcrowding continue to progress. Due to this societal background, domestic animals are concentrated in drinking grounds and pollution of river water due to manure is beginning to occur[2].

In this study, starting in 2012, we investigated monitoring of and water pollution by pathogenic microorganisms originating from the feces of

Correspondence: Chika Tada (e-mail: chika.tada.e1@tohoku.ac.jp)

domestic animals in the basin of the Tuul River, which flows through the centre of Ulan Bator, the capital city of Mongolia.

MATERIALS AND METHODS

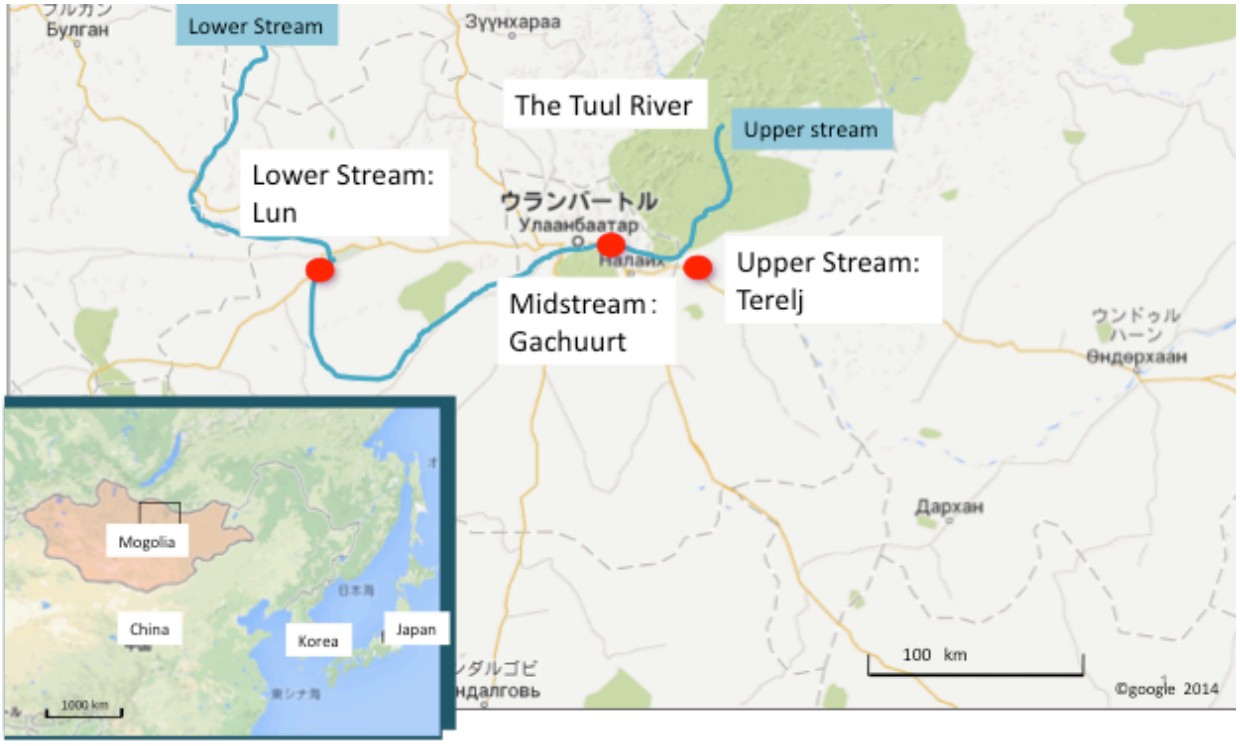
1. Fecal samples and water samples

Sampling was conducted in the Tuul River Basin of Mongolia from July to August 2012, August 2013, and August 2014. The places surveyed are shown in Map 1. The 3 survey points of the river are Terelj (47 ° 52.656 latitude, 107 ° 36.733 longitude) along the Tuul River, Gachuurt at midstream (47 ° 55.535 latitude, 107 ° 9.824 longitude), and Lun downstream (47 ° 51.772 north latitude, 105 ° 12.248 longitude).

