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Bovine trypanosomosis in Bukanga County, Western Uganda: prevalence, farmers knowledge and livestock management practices

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ABSTRACT

Trypanosomosis is considered as a threat to the ongoing efforts on poverty alleviation in Uganda despite attempts to control it. Understanding current prevalence and level of knowledge of local farmers influences the extent to which they adhere to and the success of existing control interventions. Prevalence of bovine trypanosomosis was determined from a total of 200 cattle blood samples randomly selected and screened for trypanosomes by microhaematocrit centrifugation method (HCT) and blood smear microscopy; a questionnaire was used to establish the level of knowledge and livestock practices. STATA software was used for descriptive data analysis and Ashur's scale to assess farmers' knowledge. Four blood samples (4/200) were positive for trypanosomes by both HCT and smear microscopy techniques giving a prevalence of 2% (95% CI: 1.94 – 2.06); *T. congolense* and *T. vivax* species were identified with *T. congolense* contributing 75% of all infections. Farmers level of knowledge was low and nomadic farming system was mainly practiced. Trypanosomosis remains an economically important challenge in the study area. There is need to encourage farmers to adopt integrated tick and tsetse control practices by using acaricides that kill both ticks and tsetse and there is need for community sensitization and enhancement of the prevailing trypanosomosis control interventions.

Keywords: Bovine trypanosomosis; prevalence; management practices; African Animal Trypanosomiasis

INTRODUCTION

African Animal Trypanosomosis (AAT) a disease of livestock & game animals is caused by an infection with different species of the genus *Trypanosoma*. *T. brucei*, *T. congolense* and *T. vivax* being species of livestock economic importance (Lelisa et al., 2015). It is a zoonotic disease transmitted cyclically by blood sucking flies of the genus Glossina, commonly known as tsetse (Holt et al., 2016). It is a problem in the livestock industry in Western Uganda (Alingu et al., 2014). The natives of Bukanga County in Isingiro district are traditionally pastoralists with 180,345 herds of cattle (National population and housing

census report, 2014). This disease has been tentatively diagnosed in Bukanga County (Personal communication from District Veterinary Officer, Isingiro district).

The ecological system in the area is prone to chronic drought and the terrain is characterized by bare hills and rangelands. The natives are traditionally pastoralists who mainly keep Ankole long horned cattle, goats and sheep (Wurzinger and Sölkner, 2008). Understanding prevalence of trypanosomosis, farmers knowledge and livestock practices is critical to knowing the epidemiology of the disease; and acts as a guide in designing suitable and locally acceptable control programmes. However, the above critical information was missing in Bukanga County located in south western Uganda; an information gap this study sought to bridge.

The aim of the study therefore was to determine prevalence of bovine trypanosomosis, farmers knowledge and livestock management practices in Bukanga County, Isingiro District; Southwestern Uganda.

MATERIALS AND METHODS

Study area and design

A cross-sectional field and laboratory based study was carried out to determine the prevalence of bovine trypanosomiasis, farmers knowledge and livestock management practices in Bukanga County, Isingiro district located in south western Uganda (Fig. 1) in August 2018.

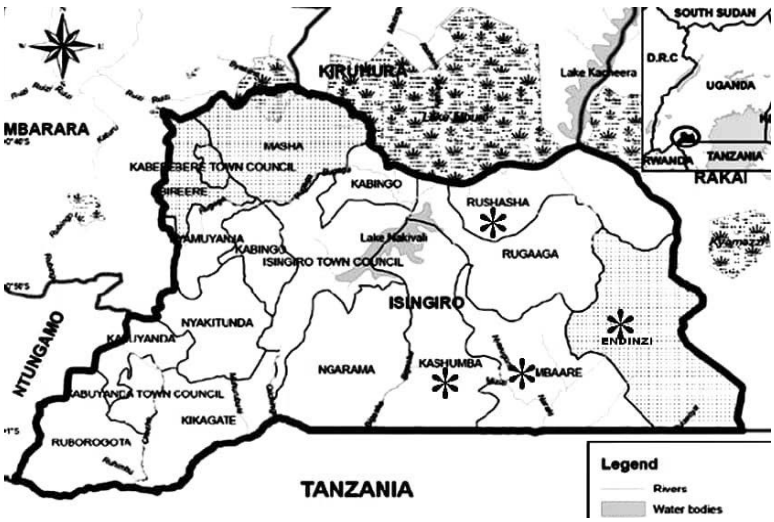

Fig. 1. A map showing sub counties in Isingiro district; Western Uganda. * Studied sub counties.

Study population and sample size

The target populations included local breeds of Ankole long horned cattle, exotic cattle and cross breed of all age groups raised under different farming systems. The sample size was estimated using Kish formula (Kish L, 1965) taking precision assumed to be +/-

0.05 at 95% level of confidence. Expected prevalence of 15.3% was used to calculate the sample size (Dennis et al., 2014). Two hundred cattle selected from 200 herds were sampled and seventy farmers were recruited for assessment of knowledge and livestock management practices.

Sampling technique

The study area was stratified into sub counties, parishes and villages and herds. Herds and farmers were selected using simple random sampling method. The study area was stratified into sub counties and selected a minimum of fifty heads of cattle were randomly selected from each of the four sub counties. After identifying animals with unexplained poor body condition in each sub county, a sampling frame was constructed and fifty animals were selected from each sub county.

Sample collection and processing

Laboratory reagents and materials were purchased from Joint Medical Stores, Uganda. Blood samples were collected aseptically from two hundred cattle selected from 200 herds, 50 blood samples from each of the four sub counties for laboratory examination. Wet blood films were used for identification of the *Trypanosoma* species based on morphological descriptions as well as motility. Thin and thick smears were made on separate slides, air dried and thin smear fixed in absolute methyl alcohol for three minute and later stained using 3% Giemsa staining protocol for 30 minute and both were examined using a light microscope using an oil emersion objective lens. Thick smears were used to detect parasites and thin smears for species typing and identification based on morphology.

Packed cell volume (PCV) determination was carried out using standard operating procedure for microhaematocrit centrifugation method and individual results were then read from a hematocrit reader and recorded in percentages. Animals with PCV ≤ 24% were considered to be anaemic, this lower limit has been previously used by Gemeda F (2015). The Buffy coat was examined microscopically for trypanosome motility under × 40 objective and ×10 eye piece.

Data analysis and management

Data obtained was entered into MS Excel spreadsheets, cross checking and editing done; validation done to ensure correctness of entry. STATA software was used for descriptive data analysis and outputs were presented in form of charts, graphs, bar graphs and tables. The prevalence was calculated as the number of infected individual animals divided by the number of cattle examined and multiplied by 100 using 95% confidence interval. Level of knowledge about AAT was measured using Ashur's knowledge measurement method (Ashur, 1977).

Ethical considerations

The study was approved by Research Ethics Committee (Ref: MUREC 1/7) and Faculty of Medicine Research Ethics committee (Ref: DMS-6) at Mbarara University Science and Technology.

RESULTS

Demographic characteristics

Two hundred cattle from two hundred farms or herds from four sub counties in the study area were sampled and a data collection guide was used to assess farmers the level of awareness about AAT and livestock management practices for AAT. The sub counties in the study area were Rushasha, Mbare, Kashumba and Endizi. The study recruited seventy farmers of different levels of formal education with the majority 91.43% (64/70) having primary as their highest level of formal education. Farmers who were recruited into the study had different tenures in the livestock industry with the majority 91.43% (64/70) with over 6 years' in the livestock industry. Besides cattle, farmers who were recruited into the study also kept goats, sheep, dogs and pigs.

General prevalence of Bovine trypanosomosis and isolated trypanosome species

Of the 200 blood samples that were collected and examined, 4/200 were positive for trypanosomes giving a prevalence of 2.0% (4/200) (95% CI: 1.94 – 2.06); the mean PCV result for all cattle sampled was 20.85% ± 4.46% (Mean ± Standard deviation, range 7 – 33%) below the lower limit of 24% (Gemeda, 2015); this signified that cattle recruited into the study were anaemic on average. Infections were identified from two sub counties of Rushasha and Mbare; each 4 % (2 out of 50) of cattle in Rushasha and Mbare sub-county were infected with trypanosomes, while none of trypanosome positive cattle were identified in Kashumba and Endizi sub-county. Rushasha is bordered by Lake Mburo National park while Mbare is bordered by Tanzania. From thin blood smear microscopy findings, only two trypanosome species (*T. congolense* and *T. vivax)* were isolated with different prevalence's at 75% (3/4) and 25% (1/4) respectively with *T. congolense* being more prevalent. No mixed infections were identified.

Farmers level of awareness about AAT

Levels of knowledge regarding identification of signs and symptoms and preventive measures were assessed following the Ashur's knowledge measurement scale (1977). According to Ashur, a score of less than 40 percent of the total correct responses is taken as an indicator of low level of knowledge, 40 – 59 percent is considered average and 60 – 80 per cent is considered good, while over 80 percent is regarded as excellent level of knowledge. Farmers knowledge about signs, symptoms and prevention was found to be low as per Ashur's knowledge level scale (1977), with only 33.30% and 22.10% correctly identifying right options about signs and symptoms and preventive measures, respectively. Only 14.28% (10/70) of study participants knew that avoiding tsetse infested areas and bush burning were important practices to prevent their animals against AAT as shown in Table 1.

Table 1. Respondent's level of knowledge about AAT infections

Variable	Frequency (n = 70)	Percentage (%)
Recognition of signs and symptoms of AAT		
a. Poor body condition	36	51.4
b. Weight loss	14	20.0
c. Standing hairs	44	62.9
d. Fever	36	51.4
e. General body weakness	14	20.0
f. Low milk production	18	25.7
g. Abortion	24	34.3
h. Loss of appetite	2	2.9
i. Lacrimation	24	34.3
j. Diarrhoea	26	37.1
Mean value		**33.3**
Awareness about appropriate preventive & control measures		
a. Spraying/ dipping	64	91.4
b. Avoiding tsetse infested areas	4	5.7
c. Bush burning	6	8.6
d. Prophylactic treatment	12	17.1
e. Treatment of cases	4	5.7
f. Use of tsetse fly traps	2	2.9
Mean value		**22.1**
Cumulative mean		**27.7**

Livestock management practices

From the study findings, all farmers 100% (70/70) could move with their cattle in search for either pasture or water Mburo National park and sprayed their animal against flies and ticks; 94.29% (66/70) reported seeking professional advice and treatment for only sick animals, 87.14% (61/70) spray their cattle, 28.57% (20/70) reported avoiding infested areas, 14.29% (10/70) use tsetse traps and 17.14% (12/70) practice bush burning as shown in Fig. 2.

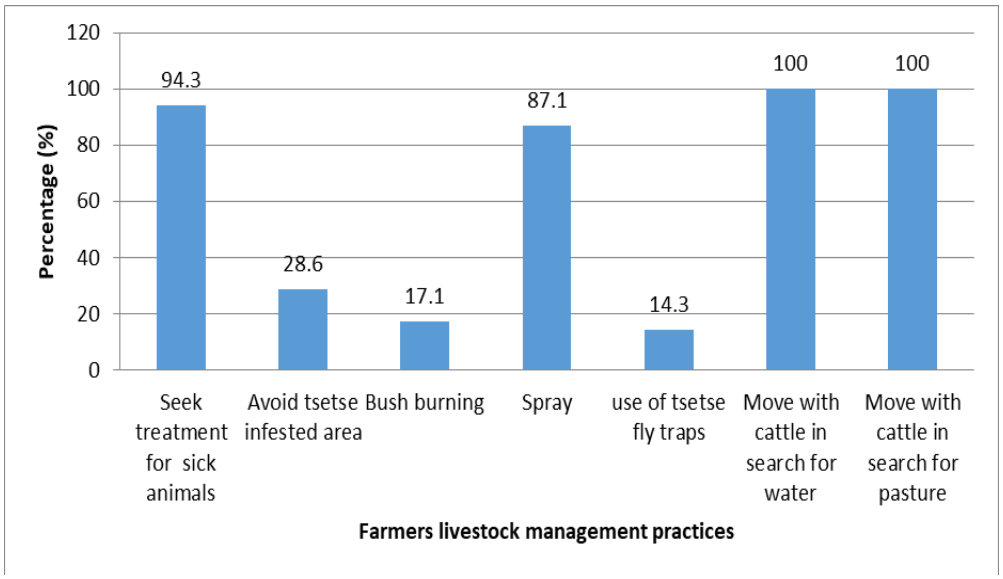

Fig. 2. A column graph showing farmers livestock management practices against AAT.

