Seropepidemiological study of *Toxoplasma gondii* and *Neospora caninum* in livestock in Mongolia and characterization of antiprotozoal compounds from soil bacteria in Mongolia

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モンゴルの家畜における
トキソプラズマ・ゴンディと
ネオスポラ・カニナムの血清疫学調査および
モンゴルの土壌細菌由来抗原虫化合物の解析

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Contents **Pages**

Abbreviations

A

B BALB/C - Inbred strain of mouse

BCA - Bininochoninic acid

BLAST - Basic Local Alignment Search Tool

BSA - Bovine serum albumin

C CCK 8 - Cell counting kit 8

D DNA - Deoxyribonucleic acid

DMEM - Dulbecco's modifies Eagle's medium

DMSO - Dimethyl sulfoxide

Dpi - Days post infection

E ELISA - Enzyme linked immunosorbent assay

F FBS - Fetal bovine serum

G GRAs - Protein secreted from dense granule organelles

GST - Glutathione S-transferase

H HFF - Human foreskin fibroblast

HRP - horseradish peroxidase

I IC_{50} - the half maximal inhibitory concentration

iELISA - indirect ELISA

Ig - Immunoglobulin

IgG - Subtype of immunoglobulins

K kDa - Kilodalton

L LAT - Latex agglutination test

M MEM - Minimum essential medium eagle

O OD - Optical density

OR - Odd ratio

P PBS - Phosphate-buffered saline

PBS-SM - PBS containing 3% skimmed milk

PCR - Polymerase chain reaction

PO - Per oral route

R RH-GFP - A green fluorescent protein expressing RH strain

RNA - Ribonucleic acid

rRNA - Ribosomal RNA

RT - Room temperature

S SI - Selectivity index

SDS-PAGE - Sodium dodcyl sulfate- polyacrylamide gel electrophoresis

16S rRNA -16S ribosomal RNA

Unit abbreviations

µl -Microliter

ml - Milliliter

L - Liter

µm - Micromolar

nM - Nanomolar

mg/kg - Milligram/kilogram

mg/ml - Miligram/ milliliter

µg/ml - Microgram/ milliliter

ng/ml - Nanogram/ milliliter

General introduction

1. Toxoplasmosis

1.1. Ultastructure and life cycle of *Toxoplasma gondii*

Toxoplasmosis caused by *Toxoplasma gondii* which is an intracellular protozoan parasite classified in the phylum *Apicomplexa*, capable to infect in various warm-blooded animals including man. There are characterized by three infectious stages: tachyzoites, bradyzoites and sporozoites. The all stages are involved in complex life cycle. Tachyzoite are crescent or oval shaped, approximately 2 to 6 µm wide and 4 to 8 µm long which contain various organelles such as apical rings, polar ring, conoid, rhoptries, micronemes, micropore, mitochondrion, endoplasmic reticulum, Golgi complex, dense granules and apicoplast. The actual functions of micronemes, conoid, rhoptries and micropores are not fully understand but they are associated with parasite growth, invasion and development in the host cell (Dubey et al., 1998, Remington et al., 2011). The rhopteries are club-shaped, secretory unique organelles play major role in *T. gondii* penetration, host cell interaction and also their contents are associated with the moving junction during the invasion (Dubremets, 2007). Also, the micronemes, secretory organelles localize in the apical tip of the parasites which has a crucial role in the host cell invasion and motility (Soldati et al., 2001). The apicoplast is an endosymbiotic organelle has been found in most of the apicomplexan parasites including *T. gondii* and *Plasmodium* spp., which has an essential function for the survival of parasites (Lim et al 2010). Also, the dense granules are secretory organelles of *T. gondii* that characterized protein for the structural modifications of the parasitophorous vacuole (PV) (Cesbron-Delauw, 2003).

The *T. gondii* has a complex life cycle as a sexual cycle in the final host belongs to the domestic cats and other family of the of *felidae*. Asexual stages can develop in all warm-blooded animals including most livestock and human that can serve as an intermediate host of *T. gondii* (Tender et al., 2002, Dubey 2010). The definitive host can acquire *T. gondii* infection ingesting by three stages (tachyzoites, bradyzoites and oocyst). Tachyzoites are rapidly replicating form which can transmit infection (Halonen and Weiss 2014). Bradyzoites are slowly multiplying stage found in tissue cysts in muscle and brains of the infected animals. Oocysts are around 10 to 12 μm in diameter which have walls with two layers and sporulation found in the outside of the cat after excretion 1 to 5 days (Dubey et al., 1998). Sporulated oocysts excrete by the definitive host's feces and cause wide-ranging environmental contamination (Hill et al., 2002, Dubey 1996).

1.2. Pathogenesis and diagnosis

T. gondii infection characterized by major categories: acquired toxoplasmosis, congenital toxoplasmosis, ocular toxoplasmosis and cerebral toxoplasmosis (Halonen and Weiss, 2013). Humans can become infected in most cases of toxoplasmosis by ingestion of tissue cyst in infected meat and oocyst in contaminated water, vegetables and foods with cat's feces. Toxoplasmosis causes adversely affects in immunocompromised hosts and in newborns with congenital toxoplasmosis. The congenital *T. gondii* infection widely occur through vertical transmission and its recognized public health importance due to the severe damage to the fetus such as stillbirth and death (Remington et al., 1994). The active infection becomes chronic stage of infection and tissue cyst are mostly formed in brain, muscles, liver and eye (Pavesio and Lighman, 1996).

The diagnosis of toxoplasmosis is beneficial for the control and prevention as an important zoonosis in the world. Therefore, many detection approaches such as microscopic, bioassay,

serological, molecular and imaging technique have been developed and improved (Liu et al., 2015). Clinical diagnostic approaches of infection have been done by the serological methods including indirect hemagglutination test (IHA), indirect fluorescent antibody test (IFAT), enzyme linked immunosorbent assay (ELISA), latex agglutination test (LAT), immunochromatographic test (ICT) and avidity test to detect different antibodies and antigens. The molecular approaches for detections of *T. gondii* as follows: conventional PCR, real time PCR, loop-mediated isothermal amplification (LAMP) and microsatellite analysis. Diagnosis of *T. gondii* based on molecular methods is appropriate technique due to the rapidly and high sensitivity, specificity in clinical samples (Switaj, 2005). The LAT is also a widely used simple test and available to detect IgG antibodies against *T. gondii* (Hill et al., 2002).

1.3. **Transmission**

Toxoplasma infection is transmitted by three ways: from mother to fetus (congenital transmission), consumption of undercooked meat containing tissue cysts and ingestion of food or water contaminated with oocysts (Hill and Dubey, 2002). Raw or undercooked meat from infected animals is potentially hazardous if ingested by humans or other animals. Consuming contaminated water, unwashed vegetables, fruits and environmental contamination directly cause *T. gondii* infection in human (Hill and Dubey 2016).