After the livestock near the water field left, feces on the ground were visually checked for freshness, collected in a polyethylene bag, and stored at 4 ℃ until DNA extraction could be performed. Animal species were determined based on the shape of feces.

In 2012, 28 samples of cattle feces, 19 samples of horse feces, and 12 samples of goat and sheep feces were obtained. In 2013, 25 samples of cattle feces, 46 samples of horse feces, and 56 samples of goat and sheep feces were obtained.

For the water sample, 10 liters of surface water was sampled from a depth of about 5 cm, transferred to a polyethylene tank, and stored at room temperature until pretreatment could be performed. The sampled water was filtered through a membrane filter (Merck Millipore, Darmstadt, Germany) with a pore size of 0.2 μm, and microorganisms in the sample water were collected on the filter. After collection, the filter was placed in a 15 ml centrifuge tube, the filter was permeated with sterilized water, separating the filter and the microorganisms. Afterwards, the suspended microbial solution was



Map1 : Survey areas of the Tuul River, in Mongolia

Table1 Objective genes used for detecting pathogenic microorganisms

Object of detection	Objective gene	Product length	Reference
Cryptosporidium sp.	18S rRNA	830 bp	Xiao et al., 1999
Giardia sp.	18S rRNA	292 bp	Hopkins et al., 1997
	gdh	432 bp	Read et al., 2004
Escherichia coli	*malB*	585 bp	Wang et al., 1996
EPEC	*eae*	454 bp	Yu et al., 1992
	bfpA	254 bp	Roberto et al., 2004
EIEC	*invE*	382 bp	Bii et al., 1993
EHEC	*Stx 1*	350 bp	Pal et al., 1998
	Stx 2	110 bp	Pal et al., 1999
ETEC	*estA1*	239 bp	Nada et al., 2010
	estA2-4	133 bp	Nada et al., 2011
	eltBI	402 bp	Nada et al., 2012
EAEC	*aggR*	308 bp	Nataro et al., 1995
	pCVD432	630 bp	Schmidt et al., 1995

centrifuged at 5000 × g for 10 minutes to precipitate microorganisms, and after discarding the supernatant, the precipitate was subjected to DNA extraction.

2. DNA extraction

FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Corporation, Taiwan) was used to extract DNA from fecal samples and water samples. The extracted DNA samples were stored at -20ºC until they could be analyzed.

3. PCR

The obtained DNA sample was subjected to PCR amplification using primers specific to pathogenic *Escherichia coli, Cryptosporidium, Giardia* (Table 1). Pathogenic *E.coli* were Enteropathogenic *Escherichia coli* (EPEC), Enteroinvasive *E.coli* (EIEC), Enterotoxigenic *E.coli* (ETEC), Enterohaemorrhagic *E coli* (EHEC) and Enteroaggregative adherent *E.coli* (EAEC). The presence or absence thereof was determined using band detection by electrophoresis.

Emerald Amp PCR Master Mix (Takara Bio Inc, Otsu, Shiga, Japan) was used for PCR amplification.

PCR was performed on the primer set of the V3 region of eubacteria 16S rRNA gene (Forward: 5'-CCT ACG GGA GGC AGC AG-3 ', Reverse: 5'-CCG TCA ATT CCT TTR AGT TT-3'). A template of 1 μl was added. 30 cycles were performed with a thermal denaturation of 98° C for 10 seconds, annealing at 56° C for 45 seconds, and an extension reaction at 72° C for 1 minute. For PCR amplification reaction of *Cryptosporidium*, Emerald Amp PCR Master Mix, final concentration of 0.2 μM primer pair, 1 μl template was used. 35 cycles were performed with a thermal denaturation of 98° C for 10 seconds, annealing at 55° C for 45 seconds, and an extension reaction at 72° C for 1 minute. A DNA solution extracted from *Cryptosporidium parvum* HNJ-1 strain was used as a positive control for PCR reaction, and sterilized Milli-Q water was used as a negative control. 3 μl of the amplified

product was subjected to electrophoresis using 1.5% agarose gel and stained with ethidium bromide. Amplification was confirmed using a UV transilluminator. For some of the amplified products, amplification was confirmed using the electrophoresis apparatus MultiNa (SHIMADZU, Tokyo, Japan).