DISCUSSION

The prevalence of bovine trypanosomiasis in this study was 2%. These findings are in agreement with similar studies in Kashaari, Uganda 2.4 % (95% CI; 1.0% - 4.8%) (Alingu et al., 2014), Nigeria (1.8%) (Fajinmi et al., 2011) and West Ethiopia (2.86%) (Tadesse et al., 2015). This could be attributed to similarities in utilization of AAT livestock management practices, diagnostic methods used and good average knowledge levels exhibited by farmers. These findings however are in disagreement with those observations from other similar studies to determine the prevalence of trypanosomosis in Tororo District 15.3% (Dennis et al., 2014), trypanosomiasis in the districts of Amuru and Nwoya, Northern Uganda was 41% (Angwech et al., 2015). This could be attributed to the differences in the diagnostic methods used where more sensitive method (PCR) was used while the current study used microhaematocrit centrifugation technique, thick and thin smear microscopy where some cases could have been missed. This could also be attributed to variation in utilization of AAT livestock control practices and average levels of knowledge exhibited by farmers in his specific studies and differences in climatic conditions and vegetative cover.

In the current study, two *Trypanosoma* species were identified that is *T. congolense* and *T. vivax*; 3/4 (75%) infections were due to *T. congolense* and 1/4 (25%) infections due to *T. vivax* species; *T. congolense* was the most predominant species (75%) followed by *T. vivax* causing 25% of the infections with no mixed infections. The current findings are in agreement with observations from other studies in Mbarara (Waiswa, et al., 2013), North West Ethiopia (Tadesse et al., 2015) and Western Oromia (Kassaye and Tsegaye. 2016). This could be attributed to bushy forest cover, climatic changes and the presence of water bodies in these areas which provides suitable breeding environment for the vectors.

However, this finding disagrees with observation in Tororo District, Uganda (Dennis et al., 2014) where *T. vivax*, *T. brucei* and *T. rhodesiense* were the species isolated. Presence of *T. congolense* transmitted cyclically by tsetse flies and *T. vivax* which can be transmitted by tsetse flies and mechanically by Tabanidae and Stomoxys suggests the presence of these vector flies in the ambits of the study area and calls for their control. This could be attributed to bushy forest cover, climatic changes and the presence of Lake Nakivale and River Kagera surrounding the study area which provide suitable breeding environment for the vectors.

Most prevalent *Trypanosoma* species identified in this current study was *T. congolense*. These findings are in agreement with the studies in south Eastern Uganda (Magona et al., 2005), Western Oromia (Kassaye and Tsegaye, 2016) and Kwale District, Kenya (Ohaga et al., 2007). However, the current findings differ from observations from similar studies in Mbarara (Waiswa and Katunguka-Rwakishaya, 2013), Tororo District, Uganda (Dennis et al., 2014), in Amuru and Nwoya district, Northern Uganda (Angwech et al., 2015), west Nigeria (Tadesse et al., 2015), Bure and Womberma districts of West Gojjam zone, North West Ethiopia where *T. vivax* was the most prevalent trypanosome species. This could be attributed to differences in the relative abundance of cyclical and mechanical vectors, differences in climatic changes, vegetative cover and presence of infected animals in close proximity of many other susceptible animals.

Farmers exhibited low level of knowledge about bovine trypanosomosis scoring 27.7% on average. Most farmers' showed low level of knowledge about recognition of sick animals and understanding of control and preventive measures of AAT as per Ashur's knowledge method (Ashur SS, 1977) with only 33.3% and 22.1%, respectively, correctly identifying right options (Table 2). These findings are in agreement with studies in Western Oromia (Kassaye and Tsegaye, 2016) and Kenya (Ohaga et al., 2007). The low level of knowledge about signs, symptoms, preventive and control measures could be attributed to low level of formal education since most of them of the participants stopped at primary level for formal education and lack of community based programmes against AAT.

These findings however, differ from findings of the survey in Burkina Faso, Mali and Guinea that indicated that most farmers (96%) recognized common signs of trypanosomiasis (Grace et al., 2009) compared to the mean recognition value of 33.30% for signs and symptoms of AAT this study. This could be attributed to differences in levels in formal education levels where most farmers in these particular studies had stopped at secondary schools as their highest level of formal education as compared to primary level in the current study and presence of community based programmes against AAT in these areas and difference in livestock tenure.

From the current study, most farmers spray/dip, sought treatment for sick cattle and administer prophylactic treatment as major livestock practice to manage AAT on their farms. These findings are in agreement with observations from similar studies in Mbarara (Waiswa and Katunguka-Rwakishaya, 2004), Tanzania (Fox et al., 1993) in Kenya (Baylis and Stevenson, 1998) and Ethiopia (Leak et al., 1995). These could be attributed to similarities in area settings, availability and accessibility of these particular services by farmers, level of knowledge about these methods. Few farmers used tsetse traps, avoided tsetse infested places and practiced bush burning to manage AAT on their farms. These findings are in agreement with the study in Bugiri District, Uganda (Okoth, 1991). This could be attributed to low level of knowledge about these methods and lack of community based programmes against AAT, similarities in climatic conditions shared by the two study areas where long dry spell forces farmers to move in search for pasture and water for their animals.

CONCLUSIONS

Bovine trypanosomiasis remains an economically important disease in Bukanga with a prevalence of 2%. Trypanosome species identified were *T. congolense and T. vivax*; *T. congolense* was the most prevalent trypanosome species responsible for most infections in the area. The persistence of the disease in this area is perpetuated by seasonal movement of farmers with their cattle to tsetse infested Lake Mburo National Park and across the Tanzanian border while in search of pasture and water for their animals during the dry season and back during the rainy season. The farmers knowledge about AAT disease recognition, control and prevention was low with only 27.7% of respondents' correctly identifying right options. Nomadic system of farming where they could move with their animals in search for pasture and water and spraying were the main livestock practices; however, only few farmers reported avoiding infested areas, using tsetse traps or practiced bush burning.

RECOMMENDATIONS

Before moving with animals in search for pasture and water, farmers should have their herds covered with blanket prophylactic treatment against AAT and when coming back to their residences, they should give blanket sanative treatment to cover any cattle that could have been infected. Farmers should be encouraged to adopt integrated tick and tsetse control practice by using acaricides that kill both ticks and tsetse to contain any flies that stray from the park or across the border to their permanent place of residence. There is need for community sensitization and enhancement of the current AAT control interventions if the condition is to be eliminated in this area.

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CONFLICT OF INTEREST

All authors declare no conflict of interest in the study.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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Usefulness of *Neospora caninum* dense granule 1 based-iELISA for the detection of canine neosporosis

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ABSTRACT

Neospora caninum is an apicomplexan parasite that causes abortion in cattle and sheep. Dog is the main host of *N. caninum* and infected one shows neurologic disorders. Accurate diagnosis is important particularly under lacking of treatment and vaccination measures. Herein, we targeted the evaluation of diagnostic ability of recombinant protein NcGRA1(rNcGRA1) for detection of specific antibody (IgG) against *N. caninum* in mice and dogs. The rNcGRA1 was expressed using the *E. coli* expression system. Diagnostic ability of rNcGRA1 was tested against experimental mouse and dog sera, as well as field dog sera. Simultaneously, same serum samples were tested against rNcSAG1 and *Neospora* lysate antigen (NLA)-based iELISA. In the *N. caninum* experimentally infected mice, specific IgG antibodies to NcGRA1 were detectable from 14 days post infection (dpi), and increased at 28 dpi with no detectable antibody levels in the negative control or *Toxoplasma gondii* infected-mouse sera. In experimentally infected dog sera, the performance of rNcGRA1 was proficient in demonstrating IgG antibody at 28 dpi. In case of field dog sera (N=18), seropositive rate was 44.4% and 22.2% for rNcGRA1 and rNcSAG1-based iELISAs, respectively. These results show that the rNcGRA1 is a novel useful diagnostic antigen for serological detection of *N. caninum* in dogs.

Keywords: *Neospora caninum*; Neosporosis; NcGRA1; ELISA; Dog

INTRODUCTION

Neospora caninum is a coccidian parasite with a wide range of intermediate hosts. Family Canidae is the final host that harbors the sexual stage of the parasite, sheds the oocyst in its feces, which is the only source of horizontal infection for the intermediate host. Ruminants and equids have been reported with some other wild and domestic animal species as intermediate hosts. The parasite transmitted horizontally in cattle and dogs via ingestion of infective oocyst or tissue cyst, respectively, while vertical transmission from the dam to fetus plays a key role in the epidemiology of bovine neosporosis (Dubey and Schares, 2011). Abortion and fetal abnormalities are the main signs of the disease in cattle. In congenitally infected pups, the parasite destroying the neural cells, causing neuromuscular paralysis. Sulfonamides and/or pyrimethamine, and clindamycin can be used successfully in the treatment of canine neosporosis, depending on the stage of infection (Dubey, 2003).

Various diagnostic methods have been developed to evaluate the infection status in bovine or canine neosporosis. The indirect fluorescent antibody test (IFAT), Indirect enzyme-linked immunosorbent assay (iELISA), immunoblotting and various agglutination tests were employed to detect anti-*N. caninum* antibodies in sera of infected animals. However, iELISA has many advantages including the time saving and capability of using for high number of samples. iELISA using single antigen provides an excellent opportunity to avoid cross-reactivity with closely related parasites (Dubey et al., 1996). Surface antigens (NcSAG1, NcSRS2) and numerous of dense granule antigens (NcGRA2, NcGRA6, NcGRA7) have been successfully used for detection of specific antibodies in infected cattle or dog mostly using iELISA (Sinnott et al., 2017).

Neospora caninum dense granule 1 (NcGRA1) was identified from tachyzoites by proteomics analysis (Lee et al., 2003). NcGRA1 secreted and accumulated in the cyst wall, it could be involved in formation of the tissue cyst wall during tachyzoite to bradyzoite conversion stage (Vonlaufen et al., 2004). The polypeptides encoded by NcGRA1 target for the host immune system during chronic infections of *N. caninum* in mouse and cattle (Atkinson et al., 2001). The antigen was evaluated as a vaccine candidate using pregnant mice model with low efficacy (Ellis et al., 2008). The diagnostic performance of rNcGRA1 was reported against sheep and donkey sera using DOT- ELISA test (Joaquin Patarroyo et al., 2013; Blanco et al., 2014). However, there is no report regarding its use as a diagnostic antigen for canine neosporosis or as a based antigen for the development of iELISA test.

Several studies have been conducted to identify and characterize the antigens of *N. caninum*, in order to improve the performance of serological diagnostic tests, and finally to participate in the control measures against *Neospora* infection (Bjerkas et al., 1994; Sinnott et al., 2017). However, further studies are required to identify more diagnostic antigens for detection of neosporosis, particularly in dog, for its epidemiological importance of disease transmission. In the current study, we have expressed the recombinant NcGRA1 (rNcGRA1) using glutathione S-transferase (GST) fusion protein in the *E. coli* expression system and evaluated its diagnostic ability against a number of experimental and field dog serum samples using the iELISA. The obtained results provide a promising novel serodiagnostic tool for specific antibody detection of *N. caninum* in dog.

MATERIALS AND METHODS

Ethics statement

All animal experiments strictly followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The study protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permission numbers: 24-17, 25-66, 18-15). For mice, general anesthesia with isoflurane was conducted before painful experiments.

Experimental animals

Female BALB/c mice aged 6–7 weeks were obtained from Clea Japan (Tokyo, Japan). The mice were housed in cages in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan under specific-pathogen-free conditions. Experimentally infected dog sera, we evaluated the same samples used in a previous study (Hiasa et al., 2012). Briefly, four specific-pathogen-free (SPF) female beagle dogs (14 to 15 months old) were purchased from Chugai Medical Animal Institute (Nagano, Japan) and were housed in separate rooms. All dogs were tested by iELISA based analysis of NLA and rNcSAG1 to prove that they are free from *N. caninum*-specific antibodies.

Parasites and host cell cultures

The *N. caninum* (Nc-1 strain) was maintained in African green monkey kidney epithelial cells (Vero cells) cultured in Eagle's minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan) and 1% streptomycin–penicillin (Sigma). For purification of tachyzoites, parasites were washed in sterile phosphate-buffered saline (PBS), and then a cell scraper (BD Bioscience, San Jose, CA, USA) used to separate the infected cell monolayer. Finally, the cell pellet was resuspended in RPMI 1640 medium (Sigma) using a 27-gauge needle and passed through a 5 μm filter (Millipore, Bedford, MA, USA).