1.4. **Epidemiology**

T. gondii is a most successful protozoan parasite that infects virtually all warm-blooded animals, including man, livestock and marine mammals. *T. gondii* infection distributed worldwide.

Human infection rates are influenced by climate, geographical condition, food consumption such as raw and undercooked meat and presence of cats. In humans, *T. gondii* infection presents all part of the world and approximately 25 to 30% of the world's human population have been infected (Montoya et al., 2004). Additionally, many studies have been conducted to investigate the seroprevalence of *T. gondii* in livestock in different area of the world (Dubey, 2009).

1.5. **Treatment**

T. gondii infection in immunocompetent hosts usually shows asymptomatic and medication is rarely required. Currently, several drugs are available for the medication of toxoplasmosis. Recommended regimens during toxoplasmosis: pyrimethamine used in combination with sulfonamide and administration of folic acid which can prevent bone marrow from the toxic effects of pyrimethamine (Halonen and Weiss, 2014). However, combination of pyrimethamine and sulfadiazine therapies cause high rates of toxic side effects leading to discontinuation of therapy (Alday and Doggett, 2017). Sulfadiazine can be replaced with clindamycin or azithromycin for patients who have an allergy to sulfa drugs (Andrews et al., 2014).

2. Neosporosis

2.1. Ultrastructure and life cycle of *Neospora caninum*

Neosporosis caused by intracellular tissue cyst-forming coccidian parasite *Neospora caninum* belong to the phylum *Apicomplexa*, which is a *Toxoplasma*-like organism. *N. caninum* has been identified as a serious disease in cattle and dogs in a number of counties all around the world. *N. caninum* infection causes huge economic losses in animal industries worldwide. The

main problems of *N. caninum* infection are considered by abortion, stillbirths and neonatal mortality in cattle (Dubey et al., 2007). Dogs are definitive hosts of *N. caninum* infection (McAllister et al., 1998). Recently, other canids, such as the Australian dingo (*Canis lupus dingo*) (King et al., 2010), coyote (*Canis latrans*) (Gondim et al., 2004), and gray wolf (*Canis lupus*) (Dubey et al., 2011) are reported as the definitive hosts and oocysts are shed in their feces. Tachyzoites are measured around 2 × 6 μm in diameter. Ultrastructurally, *N. caninum* and *T. gondii* are similar and parasite's zoites containing apical ring, polar ring, a conoid, micronemes, rhoptries, mitochondria, a nucleus, Golgi complex, ribosomes, dense granule proteins, lipid bodies, vesicles and endopasmic reticulum (Speer et al., 1999).

N. caninum is recognized by complex heteroxenous life cycles involved in three known infectious stages: tachyzoites, bradyzoites and sporozoites. The tachyzoites and bradyzoites are found in the tissues of infected animals. The tissue cysts are found in muscle and brains of the infected animals. The oocysts are environmentally resistant stage of the parasites excreted into definitive host's feces, and maturation of oocysts containing four sporozoites occurs within 24 h under suitable condition (Dubey et al., 2006, Dubey et al., 2007, Lindsay et al., 1999).

2.1. Pathogenesis and diagnosis

Abortion, stillbirth and neuromuscular disorders are the major clinical signs of infected animals, but the pathogenesis of neosporosis is complex (Dubey et al., 2006). Abortion is an important issue in livestock industries in the word. In addition, asymptomatic congenital infections are mainly occurred. Several diagnostic assays have been employed for *Neospora* diagnosis, such as histopathology, ultrastructural analysis, serological techniques and specific DNA using PCR-based molecular techniques (Dubey et al., 2007, Donahoe et al., 2015).

2.2. Transmission

Currently, *N. caninum* can be transmitted by both horizontal and vertical routes of infection. The horizontal transmission occurs when animals ingested sporulated oocyst and vertical transmission is considered from the infected dam to the fetus via the placenta The exogenous transplacental transmission and endogenous transplacental transmission have been described as a route of infection of the fetus. Until now, cattle to cattle transmission of *N. caninum* infection has not been documented (De Marez et al., 1999; Dubey et al., 2006).

2.3. Epidemiology

Many study indicated that a wide range of domestic and wild animals have been exposed to *N. caninum* and also viable isolates has been found in some hosts (Dubey and Schares, 2011). The seroprevalence of *N. caninum* was reported in dogs, dairy cattle, domestic animals, wildlife and zoo animals in the worldwide (Dubey et al., 2007). Humans are not considered as the intermediate host of *N. caninum* (McCann et al., 2008). However, anti-*N. caninum* antibodies were detected in humans in some areas (Ibrahim et al., 2009; Tranas et al., 1999).

2.4. Treatment

The control strategies such as chemotherapeutic agents are important in order to decrease economic impact of neosporosis. However, there are no approved effective drugs for neosporosis in cattle (Dubey and Schares, 2011). Currently, drugs which can be prevent abortion or transmission from dam to fetus are not available. Some chemotherapeutic agents including

toltrazuril (Strohbusch et al., 2009) and artemisinin (Kim et al., 2002) show exhibition activity against *N. caninum* infection.

3. Malaria

3.1. Ultrastructure and life cycle

Millions of people are infected by malaria parasites annually and many people die. Therefore, eradication of malaria has been becoming global problem all over the world. Human malaria is caused by five species of *Plasmodium* such as *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*. *P. falciparum* is the most pathogenic species due to its highest mortality rate, whereas *Plasmodium knowlesi* is the fifth zoonotic malaria parasite, capable to infect in *Macaca fascicularis* as a natural vertebrate host (WHO, 2017; Cox-Singh et al., 2008).

The protozoan parasite *P. falciparum* characterized by developmental stages: asexual blood stage and sexual gametocyte (Krungkrai et al., 2000). The human is infected by bloodsucking with the infected female *Anopheles* mosquito and *Plasmodium* sporozoites quickly migrate into the human liver. The sporozoites asexually multiply in the liver and differentiate into schizonts containing thousands of hepatic merozoites. The merozoites are release into the blood stream and invade new erythrocytes for their multiplying. Moreover, the merozoite develop into the sexual multiplication cycle (gametocytes) which is called a gametogenesis (Soulard et al., 2015). Then, the gametocytes are transferred into the mosquito vector via bloodsucking. *P. falciparum* gametocytes become to mature after 8 to 10 days and the life cycle continued in female *Anopheles* mosquito (McRobert et al., 2008).