4. Cloning

Amplified products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co, Ltd, Tokyo, Japan). The purified product was introduced into pGEM-T Easy Vector (Promega, Tokyo, Japan) and was subjected to phenotypic transformation into *E. coli* Competent Quick DH 5α (TOYOBO, Osaka, Japan). For the colonies formed on the plate, insertion of the target fragment was confirmed by using colony direct PCR.

M13 primer F4 and RV were used to perform colony direct PCR. For the PCR reaction, PCR was carried out by means of an Emerald Amp PCR Master Mix and composition of a final concentration of 0.2 μM primer pair. The amplification products were electrophoresed, and then the insertion of the target gene was confirmed.

5. DNA sequencing

The PCR product for which amplification had been confirmed was purified by ExoSAP-IT (USB, Cleveland, Ohio, USA) treatment. 1 μl of the purified product was subjected to a cycle sequence reaction by means of a Big Dye Terminator Cycle Sequencing Kit ver 3.1 (Applied Biosystems, Tokyo, Japan), and the nucleotide sequence was determined by using a 3130 xl genetic analyzer (Applied Biosystems, Tokyo, Japan). The base sequence that had been obtained was identified by homology search with BLAST.

Results and discussion

1. Pathogenic *E coli* detected from horse feces around the Tuul River

Table 2 shows the detection results for fecal pathogenic *E coli* collected at each site. In the 2012 survey, EPEC and EHEC were detected from horse feces collected around the Tuul River, and ETEC and EAEC were not detected. EPEC was detected twice from 10 horse feces at all upstream, midstream, and downstream points, and the positive rate was 20%. EHEC was also detected twice, and the positive rate was 20%. However, while EPEC was detected in Terelj and Gachuurt upstream and midstream, there was a difference detected in EHEC in Gachuurt and Lun.

In 2013, in addition to EPEC and EHEC, ETEC (2/33) and EAEC (1/33) that had not been detected in 2012 were also detected. The positive rate for EPEC

Table2 Positivity rate of pathogenic *Escherichia coli* in sheep, goat and cattle feces in 2012 and 2013

		Horse			Sheep,Goat			Cattle		
		Tereli	Gachuurt	Lun	Tereli	Gachuurt	Lun	Tereli	Gachuurt	Lun
EPEC										
	2012	1/3(33%)	1/6(16.7%)	0/1(0%)	-	-	0/5(0%)	0/8(0%)	0/9(0%)	0/3)0%)
	2013	3/12(25%)	5/16(31.3%)	0/5(0%)	2/12)16.7%)	1/17(5.7%)	0/10(0%)	3/8(37.5%)	0/3(0%)	2/7(29%)
EHEC										
	2012	0/3(0%)	1/6(16.7%)	1/1(100%)	-	-	5/5(100%)	8/8(100%)	4/9(44%)	3/3(100%)
	2013	2/12(16.7%)	0/16(0%)	2/5(40%)	5/12(41.7%)	10/17(58.8%)	1/10(10%)	1/8(12.5%)	0/3(0%)	0/7(0%)
ETEC										
	2012	0/3(0%)	0/6(0%)	0/1(0%)	-	-	0/5(0%)	0/8(0%)	0/9(0%)	0/3(0%)
	2013	1/12(8.3%)	1/16(6.3%)	0/5(0%)	0/12(0%)	0/17(0%)	0/10(0%)	0/8(0%)	1/3(33%)	1/7(14%)
EAEC										
	2012	0/3(0%)	0/6(0%)	0/1(0%)	-	-	0/5(0%)	0/8(0%)	0/9(0%)	0/3(0%)
	2013	0/12(0%)	1/16(6.3%)	0/5(0%)	0/12(0%)	0/17(0%)	0/10(0%)	0/8(0%)	0/3(0%)	0/7(0%)