Cloning of the NcGRA1 gene into pGEX-4T-1 vector

To clone the *NcGRA1* gene (ToxoDB: NCLIV_036400), a pair of oligonucleotide primers with restriction enzyme sites was designed and used for the amplification of the *NcGRA1* gene from the *N. caninum* (Nc-1 strain) cDNA (forward primer: 5′- AA GGA TCC ATG GTT GCA GCC TCG GTG GTT -3′; reverse primer: 5′- GC GAA TTC TTA ATG TTG CCC TTG AAG -3′). The PCR product was ligated to the *BamH*I (underlined sequence in forward primer) and *EcoR*I (underlined sequence in reverse primer) restriction sites of the pGEX-4T-1 expression vector (GE Healthcare Life Sciences, UK). The resulting plasmid construct was checked for accurate insertion by digestion with restriction enzyme*s* according to the manufacturer instructions (ROCHE Diagnostics, Germany), and by sequencing using a Big Dye Terminator Cycle Sequencing Kit, and an ABI PRISM3100 genetic analyzer (AB Applied Biosystems, Carlsbad, CA, USA).

Expression and purification of rNcGRA1 in *E. coli*

An open reading frame (ORF) of the *NcGRA1* gene was expressed as GST fusion protein in the *E. coli* BL21 (DE3) strain according to the manufacturer's instructions (New England BioLabs Inc., Ipswich, MA, USA). Expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan) for 6 h at 37°C. Bacterial cells were harvested and the pellets were suspended in sonication buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol) then centrifuged at 7,180 × *g* at 4°C for 10–15 min. Lysozyme (final concentration of 500 μg/mL) and Triton X-100 (10%) in PBS were added followed by incubation on ice for 1 h. The lysate

was applied to Glutathione Sepharose 4B beads (GE Healthcare Life Sciences, Buckinghamshire, England) according to the manufacturer's instructions. Briefly, the supernatant was incubated with washed beads overnight at 4°C with gentle rotation. GST fusion proteins were eluted with elution buffer (100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 5 mM EDTA, and 25 mM reduced glutathione powder; Wako Inc). The quantity and purity of each protein were determined by Sodium dodecylsulfate polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue R250 (MP Biomedicals Inc., Illkirch-Graffenstaden, France). The protein concentrations were assayed with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The recombinant proteins, NcGRA1-GST, and GST, were expressed abundantly with apparent molecular weights, 45 and 26 kDa, respectively, consistent with the expected molecular weights (Fig. 1).

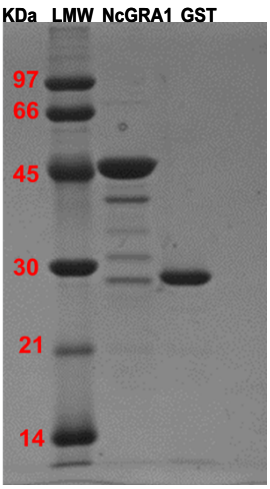

Fig. 1. SDS-PAGE of purified recombinant antigens. All recombinant proteins were expressed as GST fused protein and obtained with apparent molecular weights consistent with the expected molecular sizes of each protein. KDa; kilodalton, LMW; low molecular weight marker.

Preparation of lysate antigen

Lysate antigen from purified tachyzoites of *N. caninum* was prepared as described previously (Liao et al., 2005). The obtained extract was filtered through a 0.45 μm low-protein binding Supor® membrane (Pall Life Sciences, Ann Arbor, MI, USA), and the concentration was measured using a BCA protein assay kit.

Sera from experimentally-infected mice

A total 18 mice were divided into three groups representing non-infected (n = 6), acute (n = 6), and sub-acute infection (n = 6) groups. For infected sera, samples were collected from mice at 14 dpi, and 28 dpi. Blood samples were collected via cardiac puncture and added in tube without anticoagulants. The blood was incubated for 30 min at room temperature, and then centrifuged at 5,000 × *g*, 20 min, 20°C. After separation of serum from coagulated blood, it was centrifuged again at higher speed to harvest high purity serum and kept at -30°C until use.

Experimental and field dog serum samples

The samples evaluated in our study had been prepared and used in our previous study (Hiasa et al., 2012). In brief, four females of SPF beagle dogs (14–15 months) were intravenously inoculated with *N. caninum* Nc-1 strain tachyzoites (2×10^6). Clinical serum samples from dogs (n=18) were collected from animal hospitals located in Japan. The reactivity of the sera from experimentally infected animals was confirmed by a commercial immunofluorescent antibody test slide (VMRD, Pullman, WA, USA) and iELISA based on

rNcSAG1 (Hiasa et al., 2012; Abdelbaky et al., 2018). Sera collected at 0- and 28-days post infection were used in this study.

iELISA

iELISAs were performed as reported previously (Abdelbaky et al., 2018) with slight modifications. An amount 50 μL of the recombinant proteins (0.1 μM) and GST (0.1 μM) and NLA (5 μg/mL) were added to each well of a 96-well microtiter plate (Nunc, Roskilde, Denmark). All used antigens were prepared in coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C in the plate. On the next day, the plates were washed once with PBST (0.05% Tween-20 in PBS) and blocked with 100 μL of 3% skim milk prepared in PBS (PBS-SM) for 1 h at 37 °C. The plates were washed once, then 50 μL of the test sera diluted with PBS-SM at 1:600 for mouse sera and 1:200 for dog sera was added to the wells. The plates were incubated for 1 h at 37°C. After washing with PBST six times, 50 μL of horseradish peroxidase-conjugated goat anti-mouse and goat anti-dog IgG antibodies (Bethyl Laboratories, Montgomery, TX, USA) diluted with PBS-SM at 1:15,000 and 1:4,000 in PBS-SM, respectively, was added to plates at 37°C for 1 h. An amount 100 μL of substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/mL 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); Sigma–Aldrich, St. Louis, MO, USA] were added to each well after further six times of washing. The plates were incubated at room temperature for 1 h. Then, absorbance at 415 nm was measured using a microplate reader (MTP-120; Corona, Tokyo, Japan). Absorbance values for rNcSAG1 and rNcGRA1 antigens were determined after subtraction of the optical density for GST at 415 nm. Cutoff values for iELISA were estimated using negative control *N. caninum* dog sera (n = 4), using formula of cutoff value = mean + 3 × standard deviation.

Statistical analysis

Data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). For statistical analysis, one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test, or Student's *t*-test were performed. A P value < 0.05 was considered statistically significant. Degrees of statistical significance are shown as asterisks (*) defined in each figure legend.

RESULTS

Assessment of recombinant antigens using sera from experimentally infected mice

First, the diagnostic performance of rNcGRA1 as a test antigen, in addition to rNcSAG1 and NLA as control antigens, was evaluated using control mouse sera using iELISAs. In case of mice, antigens were assayed against sera on non-infected, 14 dpi (acute infection), and 28 dpi (sub-acute infection). The highest IgG level was observed against rNcSAG1 followed by rNcGRA1 and NLA in *N. caninum*-infected mouse sera at 14 dpi. While in case of sub-acute infection, the antibody level of NcGRA1 was comparable to NcSAG1 and to lower extent to NLA. Noteworthy, no reactivity for the recombinant antigens was observed against sera from non-infected or *T. gondii*-infected animals, unlike NLA that

showed slight cross- reactivity against related parasite *T. gondii* (Fig. 2). This result indicates the ability of rNcGRA1 for detection of anti-*Neospora* antibodies either in acute or sub-acute infections with no cross-reactivity against *T. gondii*-infected sera in mice.

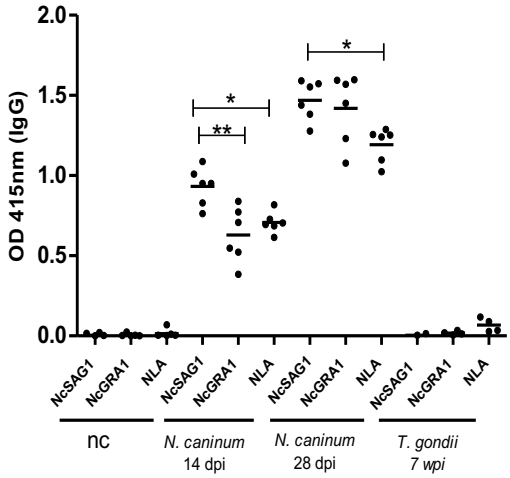

Fig. 2. Production of IgG antibodies in mouse sera against rNcGRA1, rNcSAG1 and *Neospora* lysate antigen (NLA). The mean optical density (OD) was determined at a wavelength of 415 nm. Each bar represents the mean of the tested sera (nc; sera from uninfected mice, n = 6, *N. caninum* 14 dpi; infected mice at 14 days post infection, n = 6, *N. caninum* 28 dpi; infected mice at 28 days post infection, n = 6, *T. gondii* 7 wpi; infected mice at 7 weeks post infection, n = 4). Statistically significant differences among the tested groups are shown as asterisks (*$P < 0.05$, one-way ANOVA plus Tukey–Kramer post hoc analysis).

Assessment of recombinant antigens using sera from experimentally and naturally infected dogs

Using sera from experimentally infected dog at 28 dpi, the three antigens, rNcSAG1, rNcGRA1 and NLA, were recognized by the specific IgG antibody (Fig. 3A). Although the levels of anti-NcSAG1 antibodies were higher than those of anti-NcGRA1 antibodies ($P < 0.05$), only antibody against NcGRA1 was not detectable in sera of non-infected control dogs (Fig. 3A). Regarding field dog sera (n = 18), these samples were tested using rNcGRA1 and rNcSAG1 as a control antigen. The prevalence rate based on rNcGRA1-iELISA (8/18; 44.4%) was higher compared with rNcSAG1-iELISA (4/18; 22.2%) (Fig. 3B). The two aforementioned antigens showed differences in the seropositive rates and antibody titers against IgG antibodies.

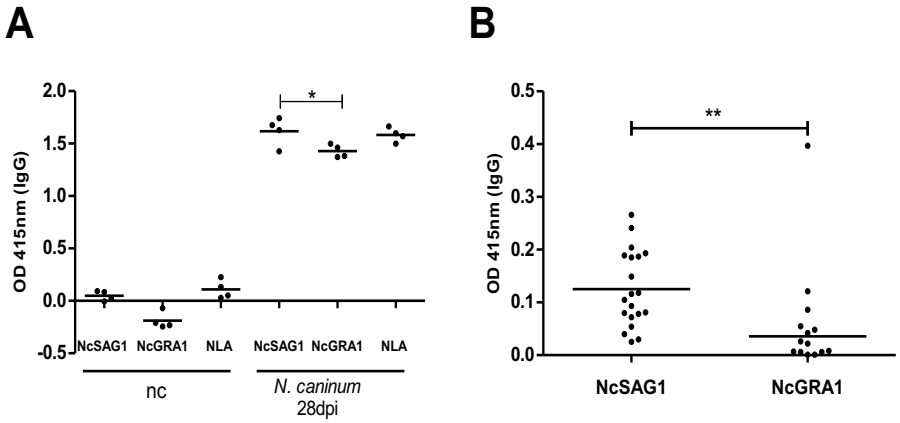

Fig. 3. The reactivity of rNcGRA1 and rNcSAG1 antigens against dog sera. (A) Performance of antigens against experimentally infected dog. Each bar represents the mean of tested sera (nc; uninfected dog sera, n = 4, *N. caninum* 28 dpi; infected dog sera collected at 28 days post infection, n = 6). Statistically significant differences among the tested groups are shown as asterisks (*$P < 0.05$, one-way ANOVA plus Tukey–Kramer post hoc analysis). (B) Performance of antigens against naturally infected dog sera. Each bar represents the mean of tested sera. Statistically significant differences among the tested groups are shown as asterisks (*$P < 0.05$, Student's *t*-test). The mean optical density (OD) was determined at a wavelength of 415 nm.

DISCUSSION

Regarding the definitive host and susceptibility to neosporosis, dog is epidemiologically important for transmission of *N. caninum*. Thus, the optimization and standardization of the diagnostic assays for detection of *N. caninum* infection are significant importance. To develop reliable diagnostic tool against *N. caninum* infection, diagnostic approaches using many antigenic proteins have been studied. Although there are numerous recombinant antigens can be used for iELISA to detect *N. caninum* antibodies in dogs, more serological assays are still needed for accurate diagnosis and further epidemiological investigations of risk factors and disease transmission.