3.2. Pathogenesis and diagnosis

A man who infected with malaria parasite may have a flu-like symptoms. The associated symptoms are including fever, headache, nausea, shaking chills, weakness, muscular pain and spleen enlargement. Anemia is commonly occurred in patients with malaria (Mawson, 2013). In case of *Plasmodium knowlesi*, gastrointestinal, abdominal pain, cough and diarrhea are additionally reported (Singh et al., 2013).

In malaria, prompt and accurate diagnosis is the important disease management. The diagnosis of malaria has been relied on parasite detection by microscopy, molecular detection and antigen-based rapid diagnostic tests (RDT) (WHO, 2000). Microscopy detection of malaria parasite is mostly used method which has advantages with rapid, quantitative and sensitive. Detection of the human malaria parasites using molecular methods has been developed and established since the 1990s. Molecular methods such as a nested PCR, real-time PCR and LAMP have been shown sensitively and potential detection. The RDT detects malaria antigen in a small amount of blood and the principle of immunochromatography has opened up possibility of a more rapid and labor-intensive. RDT of malaria parasite is specific for histidine-rich protein 2 (HRP-2), lactate dehydrogenase (pLDH) and aldolase. The pLDH-based RDT is able to differentiate *P. knowlesi* from *P. malariae* and *P. ovale*, it cross-reacts with both *P. falciparum* and *P. vivax* specific pLDH antibodies and therefore it cannot be used to differentiate between *P. knowlesi* and mixed infections of *P. vivax* and *P. falciparum*. (McCutchan et al., 2008, Moody et al., 2000, Lee et al., 2002).

3.3 Transmission

Malaria is a life-threatening disease caused by *Plasmodium* sp. that are transmitted by the bite of an infective female *Anopheles* mosquito. The malaria transmission process in complex. Mosquito to human transmission occurs when sporozoites from the salivary gland of the mosquito are injected during their blood feeding. There are more than 400 different species of the *Anopheles* mosquito and around 70 of species have been recognized as potential major vectors (Sinka et al, 2012). The mature gametocytes of *Plasmodium* are the essential stages for malaria that are able to mediate the transmission from human to the mosquito. The parasite's development starts when the *Anopheles* mosquito ingested an infected blood meal that containing gametocytes, known as *Plasmodium* sexual form (Smith et al., 2014). *Plasmodium* gametocytes can be divided into 5 morphological recognizable stages (I-V) and only mature stage (V) are released in the blood stream and ability to circulate (Baker, 2010).

3.4. Epidemiology

Annually, over 200 million cases of malaria have been reported by World health organization (WHO) and 655,000 deaths are caused by malaria in 2010. According to the World malaria report in 2017, the infection rates are increasing. There are 219 millions of people infected by malaria parasites in 87 countries and 435,000 number of people die caused by this infection (WHO, 2017). Thus, eradication of malaria has been becoming global problem all over the world. The highest malaria cases are reported in African region estimated by 200 million cases (around 92%). On the other hand, malaria prevalences is 5% and 2% in South-East Asia Region and Eastern Mediterranean Region, respectively (World Malaria report, 2018).

3.5. Treatment

Antimalarial drugs play essential role for malaria control. Currently, chloroquine, sulfadoxine-pyrimethamine and artemisinin-based combination therapies (ACTs) are recommended for treatment in most areas of the World. The WHO recommends artemisinin-based combination therapies (ACTs) for the treatment which consists of potent drugs such as artemether/lumefantrine, artesunate / amodiaquine, artesunate / mefloquine, dihydroartemisinin / piperaquine, artesunate / pyronaridine, and artesunate / sulfadoxine–pyrimethamine (Cui et al., 2015). All schizontocidal antimalarial drugs are currently employed in clinical practice in patient infection with *P. knowlesi*. However, increasing resistance of parasites to standard drugs has been widely emerging in South Asian countries (Ashley et al., 2014). Therefore, it is urgently needed to discover novel drugs against malaria.

4. Natural products

Natural products play an important role as major source of bioactive compounds for the development of new chemotherapies against infectious in human diseases (Lahlou, 2017). The natural products come from various sources including animals, plants, marine organisms, and microorganisms (Barka et al., 2016). Many drugs derived from natural products have been used for the treatment of life-threatening diseases including parasitic diseases such as malaria and toxoplasmosis. The biological active compound from plant resources like alkaloids, terpenes and phenolics have been considered for their antiparasitic properties (Kayser et al., 2003). Many plant extracts and fractions show anti-*Toxoplasma* activity (Sepúlveda-Arias et al., 2014).

One of the important natural sources with potential bioactive compounds are the microorganisms. Nowadays, discovery of bioactive compounds from microorganisms are

challenging. The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000, of which 10,000 are produced by actinomycetes, representing 43.5% of all bioactive microbial metabolites discovered. The *Actinomycetes* are filamentous gram positive bacteria, characterized by a complex life cycle belongs to the phylum of *Actinobacteria*. Approximately, 7,600 bioactive compounds are produced by *Streptomyces* species belong to the actinomycetes (Berdy et al., 2005).

In previous study, antiparasitic compounds isolated from *Streptomyces* spp. (i.e., valinomycin, staurosporine and butenolide) which are reported active against *Leishmania major*, *Trypanosoma brucei* and other *Trypanosoma* spp (Pimentel-Elardo et al., 2010). Also, amphothericin B and ivermectin are important anti-parasitics, isolated from microorganisms like a *Streptomyces* sp. (Kayser et al., 2003).

5. **General objectives of the present study**

The general objectives of the present study are to reveal country-wide seroprevalence of *T. gondii* in cattle, goat and sheep, and *N. caninum* infections in cattle in Mongolia and to identify antiprotozoal compounds from Mongolian natural resource such as actinobacteria (*streptomyces* sp.) isolated from Mongolian soils.

CHAPTER 1

Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in cattle in Mongolia

1.1. Introduction

Toxoplasmosis is a zoonotic disease caused by the protozoan parasite *Toxoplasma gondii,* which infects warm-blooded animals including human and livestock as an intermediate host. Domestic cats and other members of the family *Felidae* serve as definitive hosts of *T. gondii*. At least 17 species of wild felines have been reported as definitive hosts, i.e., European and African wild cats, Pallas' cat, Bobcat, leopard cat, Amur leopard cat, Iriomote cat, Ocelot, Geoffroy's cat, Pampas cat, jaguarundi, cougar, leopard, jaguar, tiger, lion, and cheetah (Tenter et al., 2000). Oocysts shed by the definitive host cause wide-ranging environmental contamination (Hill et al., 2002). *T. gondii* tissue cysts are commonly seen in meat-producing animals such as pig, sheep, and goats, while they are rare in beef and buffalo meat (Tenter et al., 2000). Therefore, raw or undercooked meat from these animals is potentially hazardous if ingested by humans or other animals.