was 24.2% and for EHEC was 12.2%, showing some fluctuation from 2012. As was the case in 2012, EPEC was not detected in Lun downstream. EHEC, as was the case in 2012, was also detected in Lun. The positive rate was as high as 40% in Lun's feces alone, and considering that one sample was positive (1/1) in 2012, horses living near Lun were considered to have more EHEC colonized than horses living upstream (16.7%, 2013) and midstreme (16.7%, 2012).

2. Pathogenic *E coli* detected from sheep and goat feces around the Tuul River

We were unable to collect feces Terelj and Gachuurt in 2012. We were only able to collected in Lun. EHEC was detected in all the feces collected. In 2013, EPEC and EHEC were detected. EPEC was detected only in Terelj and Gachuurt, while EHEC was detected in all locations. The positive rate for EPEC was 6.8%(3/44), while the positive rate for EHEC was 47.7%(21/44). Also, in Fagan et al. (1999) [3] results, there are reports that the *stx* gene was detected in 54% of feces collected from a group of 4 sheep. From this, it can be understood that the colonization rate for EHEC is extremely high in sheep and goats. Since EHEC is highly dangerous to humans, we must take care to prevent the feces of sheep and goats from entering river water or well water.

3. Pathogenic *E coli* detected from cattle feces around the Tuul River

Only EHEC was detected in 2012 (Table 2). It was detected from cattle feces at all locations at extraordinarily high positive rates: 100% (8/8) upstream, 44.4% (4/9) midstream, and 100% downstream (3/3), for an overall positive rate of 75%. In 2013, EPEC, EHEC, and ETEC were detected. The positive rates for EPEC, EHEC, and ETEC or 27.8%, 5.6%, and 11.1%, respectively.

In our survey, EHEC had a high positive rate of 75% in 2012, but greatly fluctuated to 5.6% in 2013. In Burkina Faso (located in West Africa) in a March - August 2010 survey[4], there was 37% EHEC detection from feces collected from 304 cattle in a slaughterhouse. The *stx* gene was detected[3] in 19% of feces collected from four cattle herds in Australia. In the Japanese survey by Kobayashi et al. (2001), on 78 farms in the Kanto-Koushinetsu area in 1998, 69% of 183 dairy cattle feces samples were positive for *stx* gene, and the positive rate was high[5]. Consequently, it was concluded that there tends to be a high colonization rate of EHEC in cattle, irrespective of whether they were nomadic or barn raised.

In terms of EPEC, a result of 21.7% positive was found when atypical EPEC (eaeA+ bfpA-) was detected in the feces of 22 of the 101 healthy Inner Mongolia dairy cattle[6]. The survey in Burkina Faso, West Africa showed that the fecal matter collected from the 304 cattle in the meat processing plant tested 8% positive for EPEC[4]. By comparing these results, it can be seen that the positivity rate of 27.8% in the survey results for EPEC in 2013 was higher than in past studies.

4. The parasitic protozoa detected in the feces in the area around the Tuul River

There were no parasitic protozoa detected in 2012, but *Giardia* was found in the feces of cattle in 2013. Moreover in the study held the next year in 2014, *Cryptosporidium* and *Giardia* were found inside the feces of cattle in the river's upper stream area in Terelj. *Giardia* was also detected in the water of the lower stream area around Lun. The *Giardia* in question was found to be the same *Giardia* found in the cattle feces in Terelj. Through this, the lower stream can be thought to be contaminated from the

feces in the upper stream.