Most of evaluated antigens have been used for testing cattle sera, while those used for dog are limited (Dubey et al., 1996; Sinnott et al., 2017). Moreover, some of previously tested antigens in dog exhibited a difficulty of protein expression because of variable degrees of solubility as reported for NcGRA7 (Lally et al., 1996), and NcSAG1 (Dong et al., 2012). For example, NcGRA7, profilin (Hiasa et al., 2012) and NcGRA2 (Jin et al., 2015), as well as NcGRA6 (Ghalmi et al., 2014), mostly detect their specific antibodies in case of acute infection. On the contrary, anti-NcSAG1 antibody is detectable both acute and chronic stages of the infection in dogs (Hiasa et al., 2012).

In the current study, rNcGRA1 was expressed by *E. coli* expression system and purified as GST-fusion protein. This can be extrapolated from efficient diagnostic ability of such antigen through the testing against *N. caninum* experimentally-infected mouse and dog sera. For either mouse or dog control samples, the performance of rNcGRA1-based iELISA was proficient and comparable to the reference antigens NcSAG1 and NLA. In case of mice, high antibody level of NcGRA1 was observed in the sub-acute status in compare to the acute one, suggesting its predominant role in late stage of infection. This effect can be deduced from its high antibody level against chronically infected dog. This result also coincides with those reported before by Atkinson et al. (2001), where the authors identified NcGRA1 using chronically infected mouse and cattle sera. Together, NcGRA1 may play a role in the chronic stage of infection in mice, dogs, and cattle.

In case of sera from naturally infected dog, rNcGRA1-based iELISA detected a higher number of seropositive samples compared to rNcSAG1-based iELISA. This might be attributed to the lower cut-off value of rNcGRA1 (0.008) compared to the cut-off value of NcSAG1 (0.189). Noteworthy, cut-off value was calculated from negative control samples kept in our laboratory and tested via both antigens as experimental samples (Fig. 3A). Nevertheless, the high performance of rNcGRA1 in detecting *N. caninum* in experimentally infected mice and dog, with no reactivity against non-infected sera in such animals and also no cross-reactivity against *T. gondii*-infected mice is supposedly regarded as a promising

result for considering rNcGRA1 as a useful antigen for detecting anti-*Neospora* antibody in dog. However, formulation of rNcGRA1 antigen with some other specific antigens as rNcGRA7, rNcGRA6 and rNcGRA2 (Hiasa et al., 2012; Jin et al., 2015; Ghalmi et al., 2014), can be employed to obtain wide-spectrum diagnostic antigen preparation. Further studies are needed to confirm the diagnostic ability of rNcGRA1 using higher number of control and field sera from dogs and also other species.

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CONFLICT OF INTEREST

All authors declare no conflict of interest in the study.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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Comparison of the morphology and viability of gamma irradiated vegetative cells, wet cysts, and dry cysts of the soil ciliate *Colpoda cucullus*

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ABSTRACT
The soil ciliate, *Colpoda cucullus*, can tolerate various types of environmental stress, including 4,000 Gy gamma radiation, by forming resting cysts (encystment). In this study, we found that *C. cucullus* resting cycts also were able to tolerate 8,000 Gy gamma radiation. Irradiated wet cysts and dry cysts were morphologically indistinguishable from non-irradiated cysts and were able to successfully revert to vegetative cells (excystment). The viability (i.e., excystment) of dry cysts was higher than that of wet cysts after 8,000 Gy gamma irradiation. However, vegetative cells did not survive 8,000 Gy gamma radiation and decomposed.

Keywords: Resting cysts; *Colpoda*; Gamma radiation; Tolerance

INTRODUCTION
The soil ciliate *Colpoda cucullus* can survive in standing water and moist soil. In addition, *C. cucullus* vegetative cells can form resting cysts to enable them to tolerate various types of environmental stress; e.g., desiccation (Corliss and Esser, 1974), acid (Sogame et al., 2011), high and low temperature (Taylor and Stickland 1936), freezing (Uspenskaya and Lozina-Lozinski, 1979), UV (Uspenskaya and Lozina-Lozinski, 1979; Matsuoka et al., 2017), and gamma radiation (Saito et al., 2020).

In general, gamma radiation is harmful for biological organisms. Whole-body exposure to 10 Gy gamma radiation is lethal for most vertebrate animals (Thornley 1963; Daly 2009). Most bacteria also cannot survive a gamma radiation dose of 200 Gy (Thornley 1963; Daly 2009). However, invertebrates and some microorganisms, such as extremophiles, can tolerate exposure to a high dose of gamma radiation. For example, the fruit fly *Drosophila melanogaster* can survive for 2 days after exposure to 1,500 Gy gamma radiation (Parashar et al., 2008) and the nematode *Caenorhabditis elegans* show almost no harmful effects after exposure to 1,000 Gy gamma radiation (Jonson and Hartman, 1988). In addition, the hyperthermophilic archaeon *Pyrococcus furiosus* has been reported to survive more than 2,500 Gy gamma radiation (DiRuggiero et al., 1997), the halophilic archaeon *Halobacterium* sp. can survive exposure to 5,000 Gy gamma radiation (Kottemann et al., 2005), and the

radioresistant bacterium *Deinococcus radiodurans* survives without loss of viability after exposure to 5,000 Gy gamma radiation (Moseley and Mattingly, 1971). It is amazing that *D. radiodurans* can even survive a dose of 12,000 Gy gamma radiation (Misra et al., 2013) and even a greater dose (Daly et al., 1994; Daly et al., 2009).

We have previously shown that *C. cucullus* resting cysts tolerate 4,000 Gy gamma radiation: most cysts were able to revert to vegetative cells after irradiation and the viability of 4,000 Gy gamma irradiated wet cysts was higher than that of irradiated dry cysts (Saito et al. 2020). In the present study, we found that *C. cucullus* resting cysts have a more extreme tolerance to gamma irradiation. Therefore, we studied the viability (i.e., excystment) and morphology of gamma irradiated *C. cucullus* wet and dry cysts.

MATERIALS AND METHODS

Cell culture and induction of encystment and excystment

Colpoda cucullus R2TTYS (Sogame et al., 2019b) was cultured in a 0.05% (w/v) rice leaf infusion supplemented with 0.05% (w/v) Na_2HPO_4 (final concentration). Occasionally, bacteria (*Klebsiella pneumoniae* NBRC13277) were added to the culture medium as a food source for *C. cucullus*.

Encystment of *C. cucullus* vegetative cells was induced by suspending the cells at high cell density (> 10,000 cells/ml) in 1 mM Tris-HCl (pH = 7.2) supplemented with 0.1 mM $CaCl_2$ (final concentration).

Excystment of *C. cucullus* cysts was induced by replacing the medium with a 0.2% (w/v) rice leaf infusion supplemented with 0.05% (w/v) Na_2HPO_4 (final concentration).

Sample preparation and gamma irradiation

For vegetative cell preparations, 1 ml of a cell suspension at low cell density (1,000 cells/ml) was put in Petri dishes. For cyst preparations, 1 ml of encystment-induced cells (10,000 cells/ml) was put in Petri dishes and incubated for 1 week. Dry cyst samples were prepared by air-drying wet cyst samples for 1 week. Six samples of each preparation were used for each irradiation experiment.

Samples of vegetative cells, wet cysts, and dry cysts were irradiated with 8,000 Gy gamma radiation (444 Gy/h, 18 h irradiation) using the RE2022 (Toshiba, Japan) in the National Agriculture and Food Research Organization (NARO). The radiation source was cobalt 60 (radioactivity, 42 TBq).

Cell viability and excystment assays

The viability of vegetative cells was determined by directly counting viable cells in 100 µl samples before and after gamma irradiation. The percent viability of vegetative cells was calculated as: Percent cell viability = (Number of viable cells after irradiation / Number of viable cells before irradiation) × 100. The excystment of wet and dry cysts was measured by directly counting excysted cells and un-excysted cells (> 100 cells in a randomly chosen field) by microscopy (Zeiss Stemi 305) before and after irradiation. The percent excystment was calculated as follows: Percent excystment = (Number of excysted cysts / Number of

excysted and un-excysted cysts) × 100. The percent viability of irradiated wet and dry cysts was calculated as follows: Percent cyst viability = (Percent excystment at 60 h after induction of excystment of irradiated samples / Percent excystment at 60 h after induction of excystment of non-irradiated samples) × 100. All values were shown as the mean ± SE of 6 identical samples. Statistical analysis was performed by the Mann-Whitney U test using the Bell Curve for Excel software (Social Survey Research Information Co., Ltd., Japan).

Microscopy

Vegetative cells, wet cysts, and dry cysts were observed with an Axio Vart.A1 optical microscope system (Zeiss, Japan). The vegetative cells were concentrated 10-fold by centrifugation (2,000 rpm for 1 min) and observed at low magnification (Fig. 1A). In addition, the cell samples were fixed in an equal volume of paraformaldehyde (PA) and observed at high magnification (Fig. 1A, upper right images).

For 4',6-diamidino-2-phenylindole (DAPI) staining, samples (wet cysts, irradiated wet cysts, and dry cysts) were suspended in a DAPI solution [1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$, 2 μg DAPI/ml] for 15 min. Wet cysts were also treated with PA and NP-40 and stained as follows: wet cysts were treated with a fixative solution [2% PA, 1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$] for 1 h, then treated with an NP-40 detergent solution [1% NP-40, 1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$] for 1 h, washed with wash buffer [1 mM Tris HCl (pH 7.2), 0.1 mM $CaCl_2$], and finally stained with the DAPI solution for 15 min. The samples were observed with a confocal laser microscope (Fluoview 10i, Olympus), using a DAPI filter (emission maximum 460 nm) with an excitation peak at 405 nm. Stained and unstained cells were counted (> 100 cells) and the data was expressed as the percent of stained cells as follows: Percent stained cysts = (Number of stained cysts / Total number of cysts) × 100.

RESULTS AND DISCUSSION

Colpoda cucullus vegetative cells, wet cysts, and dry cysts were either not irradiated or irradiated with 8,000 Gy gamma radiation. They were then examined by optical microscopy and assayed for viability. After 8,000 Gy gamma irradiation, vegetative cells were decomposed and could not be seen by microscopy (Fig. 1A). However, both irradiated wet and dry cysts were morphologically indistinguishable from non-irradiated wet and dry cysts (Fig. 1). The percent viability of irradiated vegetative cells, wet cysts, and dry cysts was 0%, 12.0 ± 5.0 %, and 42.3 ± 12.0%, respectively (Fig. 2). These results indicated that *C. cucullus* wet and dry cysts could tolerate 8,000 Gy gamma radiation.

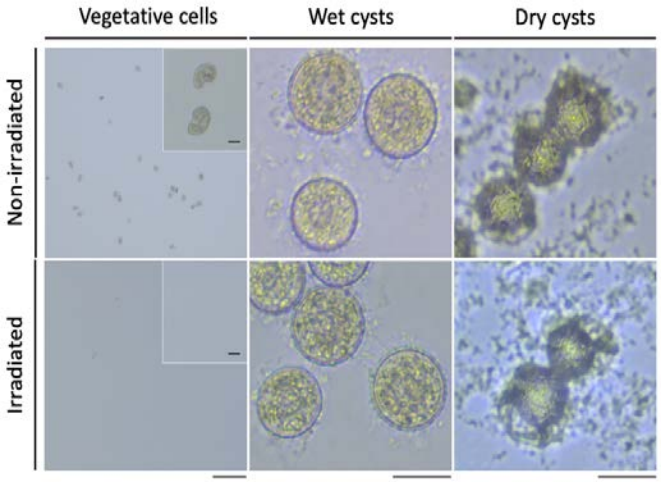

Fig. 1. Comparison of gamma irradiated and non-irradiated *C. cucullus* vegetative cells, wet cysts, and dry cysts by optical microscopy. Microscope images of non-irradiated (Top row) and 8,000 Gy gamma irradiated (Bottom row) cells and cysts. The upper right images in the vegetative cells in are higher magnifications of the cells in these samples. Scale bars mark: 250 μm and 20 μm (upper right images) for vegetative cells, and 20 μm for wet cysts and dry cysts.