Previous studies have been demonstrated that TgGRA7 based ELISA is a potential diagnostic marker for the detection of IgG in acute and chronic infection and available to differentiate *T. gondii* infection from other infection with a high specificity and sensitivity in different animals, including, goat, sheep, cattle, donkey and pigs (Selseleh et al., 2012; Terwaki et al., 2013; Wang et al., 2014; Ichikawa-seki et al., 2015; Fereig et al., 2016). In addition, GRA7 has shown high sensitivity and specificity in ruminants were as follows: sensitivity and specificity

in cattle 84% and 88%, in sheep 83% and 83%, in goat 82% and 88%, respectively (Fereig et al., 2016).

Neosporosis is a serious disease in cattle and dogs caused by the protozoan parasite *Neospora caninum*, which is a *Toxoplasma*-like organism. Major reproductive problems caused by *N. caninum* infection are abortion and stillbirths in cattle (Dubey et al., 2007). Canids, including the Australian dingo (*Canis lupus dingo*) (King et al., 2010), coyote (*Canis latrans*) (Gondim et al., 2004), and gray wolf (*Canis lupus*) (Dubey et al., 2011), are definitive hosts and can shed oocysts in their feces. *N. caninum* oocyst-contaminated food or water is considered one route of infection for cattle (De Marez et al., 1999). In addition, vertical transmission of *N. caninum* between dams and calves is another route of the infection (Thurmond et al., 1997; Schares et al., 1998). Although there is no evidence that *N. caninum* infection occurs in humans, anti-*N. caninum* antibodies are detected in humans (Ibrahim et al., 2009; Tranas et al., 1999), suggesting it as a potential zoonotic pathogen.

Several studies have been confirmed that the surface antigen 1 (SAG1) is an important diagnostic protein for the detection of specific antibodies to *N. caninum* in cattle (Chanan et al., 2003, Wilkowsky et al., 2011). Moreover, NcSAG1 has shown potential antigens to detect during acute and chronic infection to *N. caninum* in cattle and infected cows (Hiasa et al., 2012, Takashima et al., 2013, Ichikawa-seki et al., 2016).

In previous study, the seroprevalence of *T. gondii* among sheep in seven provinces of Mongolia was examined using an indirect enzyme-linked immunosorbent assay (iELISA) based on recombinant *T. gondii* matrix antigen 1 and latex agglutination test (LAT). The overall prevalence rate of *T. gondii* was 24% (42/175) and 16% (29/175) by iELISA and LAT, respectively (Tumurjav et al., 2010). In addition, seroprevalence of *T. gondii* in wild Pallas' cat, which is a small-sized

felid species (*Otocolobus manul*), was 13% (2/15) (Brown et al., 2005). These results imply the presence of *T. gondii* in Mongolia. In contrast, to my knowledge, there is no report of the prevalence of *N. caninum* in Mongolia.

The agricultural sector is the backbone of the economy in Mongolia. In particular, livestock products considered as an importance of the source of income. Mongolian livestock population (horse, cattle, camel, goat and sheep) reached to 66 million heads. Among them, cattle population was estimated to 4.3 million head in 2017 (National statistics office of Mongolia, 2017). The livestock is a subsector of agriculture which produces nearly 30% to the gross domestic product in Mongolia (Shagdar, 2002).

However, the current prevalences of *T. gondii* and *N. caninum* in Mongolia have not been well-studied. Therefore, epidemiological evidence of the prevalences of *T. gondii* and *N. caninum* in livestock is an urgent issue for reducing not only economic loss of livestock production but also public health risks. The aim of the present study was to conduct a large-scale examination of the seroprevalence of *T. gondii* and *N. caninum* infections and risk factors for such infections in cattle of Mongolia.

1.2. Materials and methods

Study area and samples

Based on economic and geographical conditions, Mongolia is divided into four major regions comprising 21 provinces: central region (Tov, Omnogobi, Gobisumber, Dornogobi, Selenge, and Dundgobi), western region (Gobi-Altai, Khovd, Bayan-Olgii, Uvs, and Zavkhan), eastern region (Sukhbaatar, Dornod, and Khentii) and khangai region (Khovsgol, Arkhangai, Ovorkhangai,

Bayankhongor, Bulgan, and Orknon). A total of 1,438 cattle sera from 20 provinces of Mongolia and the capital city of Ulaanbaatar were tested in this study (Tables 1, Figs. 1 and 2); no samples were available from Darkhan-Uul province, which is located in the central region of Mongolia. Cattle sera collected from 2014 to 2016 were obtained from the collection of serum samples at the Institute of Veterinary Medicine (IVM) in Mongolia. Blood samples were collected from each cattle in the field with venal puncture, into glass tubes without anticoagulant. Blood samples were kept on icebox and immediately transferred to laboratory of molecular genetics, Institute of veterinary medicine, Mongolian university of life sciences. They were kept frozen at −30°C until analysis. Samples were divided according to region, province, age group (1–4, 5–9, and 10–14 years), and sex for risk factor analyses of the infections.

Serological testing

In the present study, recombinant proteins of surface antigen 1 of *N. caninum* (NcSAG1) and dense granule protein 7 of *T. gondii* (TgGRA7) were used for detection of specific antibodies (Terwaki et al., 2013; Chahan et al., 2003). Purified NcSAG1 fused with glutathione S-transferase (GST) was used for detection of *N. caninum*-specific antibodies by iELISA. iELISA based on recombinant TgGRA7 protein was used for detection of anti-*T. gondii* antibodies. Fifty microliters of purified antigen were coated onto ELISA plates (Nunc, Roskilde, Denmark) with a coating buffer (carbonate-bicarbonate buffer, Sigma, St. Louis, MO, USA) at a final concentration of 0.1 μM and incubated at 4°C overnight. After incubation, plates were washed with washing buffer [0.5% Tween 20 in phosphate-buffered saline (PBS)], blocked with 100 μl of blocking solution (3% skim milk in PBS) in each well, and incubated at 37°C for 1 h. Plates were washed with washing buffer once. Cattle sera were diluted at 1:200 with 3% skim milk in PBS, and then 50 μl

of positive control, negative control, or test serum sample were added to the wells and incubated at 37°C for 1 h. After washing the plates six times with washing buffer, 50 µl of horseradish peroxidase (HRP)-conjugated anti-bovine IgG (Bethyl Laboratories, Montgomery, TX, USA) diluted at 1:10,000 (1:1 v/v in glycerol) with 3% skim milk in PBS were added to each well and incubated at 37°C for 1 h. After washing the plates six times with washing buffer, 50 µl of TMB substrate reagent (BD Bioscience, San Diego, CA, USA) were quickly added to each well and incubated at room temperature for 10 min in the dark. After incubation, 50 µl of stop solution (1 M H_2SO_4) were added to each well. Absorbance values at 450 nm (A_{450}) of each reaction were determined using an ELISA reader. The readings for recombinant antigens were subtracted from those of GST protein. The cut-off point was determined as the mean A_{450} value for standard negative sera kept in our laboratory (n = 8 negative sera) plus 3 standard deviations. The reference positive and negative sera used for *Neospora* study were confirmed by commercial IFAT slide (VMRD, Pullman, WA, USA). The sera from experimentally infected cattle were used as the reference positive sera (Nishimura et al., 2013). The reference negative sera used for *Toxoplasma* study were confirmed by commercial latex agglutination test (Toxocheck-MT, Eiken Chemical, Tokyo, Japan).