Up until now in the rivers of Japan, a survey in detecting protozoa using microscopic observation was carried out through sucrose floatation with a maximum of 100L of water from the Sagami River water system in Kanagawa Prefecture from April 1997 to December 1997, which detected 3-9 cells in 100L[7]. In the studies of the Tone River, Edo River water system in November 2000 and February 2001, *Cryptosporidium* was detected at 0-2 cells/10L in the study of November 2000. Compared to the November study, a trend showing an increase in cells detected appeared in the study in February 2001, with tens of cells/10L being found depending on the area[8].

In the surveys of this current study, *Cryptosporidium* could not be detected through the method of DNA extraction in the 10 L sample. From this, there is a possibility that the detection sensitivity of PCR is lower when compared to sucrose floatation. Improvements such as DNA extraction through filtering a larger amount of sample water and the removal of PCR inhibiting materials are necessary to improve the detection sensitivity of methods involving DNA from now on. The Tuul River in Mongolia that we studied has a contamination equal to around COD 10 mg/L, making it fall under rank E in the Ministry of Environment's Environmental Quality Standards (of Rivers) related to the preservation of living environments. The water quality of the Sagami River water in 1996 was below COD 1 mg/L [9] and found to have water quality which would be from AA to A in the Environmental Quality Standards, and there is a possibility that the difference in amount of organic matter in the river's water quality is also affecting the detection sensitivity of microorganisms. In conclusion, as *Cryptosporidium* is detected even in the rivers of Japan, it can be considered that there is ample possibility of danger in the existence of *Cryptosporidium* of the Tuul River, Mongolia where the fecal matter of nomadic livestock is found directly in the river, and continued monitoring using methods with higher sensitivity is required.

5. Detection of pathogenic *Escherichia coli* in the waters of the Tuul River

There was no *E. coli* detected from the river waters in 2012 and 2013. However, it could be thought that the detection sensitivity had possibly been low. In our survey, cultivation using the DHL Agar of the sample water from the Tuul River from 2014 led to the detection of a coliform colony of approximately 50-130 CFU/ml. There was no pathogenic *E. coli* detected through PCR in this colony that was collected. However, we found from the results of the cultivation that the river water was contaminated by a large amount of coliforms. Rank B on Japan's Environmental Quality Standards (for Rivers) is below 5,000MPN/100mL and the contamination of coliforms in the River Tuul can be seen to be equally high. In Berg Rivers, North Africa, $1.1 \times 10^3 \sim 1.4 \times 10^6$ microorganisms/100 ml of intestinal bacteria was detected[10], close to our result of 130 CFU/ml of coliforms found in the Tuul River. At that time, EIEC, EHEC and EAEC were detected in pathogenic *E. coli* using conventional PCR, and realtime PCR additionally detected EPEC as well.

We were unable to detect *E. coli* in our survey, even in the lower stream Lun area where the number of coliforms was the largest. In comparison to our method, Ndlovu et al. [10] have taken measures to improve their detection sensitivity such as having 500 ml of water centrifuged, then cultivating the pellet in 2 ml of LB broth at 37℃ for 6 hours and carrying out DNA extraction after that, or inserting

Table3 Water quality of the Tuul River at sampling areas (2013)

	The Tuul river		
	Tereli (Upper stream)	Gachuurt (Midstream)	Lun (Lower stream)
COD(mg/L)	14.9	2.3	11
PO_4-P(mg/L)	N.D.	N.D.	0.1
NH_4-N(mg/L)	0.05	0.03	N.D.
NO_3-N(mg/L)	0.24	0.14	1.24

10 μl of DNA templates at the time of PCR. From this, even though we were unable to carry out a band detection through PCR on the objective gene this time, it can be considered that there is ample likelihood of detection being possible through raising the sensitivity higher.

6. The connection between livestock and the pollution of river water by the feces and urine of nomadic livestock

As Table 3 shows, out of the upper stream (Terelj), midstream (Gachuurt) and lower stream (Lun) areas, the lower stream area of Lun is more contaminated by the others with its higher levels of nitrate-nitrogen. In addition, the COD value of Terelj and Lun was above 10 mg/L, which is water quality in a severely highly-contaminated state, making it fall under the lowest rank of E in Japan's Environmental Quality Standards of rivers.