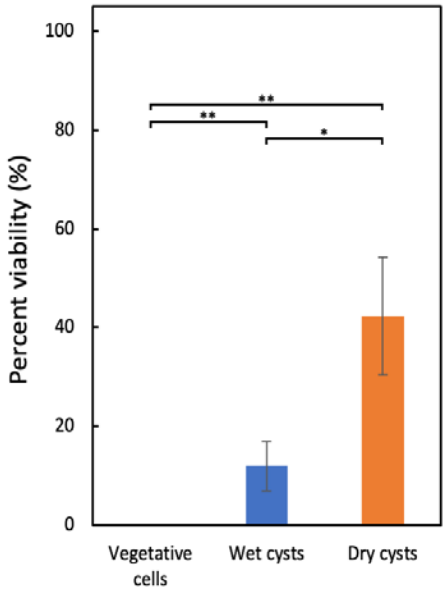

Fig. 2. Percent viability of 8,000 Gy gamma irradiated *C. cucullus* vegetative cells, wet cysts, and dry cysts relative to non-irradiated *C. cucullus* vegetative cells, wet cysts, and dry cysts, respectively. Each column shows the mean and standard error of six samples. Single asterisks and double asterisks indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively (Mann-Whitney U test).

Although both irradiated wet and dry cysts showed no apparent damage due to 8,000 Gy irradiation by optical microscopy (Fig. 1), their viability after irradiation was significantly reduced (Fig. 2). Therefore, 8,000 Gy gamma radiation produced considerable damage in wet and dry cysts that was not visible by optical microscopy. To investigate radiation damage to the cell membrane, ectocyst (the outermost layer of the cyst wall), and/or the endocyst (the inner layers of the cyst wall), wet cysts (Control), wet cysts treated with PA and NP-40 (PA+NP-40), irradiated wet cysts (Irradiated), and dry cysts (Dry cysts) were stained with DAPI (Fig. 3). Although DAPI is difficult to permeate through viable cell membrane, it passes through damaged cell membrane and preferentially binds dsDNA. Therefore, DAPI

staining can be used to investigate whether a cell membrane and/or cyst wall has been damaged. In this study, the nuclei of wet cysts were barely stained with DAPI, but the nuclei of wet cysts treated with PA and NP-40 were stained (Fig. 3A, 3B). The nuclei of 8,000 Gy gamma irradiated wet cysts were also stained with DAPI (Fig. 3A): the percent of DAPI-stained irradiated wet cysts was similar to that of DAPI-stained wet cysts after PA and NP-40 treatment (Fig. 3B). Non-irradiated dry cysts were also stained with DAPI (Fig. 3A): the percent of DAPI-stained non-irradiated dry cysts was similar to that of DAPI-stained wet cysts after PA and NP-40 treatment (Fig. 3B). These results indicated that both 8,000 Gy gamma irradiation and desiccation damaged both *C. cucullus* cell membranes and cyst walls.

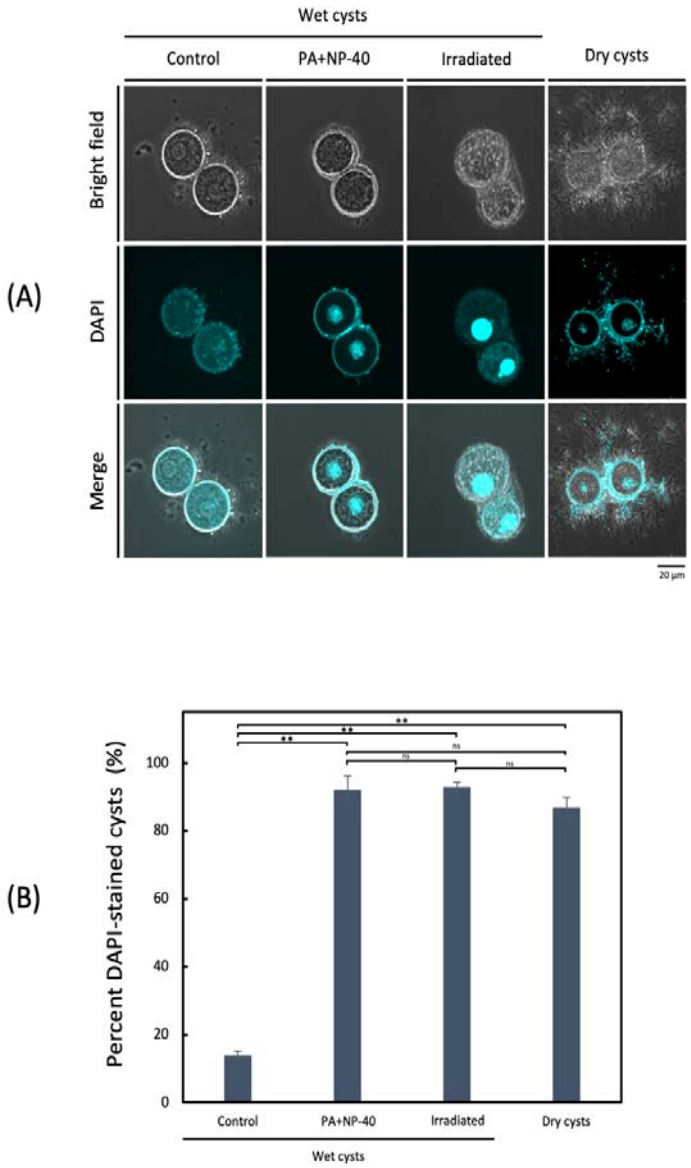

Fig. 3. DAPI stained *C. cucullus* cysts. (A) Confocal laser microscope images of DAPI-stained wet cysts (Control), wet cysts after PA and NP-40 treatment (PA+NP-40), irradiated wet cysts (Irradiated), and dry cysts (Dry cysts). Bright field images (Top row), images of DAPI-stained cysts (Middle row), and the merged images (Bottom Row) are shown. (B) Percent of DAPI-stained wet cysts (Control), wet cysts after PA and NP-40 treatment (PA+NP-40), irradiated wet cysts (Irradiated), and dry cysts (Dry cysts). Each column shows the mean and standard error of six samples. Double asterisks and ns indicate a significant difference at $p < 0.01$ and not significant among samples, respectively (Mann-Whitney U test).

After 8,000 Gy gamma irradiation and the induction of excystment, both wet cysts and dry cysts gradually reverted to vegetative cells (Fig. 4). At 60 h after the induction of excystment, 80% of the non-irradiated dry cysts had reverted to vegetative cells. However, only about 10% and 40% of the gamma irradiated wet and dry cysts, respectively, had reverted to vegetative cells at 60 h after the induction of excystment (Fig. 4). In contrast, the percent of DAPI-stained gamma irradiated wet cysts and non-irradiated dry cysts were very similar: both were about 90% (Fig. 3). Hence, 8000 Gy irradiation caused serious damage to *C. cucullus* cells in addition to direct damage to their cell membranes.

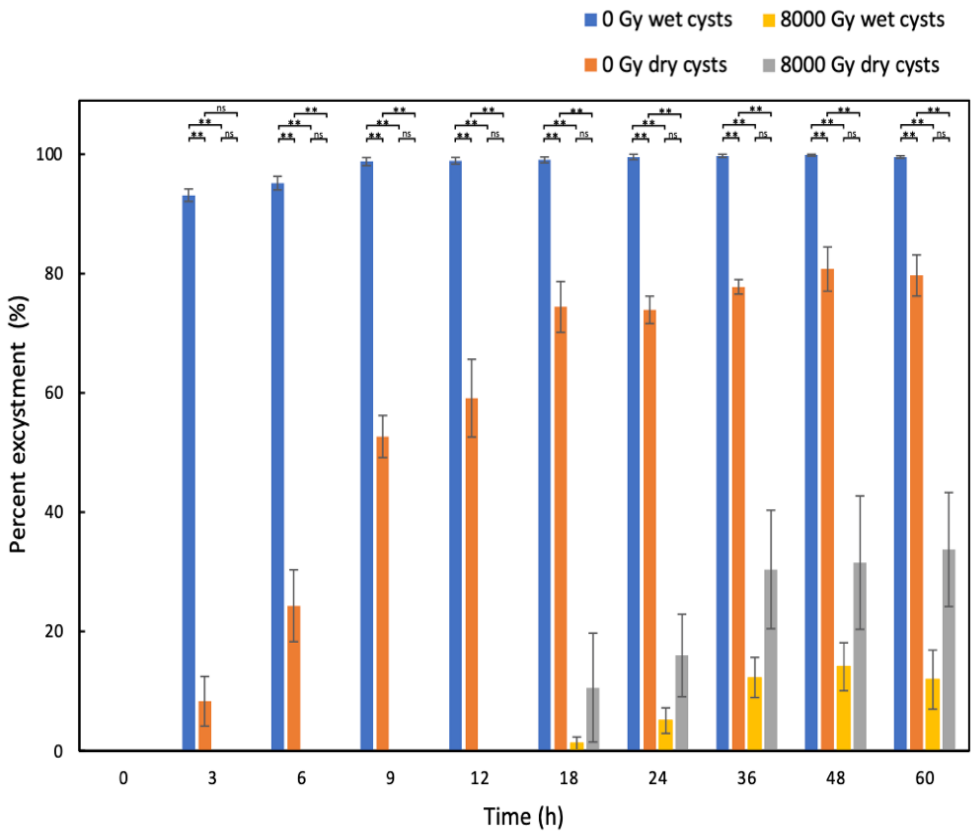

Fig. 4. Excystment of *Colpoda* non-irradiated and irradiated wet cysts and dry cysts as a function of time after the induction of excystment. Each column and bar shows the mean and standard error of six samples, respectively. Double asterisks and ns indicate a significant difference at $p < 0.01$ and not significant among samples, respectively (Mann-Whitney U test).

Radiation damage in cells (Richer et al., 2016) is mediated by reactive oxygen species (ROS) stress (Jung et al., 2017; Close et al., 2013; Imlay and Linn, 1988). In gamma irradiated cells, DNA is oxidized and proteins are carbonylated (Halliwell and Gutteridge 1999; Azzam et al., 2012). However, this damage can be repaired during the active cell phase of the hyperthermophile *P. furiosus* (Dirggiero et al., 1997). Repair of *C. cucullus* wet cysts

after 4,000 Gy gamma irradiation has been reported, but not for dry cysts due to its desiccation (Sogame et al., 2019a).

A graphical summary of our previous study (Saito et al., 2020) and this study is shown in Fig. 5. Damage in irradiated wet cysts was repaired during and after gamma irradiation, but damage in irradiated dry cysts was repaired only after the induction of excystment (Saito et al., 2020; Fig. 5). Therefore, both gamma irradiated wet and dry cysts were able to gradually revert to vegetative cells, but the excystment of wet cysts started sooner after the induction of excystment and was greater than that of dry cysts after 4,000 Gy gamma irradiation (Saito et al., 2020; Fig. 5). The repair delay in dry cysts may be due to the lack of water in these cysts, although we cannot exclud the possibility that wet cysts may be affected by radiation attenuation due to water. In this study, both wet cysts and dry cysts gradually reverted to vegetative cells after 8,000 Gy gamma irradiation, but the excystment of dry cysts tended to be faster and greater than that of wet cysts (Fig. 4). This result indicated that damage due to 8,000 Gy gamma irradiation could not be repaired during irradiation, but was repaired after the induction of excystment in both wet and dry cysts (Fig. 5). The damage in dry cysts, mediated by ROS stress produced by radiolysis of water due to the radiation (Azzam et al., 2012), was less than in wet cysts because of the lack of water in dry cysts. Hence, the excystment of dry cysts tended to be greater than that of wet cysts after 8,000 Gy irradiation (Fig. 4, 5). For non-irradiated cysts, > 90% of wet cysts had excysted and about 10% of dry cysts had started to excyst by 3 h after the induction of excystment, and almost all wet cysts and about 80% of dry cysts had excysted by 60 h after the induction of excystment (Fig. 4). However, 8,000 Gy irradiated wet and dry cysts had started to excyst at 18 h after the induction of excystment: about 10% and 40% of the gamma irradiated wet and dry cysts, respectively, were excysted by 60 h after the induction of excystment (Fig. 4).