The specificity and sensitivity between the results of commercial latex agglutination test and the TgGRA7-based iELISA were calculated. The specificity and sensitivity of the iELISA were as follows: TgGRA7 antigen: sensitivity 84%, specificity 88% (Fereig et al., 2016). In the case of NcSAG1-based iELISA, the specificity and sensitivity between the results of IFAT and iELISA were: sensitivity 100%, specificity 100% (Chahan et al., 2003).

Statistical analysis

In the present study, odds ratios (ORs) and 95% confidence intervals (95% CIs) were determined. The data analysis was performed using Graphpad Prism 6 software (GraphPad software Inc., La Jolla, CA, USA). The Chi-square test was used to analyze the data. Associations were tested using odds ratios (OR) and 95% confidence intervals (CI) after adjustments. A *P* value of <0.05 was considered statistically significant for differences using the VassarStats online tool (http://vassarstats.net/).

1.3. Results

The overall seroprevalence rate of *T. gondii* in Mongolian cattle was 18.7% (range: 2.6–42.1%) (Table 1). The highest seroprevalence rate of *T. gondii* was 42.1% in Dundgobi, which is located in the central region of Mongolia. In addition, higher seroprevalence rates were observed in Ovorkhangai of khangai region (41.7%) and the capital city of Ulaanbaatar (38.5%). Lower seroprevalence rates were seen in Bayan-Olgii (2.6%) and Khovd (5.7%) provinces in the western region (Table 1, Fig. 1). Higher seroprevalence rates of *T. gondii* were found in the central region (27.1%) and the khangai region (23.3%) compared with that of the western region (9.0%), which had the lowest seroprevalence rate (Table 2). This result suggests that geographical condition may be a potential risk factor of *T. gondii* infection in Mongolia. Although age and sex were also analyzed as potential risk factors of *T. gondii* infection, no significant differences were observed (Table 3).

The overall seroprevalence of *N. caninum* in cattle of Mongolia was 26.2% (range: 10.5%–69.2%) (Table 1). The highest seroprevalence rate was observed in the capital city of Ulaanbaatar (69.2%). In addition, Dornogobi (42.9%) and Dundgobi (44.7%) provinces in the central region

and Zavkhan province (43.9%) in the western region showed higher seroprevalence rates. The three lowest seroprevalence rates were seen in the western region, Bayan-Olgii (10.5%), Uvs (12.3%) and Gobi-Altai (13.3%) (Table 1, Fig. 2). The seroprevalence rates of *N. caninum* in the central region (30.8%) and the khangai region (30.7%) were higher than that in the western region (20.8%) (Table 4). Risk factor analysis of *N. caninum* seroprevalence showed no significant difference according to age, while a significant difference in *Neospora* seroprevalence was observed according to sex (Table 5). In particular, the seropositive rate of female animals (27.5%) was higher than that of male animals (20.4%) ($P = 0.018$).

Mixed infection with *T. gondii* and *N. caninum* were observed among cattle in Mongolia. The overall seroprevalence of mixed infection was 6.8% and infection rates ranged from 0% to 38.5% (Table 1). Relatively high rates of mixed infection observed in Ulaanbaatar (38.5%), Dundgobi (31.6%), Dornogobi (21.4%) and Ovorkhangai (21.4%), infection rates in other provinces ranged from 0% to 12.2% (Table1).

1.4. Discussion

The livestock sector plays an important economic role in Mongolia because milk and meat products derived from cattle are served as foods for daily life of Mongolians. Most consumed meat are sheep (31.5%), goat (27.7%) and cattle (21.3%) in Mongolia. Additionally, annually report 2016 of milk industries in Mongolia shows that milk production from cow, sheep and goat estimates 522.4, 99.1 and 173.6 million of liter, respectively. Mongolia (National statistics office of Mongolia, 2017). Therefore, cattle is one of the important food source in. Although *T. gondii* or *N. caninum* infection affects the livestock industry by decreasing productivity due to abortion

and stillbirth in cattle and neurological symptoms in calves, available data of the prevalences of these protozoan parasites in Mongolia are limited.

T. gondii is not main causative agents of abortion in cattle. Contribution of the milk and meat from the infected cattle on *Toxoplasma*-prevalence is unknown (Dubey, 1986). However, consumption of undercooked and raw beef and cow's milk has been considered as a risk factor for human infection. Therefore, in the present study, cattle sera from 20 of 21 provinces of Mongolia were examined for wide-ranging seropositivety of *T. gondii* and *N. caninum*.

Overall seroprevalences of *T. gondii* and *N. caninum* in cattle of Mongolia were 18.7% and 26.2%, respectively. China and Russia are neighboring countries of Mongolia. In China, prevalence rates of *T. gondii* in cattle were 5.7% in the southern part by indirect hemagglutination antibody test (Zhou et al., 2012) and 4.8% in the northwest part by modified agglutination test (Tan et al., 2015). However, there are no reports of *Toxoplasma* prevalence in cattle of Russia. Concerning seroprevalence of *N. caninum* in cattle by ELISA, prevalence rates of 13.3% in northeastern China (Wang et al., 2010), 18.9% in southern China (Xia et al., 2011), and 9.97% in Russia (Hemphill and Gottstein, 2000) were reported. Thus, the seroprevalences of *T. gondii* and *N. caninum* in cattle of Mongolia were higher than those reported in China and Russia, indicating that *T. gondii* and *N. caninum* infections may be a hazard to the livestock industry in Mongolia.

In my study, both *N. caninum* and *T. gondii* prevalence rates were significantly higher in the central region of Mongolia, suggesting that one risk factor of such infections might be climate condition, particularly warmer temperatures around this area. In contrast, the overall lowest *T. gondii* prevalence rate was observed in the western region of Mongolia. According to climate and geographical conditions, the average annual temperature of the central region is higher because most of the largest desert (Gobi) is located around this area. Conversely, the western region of

Mongolia is the coldest and has the highest elevation (Altai Mountain area). Although *T. gondii* oocysts (and likely *N. caninum* oocysts) are resistant to environmental conditions such as high temperature, drying, and freezing (Dubey et al, 1998; Dubey et al, 1970), these infections may increase in warmer and humid conditions than colder and dry climates (Tenter et al., 2000). Thus, climate and geographical conditions in Mongolia may affect both *N. caninum* and *T. gondii* seroprevalence rates.