These surveys of 2012 and 2013 did not detect pathogenic *E. coli* or parasitic protozoa from the river water, but as it was detected in 2014, this suggested that contamination of the river water was being caused by the feces and urine of livestock. At the same time, the results of the study carried out by Yoshihara et al. (2016) on drinking water used on lambs and the weight gained by the lambs showed that drinking the water in the lower stream area of Lun raised the lambs' EPEC infection rate [11].

The positivity rate of the pathogenic *E. coli* in the nomadic livestock in Mongolia that we studied showed a higher trend in comparison to past research reports. From this, the feces and urine of the nomadic livestock could be thought to be contaminating the river water, and due to the contamination, the infection was spreading further by livestock drinking it. In addition, while this study only focused on the livestock, there were scenes of nomadic farmers swimming in the river or using the water to wash their clothes in the Tuul River of Mongolia, especially in the lower stream areas such as Lun, which means there is also the possibility of having negative effects on a human health. According to the report provided by Sarantuya et al. in 2004 on the infection status of pathogenic *E. coli* in children in Mongolia, EPEC has been detected at 3.8% in diarrhetic feces [12]. Additionally, there was a 15.1% detection rate of EAEC in the diarrhetic feces. In this current study, the EHEC often detected in the feces of cattle has not been detected in the diarrhetic feces of children in 2004, but our survey in 2013 showed that the EAEC detected in the feces of horses existed in many of the children's diarrhetic feces, so it can be thought that there is ample possibility of horse feces having a negative effect on children's health. In fact, as shown in Photograph 1, in the lower stream area of Lun, horses gather at the foot of the bridge and there was a large amount of horse feces left on that riverbank. Caution from now on is necessary.

The state of nomadic farming in Mongolia has undergone a great change in present times, even in comparison to 2012 when we started our surveys. While they used to let the animals free in grassland, there are now fences and a move towards set stocking. Due to set stocking, the river water and

Photograph1 The horses at Lun in the lower stream area of the Tuul River, in Mongolia and the state of their feces.

The horses seek the shade and tens of them gather at the foot of the bridge. A large amount of horse feces are left in the river bank.

places used for drinking water can be thought to be put under even further concentrated strain due to the feces and urine, and there is a fear that the ensuing contamination of water and the infection rate of pathogenic microorganisms in the animals and people that drink the water will increase. It can be said that setting in order management systems such as a method for managing nomadic farming and drinking areas from now on is connected to the environment of the water and the health of the animals and people that live round it.

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原　著

モンゴルトゥール川における遊牧家畜糞便に起因する病原性微生物の検出調査

多田千佳、琴賀岡朋絵、高田　萌、福田康弘、中井　裕
989-6711　東北大学大学院農学研究科，宮城県大崎市鳴子温泉蓬田 232-3

モンゴルでは、過放牧とともに水飲み場で家畜が集中しており、遊牧家畜からの糞便による河川水汚染が懸念されている。 本研究では、2012 年から 2014 年まで、トゥール川の遊牧家畜（馬、羊、ヤギ、ウシ）の糞便および河川水を採取し、病原性微生物（大腸菌、クリプトスポリジウム、ジアルジア）の検出調査を行った。その結果、過去の他国の家畜の病原性微生物陽性率と比較して、モンゴルの遊牧家畜は、病原微生物陽性率が他国より高い傾向があった。 これらの結果から、健全な水環境保全のために、遊牧家畜の糞便由来による河川水質汚染を防止可能な新たな遊牧のあり方を考慮する必要性が示された。

キーワード：遊牧家畜, 糞便, 河川水, 大腸菌, クリプトスポリジウム, 鞭毛虫.

連絡者： 多田　千佳　chika.tada.e1@tohoku.ac.jp

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