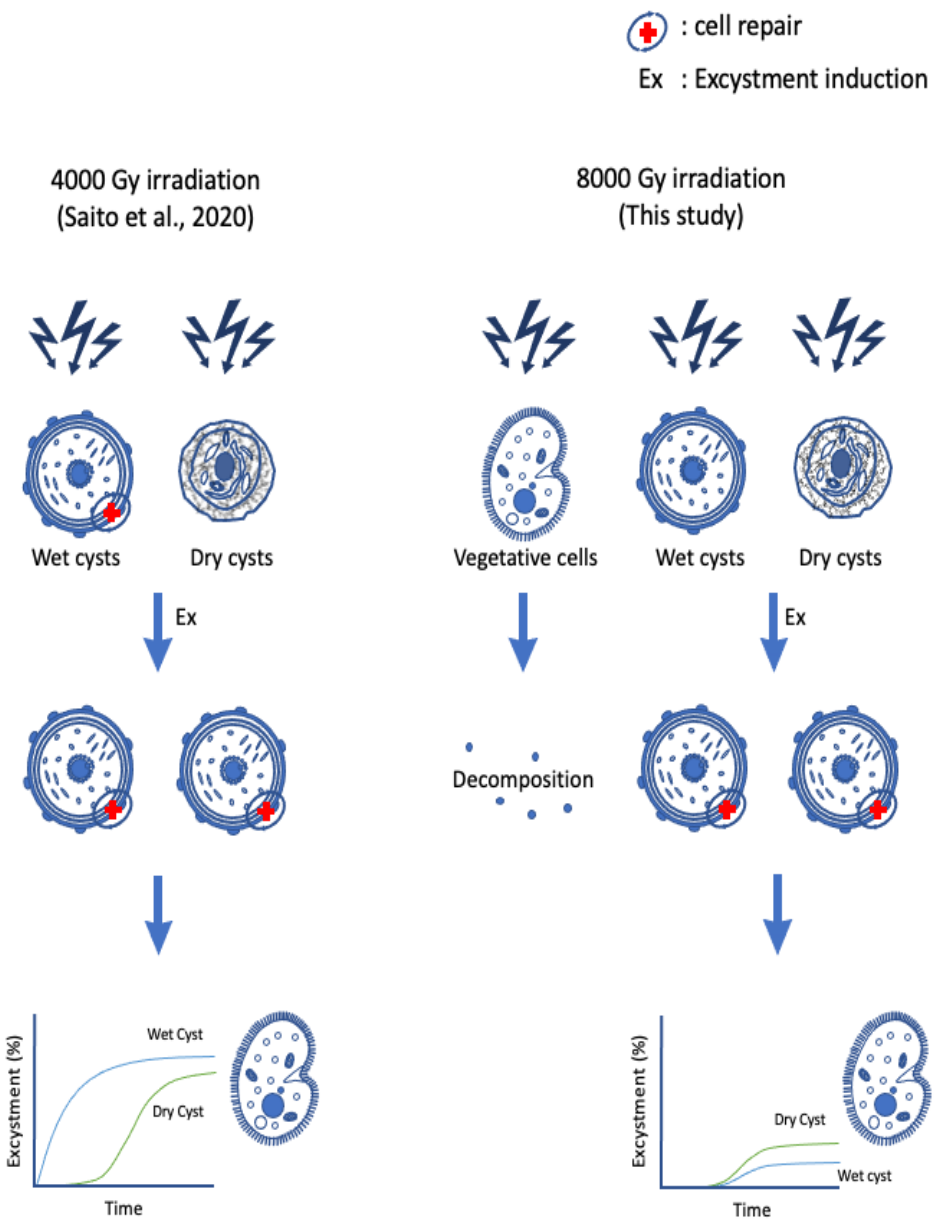

Fig. 5. Schematic summary of our current and previous results (Saito et al., 2020) on the events in the irradiation and induction of excystment of *C. cucullus* cells and cysts.

In this study, we found an extreme tolerance to gamma radiation in *C. cucullus* resting cysts. *Colpoda cucullus* vegetative cells do not have this tolerance and are decomposed by 8,000 Gy gamma radiation: the damage was lethal even at 500 Gy gamma radiation (Saito et al., 2020), similar to bacteria (Thornley 1963; Daly 2009). However, *C. cucullus* acquires

extreme tolerance to gamma radiation by forming resting cysts as cryptobiotic forms. Although *Colpoda* are not exposed to such high radiation in their habitats, they do have extreme gamma radiation tolerance. *Colpoda* may have evolved such tolerance as a strategy to adapt to terrestrial environmental stresses, such as desiccation, and to protect themselves from ROS stress (França et al., 2007). In resting cysts, the cyst wall is important to protect cysts from physical damage and for the maintenance of cell shape for cell repair (Sogame et al., 2019a). This may enable the excystment of resting cysts after environmental stress.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is approved by all authors, has not been published before, and will not be published elsewhere in the same form without the written consent of the copyright-holder.

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Seroprevalence of *Toxoplasma gondii* in Farm Animals in West Kordofan, and Blue Nile states, Sudan

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ABSTRACT

Considering the veterinary and public health significance of *T. gondii* infection and absence of epidemiological data in West Kordofan, and Blue Nile states in Sudan, a total of 392 serum samples from camel, cattle, sheep and goats in West Kordofan, and Blue Nile states were examined for *T. gondii* antibodies using Latex agglutination test. The overall seroprevalence was 22.4% (88/392). The seropositivity rate was 13.3% (6/45), 44% (33/75), 61% (22/36), 15.4% (21/136), 6% (6/100) in camels, cattle, sheep and goats and donkeys, respectively. Animal species was the only risk factor of *T. gondii* seropositivity. The significant higher seroprevalence of *T. gondii* in sheep and cattle indicates their potential role in the transmission of human toxoplasmosis in Sudan and the widespread contamination of the rural environment in Sudan with *Toxoplasma* oocysts. Further epidemiological studies, as well as special awareness and educational programs for toxoplasmosis are strongly suggested.

Keywords: Camel; Cattle; Goats; Seroprevalence; Sheep; Sudan; *Toxoplasma gondii*

INTRODUCTION

According to the official reports, the livestock population of Sudan was estimated at about 109 million heads, comprising about 4.9 million camels, 40 million sheep, 32 million goats, and 31 million cattle (FAO, 2020). The Sudanese livestock sector plays a crucial role in the national economy and welfare of the entire population via provision of essential food, cash from export earnings, means of transport and manure for soil fertility (IGAD, 2013). In spite of their significant contribution to the national economy, the livestock receive little

attention from the government and are influenced by a multitude of diseases, including toxoplasmosis (Wilson, 2018).

Toxoplasmosis is a neglected zoonosis caused by a cosmopolite protozoon, *Toxoplasma gondii*. This parasite is one of the ubiquitous parasites among humans and warm-blooded animals. Up to One-third of the world population was found to be infected with toxoplasmosis (Tenter et al., 2000). *T. gondii* infection can be acquired via ingestion of contaminated food, water or dust with sporulated oocysts, containing sporozoites, consumption of undercooked meat containing tissue cysts, and by transplacental transmission of tachyzoites from mother to fetus (Dubey et al., 2010). Despite, toxoplasmosis in humans is generally asymptomatic; it can have deleterious effects on immunocompromised patients and pregnant women (Weiss and Dubey, 2009). Toxoplasmosis is also responsible for economic and reproductive problems in livestock especially sheep and goats, resulting in stillbirth, neonatal deaths and abortion (Buxton, 1998).

In Sudan, few reports investigated the seroprevalence of *T. gondii* in farm animals (Camel, cattle, sheep and goats) and the prevalence ranged between 6.2% to 76% (Elamin et al., 1992; Khalil and Elrayah, 2011; El-Basheir et al., 2012; Elfahal et al., 2013; Medani and Kamil, 2014; Atail et al., 2017; Jomaa et al., 2017; Lazim et al., 2018). Despite the impact of *T. gondii* on animal industry, the epidemiological situation of this parasite in several states in Sudan, such as West Kordofan and Blue Nile states is unknown. Therefore, the present study aimed to determine the prevalence of *T. gondii* infection in camel, cattle, sheep and goats and the associated risk factors in West Kordofan and Blue Nile states.

MATERIALS AND METHODS

Ethical approval and consent to participate

Permission was obtained according to the standards of animal experimentation and Ethics committee of Obihiro University of Agriculture and Veterinary Medicine (Approval No. 19–19).

Study area and sample collection

The current study was performed in two states located in the south of Sudan; West Kordofan (12°0′N 28°9′E) and Blue Nile (11°16′ N 34°4′E). Camels, cattle, sheep, and goats are owned by nomadic tribes and reared in a free-range breeding. Both states selection was due to lack of epidemiological screening of *T. gondii* in farm animals.

A total of 292 serum samples were collected after obtaining oral consent from the owners in West Kordofan State (n = 151; 45 camel, 34 cattle, and 72 goats) and Blue Nile State (n = 141; 41 cattle, 36 sheep, and 64 goats). Samples were collected with jugular venipuncture into plain vacutainer tubes. Serum was separated and stored at -20ºC until analysis.

Latex agglutination test (LAT)

Serum samples were tested for the presence of *T. gondii* antibodies, via Latex agglutination test, Toxocheck-MT (Eiken chemical Co., Tokyo, Japan) according to the

manufacturer instructions. Screening of each serum sample was conducted at two-fold dilution starting from 1:8 to 1:1,024. Samples that showed agglutination at 1:32 were regarded positive.

Statistical analysis

To analyze the impact of animal species, gender and location as risk factors on the distribution of *T. gondi*i in Sudan, Fisher's exact test in GraphPad Prism 6.0 software was performed (GraphPad Software, Inc., La Jolla, CA, USA). Odds ratio (OR) and 95% confidence intervals (CI) were calculated. $P < 0.0001$ was regarded statistically significant.

RESULTS

Out of 292 animal sera surveyed for *T. gondii* antibodies among, 82 (28%) were seroreactive; being 13%, 41%, 22% in camels, cattle and goats, respectively in West Kordofan state while that in Blue Nile state was 46%, 61%, 7.8% in cattle, sheep and goats, respectively (Table 1).

Table 1. Seroprevalence of *Toxoplasma* gondii in different farm animals in West Kordofan and Blue Nile states and the titer of antibodies.

Location	Species	Total	No. of positive (%)	Antibody titers					
				1:32	1:64	1:125	1:256	1:512	1:1024
West Kordofan	Camel	45	6 (13)	5	0	0	1	0	0
	Cattle	34	14 (41)	8	2	1	1	0	2
	Goats	72	16 (22)	2	3	6	4	0	1
	Total	151	36 (23.8)						
Blue Nile	Cattle	41	19 (46)	12	3	1	3	0	0
	sheep	36	22 (61)	3	6	10	2	1	0
	Goats	64	5 (7.8)	0	0	1	0	1	3
	Total	141	46 (32.6)						
Total		292	82 (28)	30	14	19	11	2	6

Table 1 shows also the distribution of *T. gondii* antibodies among camel, cattle, sheep and goats in West Kordofan and Blue Nile states and the titer of antibodies. The antibody titeration results were as follows: 30 (1:32), 14 (1:64), 19 (1:125), 11 (1:256), 2 (1:512), 6 (1:1,024). At a dilution of 1:32 and 1:125, higher rate of seropositivity was observed.

Overall prevalence in different animal species was 44%, 13.3%, 61.1%, and 15.4% in cattle, camel, sheep and goats, respectively. Infection rate was 23.8%, and 32.6%, in West Kordofan and Blue Nile states, respectively (Table 2).

To examine the associated risk factors for *T. gondii* seropositivity, geographic location, gender and animal species were examined. The seroprevalence was significantly higher ($P < 0.0001$) in sheep (OR (95% CI) = 0.12, 0.05 - 0.3) and cattle (OR = 0.23, 0.1 -

0.4) in comparison with goats (Table 2). Nonetheless, no significant difference was discerned in relation to the gender (P = 0.8) and location (P = 0.1) (Table 2) and *Toxoplasma* seropositivity.

Table 2. Analysis of the effect of animal species, gender and location on the distribution of *T.gondii* inSudan.