Although the prevalence of *T. gondii* in cattle may be an infectious source to human, human *T. gondii* infection is not well studied in Mongolia. The TORCH test is a blood screen for *T. gondii*, rubella virus, cytomegalovirus, herpes simplex, human immunodeficiency virus, and other organisms, which lead to severe fetal anomalies and fetal loss (Kaur et al 1999). The TORCH test is recommended for pregnant women with high-risk pregnancies in Mongolia. Although TORCH infections were tested among 100 pregnant women in Mongolia, *T. gondii* infection was not detected by ELISA (Otgontsetseg et al., 2013). However, large-scale screening of *T. gondii* is required to understand the impact of toxoplasmosis in Mongolia. Meat and meat-derived products containing *T. gondii* tissue cysts are potential sources of infection in human (Tenter et al., 2009). However, consumption of undercooked and raw meat is not a typical practice among Mongolians. Therefore, intake or consumption of contaminated water, soil, and raw vegetables might be a potential risk factor of *T. gondii* infection in human. Although another source of *T. gondii* infection is cats and members of the family *Felidae*, cats are not common domestic animals in Mongolia. Generally, domestic cats do not widespread in whole regions of Mongolia because herders do not regularly maintain cat as a pet. However, small number of domestic cats are kept in household to control rodents.

Several species of wild cats inhabit Mongolia such as the wild cat (*Felis silvestris*), Eurasian lynx (*Lynx lynx*), Pallas's cat (*O. manul*), and snow leopard (*Uncia uncia*). Among them, wild cats are distributed in desert areas (Gobi) and Mongolian Altai Mountain area. However, hybridization between wild and domestic cats has been observed, which influences the genetic purity of wild cats, and competition between prey domestic cats and wild cats can lead to disease transmission. The Eurasian lynx (*L. lynx*) is widely distributed in Mongolia (Clark et al., 2006). The snow leopard is distributed in the mountainous area of western Mongolia, but they are rare in all parts of Mongolia. Moreover, the snow leopard is thought to prey on livestock (Mallon 1984; Clark et al., 2006; Shehzad et al., 2012). Thus, the transmission of *T. gondii* infection via oocysts shed by wild felines may also facilitate infection in human (especially nomads) and livestock.

In the present study, the seroprevalence of *N. caninum* was significantly higher in female cattle (27.5%) compared with male cattle (20.4%), suggesting sex is an important risk factor because of expected reproductive problems such as abortion and stillbirth in cattle. *N. caninum*-specific antibody titers increase during pregnancy and peak within a month after calving in *N. caninum*-infected dams (Ybañez et al., 2013). Therefore, the detection rate of *N. caninum*-specific antibodies in female cattle might be higher than that in male animals. Higher seropositive rate of anti-*N. caninum* antibodies was recorded in female (27.5%) compared with male (20.4%) by statistical analysis. However, this result should be considered unequal sample size (female: 1,174, male: 264).

I investigated the mixed infection with *T. gondii* and *N. caninum* in cattle in Mongolia. The overall seroprevalence rate was 6.8%, based on this data, the mixed infection with *T. gondii* and *N. caninum* are not commonly occurred in Mongolia (Table 1).

Furthermore, the *N. caninum* infection rate in cattle was shown to increase in the presence of dogs on farms as a neighborhood animal (Dubey et al., 2007). Because Mongolian herders and farmers maintain dogs as domestic animals, the high prevalence of *N. caninum* infection in Mongolian cattle may be due to contact with feces of *N. caninum*-infected domestic dogs. For the Mongolian herders, dogs are essential to keep in the households as herding dogs. Although most of the herders and farmers are maintaining several dogs in the households, they generally do not care about health check and medication for dogs. Therefore, further studies are urgently needed to determine the infection of *T. gondii* and *N. caninum* in cats and dogs. They would be help to understand of infection sources of those diseases. In addition, wild canids should be considered as the definitive host of *N. caninum*. Wild canids such as gray wolf (*Canis lupus*), Asiatic wild dog (*Cuon alpinus*), raccoon dog (*Nyctereutes procyonoides*), and corsac fox (*Vulpes corsac*) are distributed in Mongolia. Among them, the gray wolf is widely distributed and considered prey livestock in Mongolia (Clark et al., 2006). However, to my knowledge, there are no reports about the prevalence of *N. caninum* infection in these dogs in Mongolia. Further investigations are needed to assess the relationship between dogs and cattle for *N. caninum* infection in Mongolia.

1.5. Summary

Toxoplasma gondii and *Neospora caninum* are protozoan parasites that cause huge economic losses in animal industries worldwide. *N. caninum* can cause abortion storms and high culling rates in cattle, whereas *T. gondii* infection is a significant concern in both human and animals because it can induce abortion and clinical symptoms in immunocompromised hosts. The aim of this study was to determine the seroprevalence of *T. gondii* and *N. caninum* in cattle in Mongolia. Specific antibodies to *T. gondii* and *N. caninum* were detected by using an indirect

enzyme-linked immunosorbent assay (iELISA) based on recombinant antigens of dense granule protein 7 of *Toxoplasma gondii* and surface antigen 1 of *Neospora caninum*, respectively. A total of 1,438 cattle sera from 20 of 21 provinces of Mongolia and the capital city of Ulaanbaatar were tested. Overall, 18.7% and 26.2% of cattle were positive for specific antibodies to *T. gondii* and *N. caninum*, respectively. Prevalence rates were higher (*T. gondii* infection: $P < 0.0001$, *N. caninum* infection: $P = 0.002$) in the central region of Mongolia (*T. gondii* infection: 27.1%, *N. caninum* infection: 30.8 %) compared with western region, suggesting that prevalence rates might be influenced by geographical condition, particularly warmer temperatures around this area in Mongolia. The lowest prevalence rates were observed in the western region of Mongolia (*T. gondii*: 9%, *N. caninum*: 20.8%). In addition, the seroprevalence of *N. caninum* in female animals (27.5%) was significantly higher than that in male animals (20.4%) ($P = 0.018$), suggesting an important risk factor of abortion and stillbirth in cattle. The present results showed that *T. gondii* and *N. caninum* infections might be a risk for public health and economy of the livestock industry in Mongolia. This study demonstrates high seroprevalences of *T. gondii* and *N. caninum* in Mongolia and provides valuable new data for development of control measures against these infections in Mongolia.