Factor	Number of tested	Seroprevalence		Odds ratio (95% confidence interval)	*p*-value
		Positive number (%)	Negative number (%)		
Species					
Goats	136	21 (15.4)	115 (84.6)	Reference	
Sheep	36	22 (61.1)	14 (38.9)	0.12 (0.05 - 0.3)	< 0.0001
Camel	45	6 (13.3)	39 (86.7)	1.19 (0.5 - 3.2)	0.8
Cattle	75	33 (44)	42 (56)	0.23 (0.1-0.4)	< 0.0001
Gender					
Female	260	74 (28.5)	186 (71.5)	Reference	
Male	32	8 (25)	24 (75)	1.19 (0.5-2.8)	0.8
Location					
West Kordofan	151	36 (23.8)	115 (76.2)	Reference	
Blue Nile	141	46 (32.6)	95 (67.4)	0.65 (0.4 -1.1)	0.1

DISCUSSION

The absence of epidemiological data concerning toxoplasmosis in West Kordofan and Blue Nile states warranted more investigation. Our survey showed a *T. gondii* seropositivity of 13.3%, 44%, 61.1%, and 15.4% in the examined camel, cattle, sheep and goats, respectively. This is the first report about toxoplasmosis in farm animals in West Kordofan and Blue Nile states, Sudan and the results are comparable with other states in Sudan. By comparison, the current seropositivity of *T. gondii* in camels (13.3%) was relatively similar to that in a previous serosurvey conducted in Gedarif state in which seropositivity was 14.6% (Jomaa et al. 2017). Our seroprevalence results in camel were lower than those reported with other investigators who showed prevalence in camels was 76% in Butana plains, 44% in Khartoum and 20% in Butana plains (Elamin et al. 1992; Khalil and Elrayah, 2011; El-Bashier et al. 2012), respectively.

Conversely, the infection rate of *T. gondii* in cattle in the current study (44%) was higher than those reported in River Nile (6.2%), Gazira (14.9%) and Khartoum states (12.7%, 32%, 11.9%) (Lazim et al., 2018; Elfahal et al., 2013; Khalil and Elrayah, 2011; Medani and Kamil, 2014), respectively.

In sheep, our results demonstrated a prevalence of 61.1%, which was relatively close to that in previous two reports where the prevalence of toxoplasmosis in sheep was 57.5% in Khartoum and El-Gadarif states (Khalil and Elrayah, 2011; Atail et al., 2017). In addition, our results were higher than those observed in previous investigations; 40.9% at Khartoum

state, and 26.5% at River Nile state (Medani and Kamil, 2014; Lazim et al., 2018), respectively. Our results in goats showed that seroreactivity was 15.4% which were lower than those reported by previous studies; 46.5% at El-Gadarif state and 27.9% in River Nile state (Atail et al., 2017; Lazim et al., 2018), respectively.

The above-mentioned differences in *T. gondii* infection rates may be because of variations in geographical locations, serologic assays utilized, climatic conditions, animal species, sample size and sampling time (Fereig et al., 2016; Zhang et al., 2016; Abdelbaset et al., 2020).

Among the analyzed risk factors, the animal species represented the only associated risk factor for *T. gondii* seropositivity. Seroprevalence was significantly higher in sheep and cattle compared to the goats. These results were in the same line with previous reports that revealed sheep have a high susceptibility to *Toxoplasma* infection (Khalil and Elrayah, 2011; Fereig et al., 2016). These results also point out the importance of sheep and cattle in the transmission of human toxoplasmosis in Sudan, particularly when considering the Sudanese people's habit of consumption of partially cooked meat, liver, and viscera or unpasteurized milk (El-Hassan et al., 1991). Regarding the gender and geographical location, no statistical significance was observed. These findings were similar to other reports in which no significant relationship was noticed between these risk factors and *T. gondii* seropositivity (Elfahal et al., 2013; Fereig et al., 2016).

In a conclusion, this is the first report investigating the prevalence of *T. gondii* infection in camel, cattle, sheep and goats in west Kordofan and Blue Nile states in Sudan. The remarkable high prevalence of *T. gondii* antibodies in sheep and cattle suggests their potential role in the transmission of human toxoplasmosis in Sudan. Further epidemiological studies, as well as special awareness and educational programs for toxoplasmosis are urgently needed. Additional studies may determine the overall impact of toxoplasmosis on the livestock economy

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that the work described has not been published previously and that it is not under consideration for publication elsewhere. All the authors approve the publication of this manuscript and that, if accepted, it will not be published elsewhere in the same form, in English or any other language, including electronically without the written consent of the copyright holder.

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Tolerance of *Colpoda cucullus* Nag-1 wet resting cysts to extreme pH (pH 1 and 13): Implications of less permeability of the cyst membrane to H^+ and OH^-

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ABSTRACT

In parasitic unicellular eukaryotes, the tolerance to the pH (1-1.5) of gastric acid is a crucial survival strategy so that they can proliferate in the intestinal tract. We found that the resting cysts of non-parasitic soil ciliate *Colpoda cucullus* Nag-1 showed a strong tolerance to both extremely low and high pH. The purpose of this study was to explore the tolerance mechanism to extreme pH. Most cysts were alive after exposure to 0.1 M HCl (pH 1) for 4 h, or after exposure to 0.1 M NaOH (pH 13) for 3 h. Such tolerance to extreme pH is acquired gradually over several days after encystment induction. The resting cysts were reversibly dehydrated by osmotic pressure when they were transferred from water to 0.1 M HCl or 0.1 M NaOH. This result suggests that H^+/Cl^- and Na^+/OH^- may diffuse through the cyst wall to reach the plasma membrane. Acid tolerance was reduced in the presence of protonophore (CCCP), suggesting that less permeability of the cyst plasma membrane to H^+ may be responsible for acid tolerance.

Keywords: *Colpoda*; resting cysts; tolerance to extreme pH

INTRODUCTION

The formation of dormant forms or resting cysts tolerant to desiccation (Corliss and Esser, 1974), low temperature (Taylor and Strickland, 1936) or UV rays (Lonnen, *et al*., 2014; Matsuoka *et al*., 2017; Yamane *et al*., 2020) is an adaptive strategy for the survival of unicellular eukaryotes in terrestrial environments. Vegetative forms of terrestrial unicellular

eukaryotes such as the ciliate *Colpoda* promptly transform into resting cysts when they detect approaching desiccation. When favorable aquatic environments are recovered, they excyst to emerge from the resting cysts, and grow rapidly.

When *Colpoda cucullus* Nag-1 vegetative cells are induced to encyst by being suspended in a Ca^{2+}-containing medium at high cell density (Matsuoka *et al*., 2009), the cells become rounded within 2-3 h, and mucus is expelled into the extracellular space. Subsequently, small sticky globules called lepidosomes (Foissner *et al*., 2011) are extruded, trapped in a mucus layer, and form a mucous/lepidosome layer (Funatani *et al*., 2010; Funadani *et al*., 2016). The spherical cells are surrounded by a single rigid layer (ectocyst layer) in about 3 h, followed by the formation of the first-synthesized endocyst layer just beneath the ectocyst layer. Then, the mitochondrial membrane potential disappears (arrest of mitochondrial activity) (Funatani *et al*., 2010; Sogame *et al*., 2014), and autophagosomes form to digest cellular components of the vegetative cell at 3-5 h. The autophagy continues up to 24 h after encystment induction. The endocyst layers continue to form, and several layers are formed over several days, thereby creating a cyst wall composed of a mucus/lepidosome layer, ectocyst layer and endocyst layers (from outside) (Funatani *et al*., 2010). Many presumed carbohydrate grains accumulate in the cytoplasm over several days, and cilia and kinetosomal structures disappear in mature (over 1-week-old) cysts (Funatani *et al*. 2010).

We previously reported that the wet resting cysts of *C. cucullus* Nag-1 show a tolerance to HCl; most cysts survive in 0.1 M HCl (pH 1), for at least 1 h (Sogame *et al*., 2011). The tolerance to extremely low pH is a crucial survival strategy for parasitic unicellular eukaryotes in the stomach acidic pH (pH 1-1.5) of host animals, so that they can proliferate in the intestinal tract. For example, the vegetative forms (trophozoite) of the free-living pathogenic *Entamoeba histolytica* transform into cystic or infective form, which can tolerate the stomach acidic environment, and excysts in the terminal ileum (Serrano-Luna *et al*., 2013). The cysts of parasitic unicellular eukaryote *Giardia lamblia* also pass through stomach gastric acid alive (Hawrelak, 2003). *Acanthamoeba* that infects the cornea of the eye to cause *Acanthamoeba* keratitis is also resistant against 80 mM HCl, even though it does not infect the gastrointestinal tract (Lloyd, 2014). Whether parasitic or free-living, acid tolerance seems to be a common strategy of unicellular eukaryotes for survival in host animal intestines.

In the present study, we found that wet resting cysts of *C. cucullus* Nag-1 survived at least 3-4 h when they were exposed to both extremely low pH (pH 1) and high pH (pH 13). We discuss the tolerance mechanism of *Colpoda* resting cysts against extreme pH.

MATERIALS AND METHODS

Cell culture and encystment induction

Vegetative cells of *Colpoda cucullus* Nag-1 (Funadani *et al*., 2016) (18S ribosomal RNA gene: GenBank Accession No. AB918716) were cultured in a 0.05% (w/v) dried wheat leaves infusion. Two-day cultured vegetative cells were collected by centrifugation (1,500 × *g*, 2 min), and then suspended at a high cell density (> 5, 000 cells/mL) in an encystment-

inducing medium containing 0.1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2). The cell suspension (approximately 1,000 cells in 200 μL suspension) was dispensed in watch glasses under humid conditions. The resting cysts adhered to the bottom of the watch glass.

Treatment with HCl, NaOH and NaCl solutions

Prior to treatment with test solutions, the encystment-inducing medium in the cyst-adhered watch glass was discarded, washed with water 2-3 times, and then the watch glass was refilled with 300 μL of test solutions. After a certain treatment time, the test solutions were again discarded, washed with running water for 10 min, and a fresh 0.05% wheat leaves infusion was poured in to induce excystment (Watoh *et al*., 2003; Tsutsumi *et al*., 2004). Cysts were randomly chosen, and the number of vacant cysts from which vegetative cells had emerged was counted 24 h after excystment induction. The rates of excystment (Figs. 1, 2, 4) and viability (%) of vegetative cells (Fig. 2) were expressed as the percentage of the total number of observed cells (50 cells). Points and attached bars correspond to the means of 5 (Figs. 1, 2) or 6 (Fig. 4) measurements and their standard errors (SE), respectively. In each measurement set (HCl, NaOH and NaCl in Figs. 1, 2) and each series of measurement (HCl/CCCPs or NaOH/CCCPs in Fig. 4), the same lot samples were used. In Fig. 3, statistical analysis was performed using t-test. *P* values < 0.01 were considered significant.

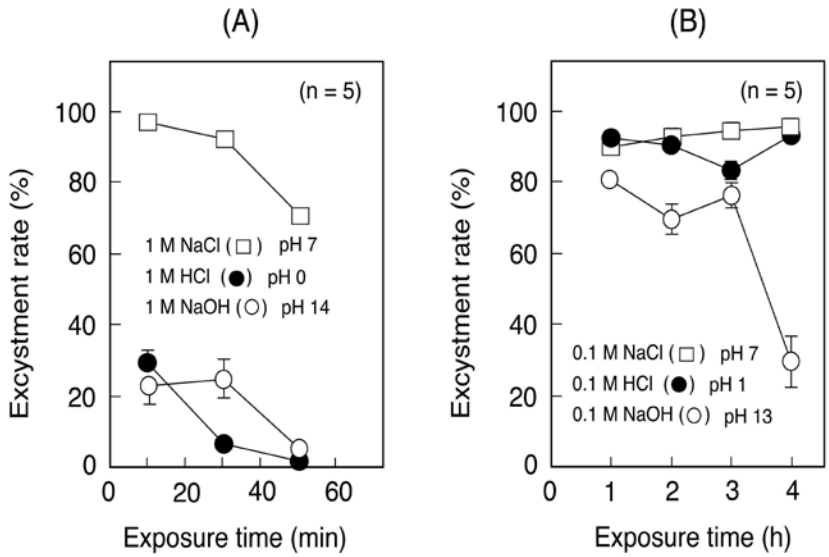

Fig. 1. Tolerance of mature (1-week-old) resting cysts of *C. cucullus* Nag-1 to 1 M (A) and 0.1 M (B) HCl and NaOH test solutions. Ordinate and abscissa indicate excystment rates (%) and the time cysts were exposed to the test solutions.

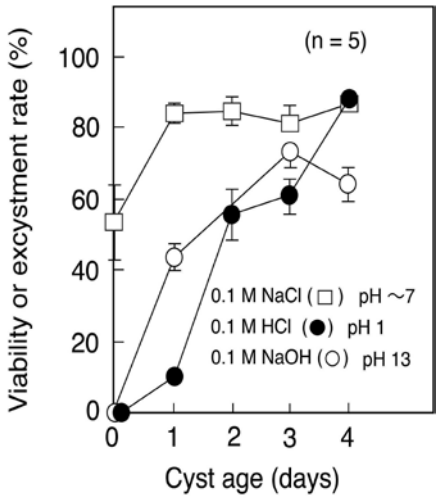

Fig. 2. Tolerance of *C. cucullus* Nag-1 encysting cells at various ages to 0.1 M HCl and 0.1 M NaOH test solutions. Ordinate indicates excystment rates (%) of immature cysts age 1 day or more or viability (%) of vegetative cells that were exposed to the test solutions for 1 h. Abscissa indicates the cyst age of encysting cells. Cyst age '0 day' is the vegetative cells first suspended in encystment-inducing medium and subsequently in the test solutions. In the measurements of viability of vegetative cells, a single lot of 50-cell samples of vegetative cells was prepared using a thin glass pipette.

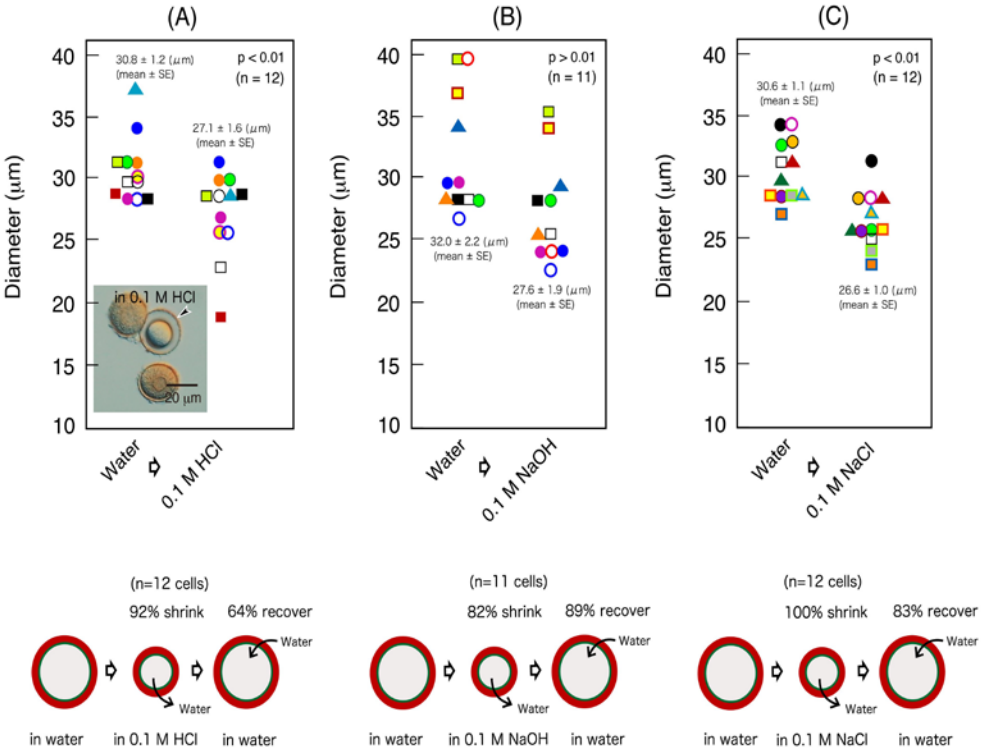

Fig. 3. Osmotic shrinking of *C. cucullus* Nag-1 wet resting cysts (1-week-old) under hypertonic condition caused by 0.1 M HCl (A); 0.1 M NaOH (B); and 0.1 M NaCl (C). The resting cysts adhered to the bottom of watch glasses were first immersed in water for 5 min to measure their diameter (left points in each figure). Thereafter, water was replaced by 0.1 M of each test solution (HCl, NaOH and NaCl), and diameter of the cyst was measured after 5 min (right points in each figure). Each point in each figure represents the diameter of each cyst's cell body, and a set of data markers with the same color and shape indicates the values obtained from the same cyst. One run of measurement in each experiment [(A) water → HCl, (B) water → NaOH, (C) water → NaCl] was done in the same cell. (A) Inset photograph. The cysts immersed for 5 min in 0.1 M HCl, and then returned to water for 5 min. In the cyst indicated by the arrowhead, cell body remains shrunk and detached from cyst wall, which is killed.

In the schematic diagrams, the 'shrink or recover (%)' indicates the percentage of the cysts which shrank by at least 10% in diameter after soaking for 5 min in the test solutions (0. 1 M HCl, 0.1 M NaOH and 0.1 M NaCl), or which recovered by at least 90% after soaking for 5 min in water. The values are expressed as the percentage of total number of observed cells (11-12 cells). One run of measurement (water → test solutions → water) was done in the same cell.

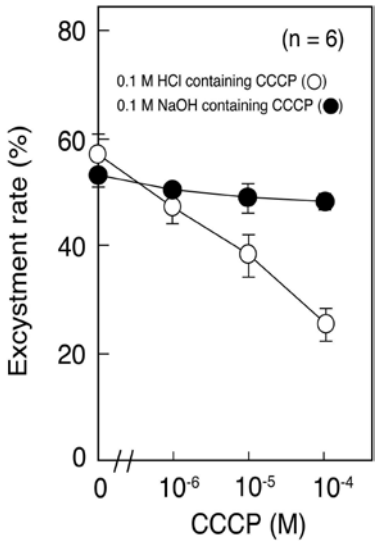

Fig. 4. Tolerance of mature (1- to 2-week-old) resting cysts of *C. cucullus* Nag-1 to 0.1 M HCl (open circles) and 0.1 M NaOH (closed circles) solutions containing various concentrations of CCCP and 0.1% DMSO. The cysts were exposed to each test solution for 1 h.

Protonophore treatment

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Wako Pure Chemical Industries) was dissolved in dimethyl sulfoxide (DMSO) to give 100, 10 and 1 μM stock solutions, and 1 mL of each stock solution was added to 1 mL volumes of test solutions to produce final concentrations of test solutions of 10^{-4} M, 10^{-5} M and 10^{-6} M, respectively (final concentration of DMSO at 0.1%).

RESULTS AND DISCUSSION

When mature (1-week-old) resting cysts of *C. cucullus* Nag-1 were exposed to 1 M HCl (pH 0) or 1 M NaOH (pH 14) solution for 10-30 min, 20–30% of the cysts survived (Fig.

1A). When the cysts were exposed to 0.1 M HCl (pH 1) and 0.1 M NaOH (pH 13), most cysts were alive at least for 4 h and 3 h, respectively (Fig. 1B). Such tolerance to extreme pH is gradually acquired over several days after encystment induction (Fig. 2).

In a previous study (Sogame *et al*., 2011), we suggested that the tolerance of *Colpoda* cysts to HCl was possibly acquired by preventing its diffusion across the endocyst layers. If H^+/Cl^- and Na^+/OH^- could diffuse through the cyst wall to reach the plasma membrane, it would be reasonable to expect the cell body of cysts to be dehydrated by osmotic pressure, because the osmolality of the cytosol of *C. cucullus* Nag-1 wet resting cysts is estimated to be below 0.052 Osm/l (Matsuoka *et al*., in press). Actually, the diameter of resting cysts transferred from water into 0.1 M HCl (Fig. 3A) or 0.1 M NaCl (Fig. 3C) decreased significantly (t-test; $p < 0.01$). When the cysts were exposed to 0.1 M NaOH, their mean diameter was reduced (Fig. 3B), although there was not a significant difference between the columns labeled with 'water' and with '0. 1 M NaOH' (t-test; $p > 0.01$). A large number of cysts were reversibly dehydrated, as shown in the schematic drawings in Fig. 3. On the other hand, some of the cysts that had been exposed to HCl or NaOH, and then returned to water remained shrunk. In some cases, the cell body was detached from the cyst wall as shown in the inset photo in Fig. 3A (arrowhead). In this case, selective permeability of the plasma membrane may be destroyed. These results suggest that H^+/Cl^- and Na^+/OH^- may diffuse through the cyst wall to affect the plasma membrane.

The membrane conductance of H^+/OH^- ($G_{H/OH}$) through the phosphatidylethanolamine planner membrane was only elevated 4-fold when H^+ concentration increased from 10^{-7} M to about 10^{-1} M (Gutknecht, 1987). This indicates that the lipid bilayer may not be destroyed even under extreme pH. In acidophiles, one adaptation strategy against extreme acidic pH is impermeability of the cell membrane, which restricts the influx of H^+ into the cytoplasm (Mirete *et al*., 2017). Judging from these previous findings, it is likely that less permeability to H^+ and OH^- may be responsible for the tolerance of *C. cucullus* Nag-1 resting cysts to extreme pH. Actually, our preliminary measurements by means of BCECF-AM [(2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester)], fluorescent probe that enables monitoring of cellular pH showed that intracellular pH of *Colpoda* cysts suspended in 0.1 M HCl solution dropped to only 5.8.

If the restriction of permeability of *C. cucullus* Nag-1 cyst membrane to H^+ were responsible for acid tolerance, an elevation of H^+ permeability of the resting cysts by the addition of protonophore (CCCP) could be expected to lose tolerance to extreme pH. Actually, acid tolerance was reduced in a CCCP-concentration dependent manner when the mature cysts were exposed to 0.1 M HCl for 1 h in the presence of CCCP (Fig. 4). On the other hand, the tolerance of cysts to 0.1 M NaOH was not diminished in the presence of CCCP (Fig. 4).

When the cysts were immersed in 0.1 M NaOH solution containing CCCP, H^+ could diffuse from the cell interior to extracellular space by the action of H^+ carrier CCCP, and it is expected that intracellular H^+ concentration drops while OH^- is elevated by the dissociation of intracellular H_2O to keep the ionic product of water at 10^{-14} $(mol/L)^2$. One of the reasons why cysts' tolerance to 0.1 M NaOH was not reduced is that the H^+ carrier CCCP may barely diffuse into the cell interior. Even if CCCP were to sufficiently diffuse into the cell interior,

H^+-transport efficiency from cell interior to exterior may be lower compared to the opposite (from exterior to interior), because the association probability of CCCP and H^+ in cytosol (10^{-7} M H^+) is 10^6-fold lower than that in the surrounding medium (10^{-1} M H^+).

As shown in Fig. 1B, the tolerance of mature cysts to 0.1 M NaOH was suddenly reduced at 4 h. In this case, an inflow of OH^- may have caused the elevation of intracellular OH^- concentration and reduction of H^+, because conductance of artificial membrane to OH^- increases in the high pH range (> pH 11) (Gutknecht and Walter, 1981).

Even in the presence of 10^{-4} M CCCP and 0.1 M HCl, some cysts survived (Fig. 4). This implies that the cyst plasma membrane is still less permeable to H^+ even in the presence of CCCP. Otherwise, the acid tolerance of the *C. cucullus* cysts might be partly attributed to intracellular buffering capacity.

The lower permeability of the *Colpoda* cyst membrane to H^+ or OH^- might be attributed to the absence of an ion channel on the plasma membrane due to the silencing of most genes expressed in vegetative cells. This is because an abrupt influx or efflux of H^+ or OH^- possibly occurs through denatured ion channels or water channels under extreme pH environments. When *C. cucullus* Nag-1 vegetative cells are induced to encyst, the amount of total mRNA contained in the cells abruptly decreases within 3 h to reach about 25% of the initial level at 5 h after onset of encystment. The amount of total proteins begins to decrease in 12 h, and reaches about 25% 1 day after encystment induction (Sogame *et al*., 2014). *Colpoda* cysts begin to acquire tolerance to extreme pH within 1 day of the start of encystment (Fig. 2). These results are not inconsistent with above-mentioned idea.

We note here that there is an association between the lipid composition of the plasma membrane and acid tolerance in some archaea (Mirete *et al*., 2017). During encystment of the unicellular parasite *Giardia lamblia*, lipid composition is altered (Ellis *et al*. 1996). It is also likely that lipid composition of the plasma membrane of *C. cucullus* Nag-1 cysts may be altered, so that membrane permeability to H^+ and OH^- may drop extensively.

The tolerance of *C. cucullus* Nag-1 against 0.1 M HCl implies that this organism may survive in the gastric juices of animals, and may enable cysts to survive in the gastrointestinal tract. It is likely that the acid tolerance of *C. cucullus* Nag-1 may be related to their widespread distribution through animals, as already suggested by Sogame *et al*. (2011).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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