Table 1: Seroprevalence of *T. gondii* and *N. caninum* in cattle in different provinces of Mongolia

Regions	Provinces	Seroprevalence of *T. gondii* % (No. positive / No. sampled)	95% CI of *T. gondii* seroprevalence	Seroprevalence of *N. caninum* % (No. positive / No. sampled)	95% CI of *N. caninum* seroprevalence	Mixed seroprevalence % (No. positive / No. sampled)	95% CI of mixed seroprevalence
Central region	Tov	10.8 (7/65)	4.8-21.5	33.8 (22/65)	22.8-46.7	0.0 (0/65)	0.0-6.9
	Omnogobi	28.7 (29/101)	20.3-38.7	17.8 (18/101)	11.1-26.9	5.9 (6/101)	2.4-12.9
	Gobisumber	20 (4/20)	0.6-44.2	30 (6/20)	12.8-54.3	5.0 (1/20)	0.2-26.9
	Dornogobi	33.3 (14/42)	20.0-49.6	42.9 (18/42)	28.0-58.9	21.4 (9/42)	10.8-37.2
	Selenge	28.2 (20/71)	18.4-40.2	25.4 (18/71)	16.1-37.3	11.3 (8/71)	5.3-21.5
	Dundgobi	42.1 (16/38)	26.7-59.0	44.7 (17/38)	29-61.5	31.6 (12/38)	18.0-48.7
Western region	Gobi-Altai	8.9 (4/45)	2.8-22.1	13.3 (6/45)	5.5-27.4	0.0 (0/45)	0.0-9.0
	Khovd	5.7 (3/53)	1.4-16.6	32.1 (17/53)	20.3-46.4	0.0 (0/53)	0.0-8.0
	Bayan-Olgii	2.6 (4/153)	0.8-6.9	10.5 (16/153)	6.2-16.7	1.3 (2/153)	0.2-5.1
	Uvs	12.3 (8/65)	5.8-23.3	12.3 (8/65)	5.8-23.3	3.1 (2/65)	0.5-11.6
	Zavkhan	20.7 (17/82)	12.8-31.3	43.9 (36/82)	33.1-55.2	12.2 (10/82)	6.3-21.7
Eastern region	Sukhbaatar	17.6 (18/102)	11.0-26.7	17.6 (18/102)	11.0-26.7	3.9 (4/102)	1.2-10.3
	Dornod	17.5 (20/114)	11.2-26.0	34.2 (39/114)	25.7-43.7	4.4 (5/114)	1.6-10.4
	Khentii	15.2 (20/132)	9.7-22.6	18.2 (24/132)	12.2-26.0	4.5 (6/132)	1.8-10.0
Khangai region	Bulgan	23 (14/61)	13.5-35.8	29.5 (18/61)	18.8-42.7	4.9 (3/61)	1.2-14.6
	Khovsgol	20 (11/55)	10.8-33.3	20 (11/55)	10.8-33.3	9.1 (5/55)	3.3-20.7
	Orknon	10 (1/10)	0.5-45.8	20 (2/10)	3.5-55.7	0.0 (0/10)	0-34.4
	Ovorkhangai	41.7 (35/84)	31.1-52.9	38.1 (32/84)	27.9-49.3	21.4 (18/84)	13.5-32
	Bayankhongor	11.8 (9/76)	5.8-21.7	36.8 (28/76)	26.2-48.7	0.0 (0/76)	0.0-6.0
	Arkhangai	17.9 (10/56)	9.3-30.8	25 (14/56)	14.8-38.6	3.6 (2/56)	0.6-13.3
Capital city	Ulaanbaatar	38.5 (5/13)	15.1-64.4	69.2 (9/13)	38.8-89.6	38.5 (5/13)	15.1-67.7
	Total	**18.7 (269/1,438)**	**16.7-20.8**	**26.2 (377/1,438)**	**23.9-28.5**	**6.8 (98/1438)**	**5.6-8.2**

95% Ci calculated according to the described method http://vassarsats.net

CI: Confidence interval.

Table 2: Seroprevalence of *T. gondii* in different regions of Mongolia.

Regions of Mongolia	No. tested	No. positive	No. negative	Seroprevalence (%)	OR (95%CI)	*P*-value
Central region	350	95	255	27.1	0.26 (0.17-0.40)	<0.0001
Western region	398	36	362	9	-	
Eastern region	348	58	290	16.6	0.49 (0.31-0.77)	0.002
Khangai region	342	80	262	23.3	0.32 (0.1-0.49)	<0.0001

OR: odds ratio,

CI: confidence interval,

-: reference group.

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Table 3: Analysis of risk factors associated with *T. gondii* infection in Mongolian cattle

Risk factors		No. sampled	No. positive	No. negative	Seroprevalence (%)	OR(95%CI)	*P*-value
Age groups (Years)	1--4	710	135	575	19	-	
	5--9	430	75	355	17.4	1.11 (0.81-1.51)	0.507
	10--14	44	10	34	22.7	0.79 (0.38-1.65)	0.543
	Unknown	254	49	205	19.2	0.98 (0.68-1.41)	0.92
Sex	Female	1,174	213	961	18.1	-	
	Male	264	56	208	21.2	0.82 (0.59-1.14)	0.248

OR: odds ratio,

CI: confidence interval,

-: reference group.

Table 4: Seroprevalence of *N. caninum* in different regions of Mongolia.

Regions of Mongolia	No. tested	No. positive	No. negative	seroprevalence (%)	OR (95%CI)	*P*-value
Central region	350	108	242	30.8	0.59 (0.42-0.82)	0.002
Western region	398	83	315	20.8	-	
Eastern region	348	81	267	23.2	0.86 (0.61-1.22)	0.427
Khangai region	342	105	237	30.7	0.59 (0.42-0.83)	0.002

OR: odds ratio,

CI: confidence interval,

-: reference group.

Table 5: Analysis of risk factors associated with *N. caninum* in Mongolian cattle.

Risk factors		No. sampled	No. positive	No. negative	Seroprevalence (%)	OR (95%CI)	*P*-value
Age groups (Years)	1--4	710	176	534	24.8	-	
	5--6	430	125	305	29.1	0.80 (0.61-1.05)	0.111
	10--14	44	13	31	29.5	0.78 (0.40-1.53)	0.479
	Unknown	254	63	191	24.8	0.99 (0.71-1.39)	1
Sex	Female	1,174	323	851	27.5	-	
	male	264	54	210	20.4	1.47 (1.06-2.04)	0.018

OR: odds ratio,

CI: confidence interval,

-: reference group.

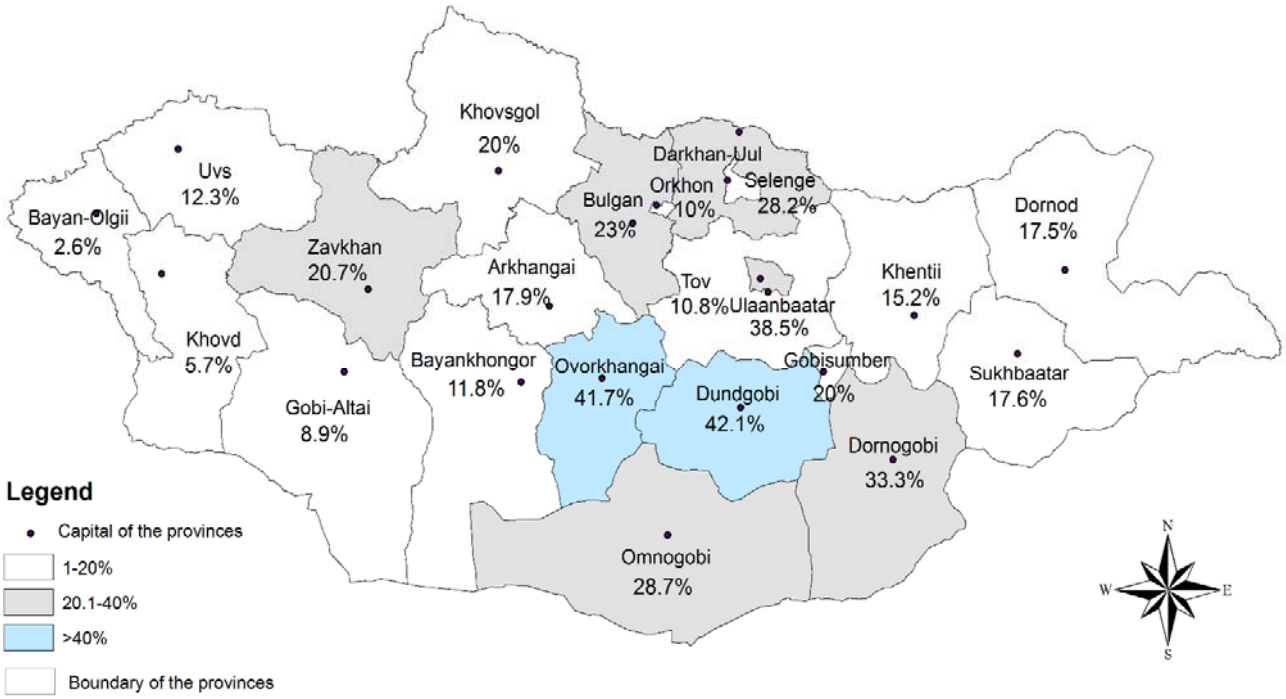


Fig. 1. Geographical distribution of *T. gondii* infection in Mongolian cattle used in this study. Infection rates are indicated as follows: white 1%–20%, gray 20.1%–40%, and light blue >40%.

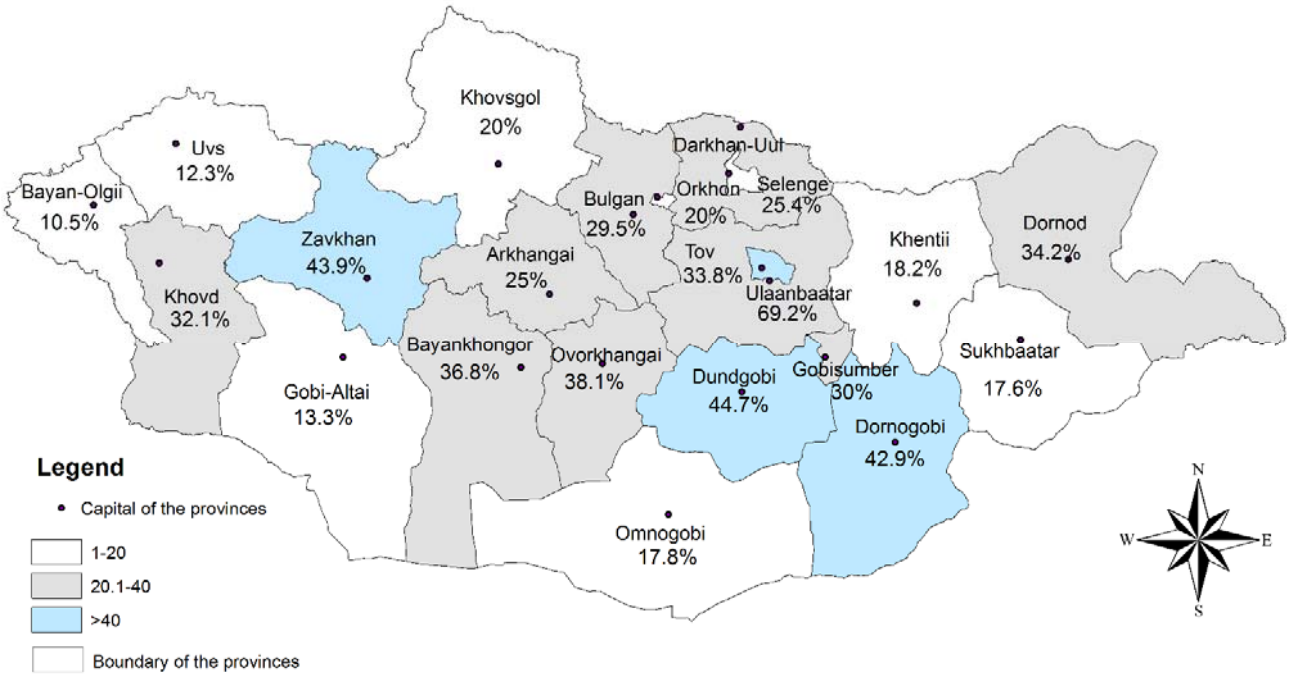


Fig. 2. Geographical distribution of *N. caninum* infection in Mongolian cattle used in this study. Infection rates are indicated as follows: white 1%–20%, gray 20.1%–40%, and light blue >40%.

CHAPTER 2

Seroepidemiological study of *Toxoplama gondii* in small ruminants (sheep and goat) in different provinces of Mongolia

2.1. Introduction

Toxoplasmosis is a disease that occurs worldwide and is caused by the intracellular apicomplexan protozoan parasite *Toxoplasma gondii*. *T. gondii* is capable of infecting almost all types of animals including humans, livestock, and wild animals. Small ruminants (sheep, goats) and cattle are the intermediate hosts of *T. gondii*. Domestic cats and wild felids are the definitive hosts that can excrete oocysts into the environment (Dubey, 2010).

Previous studies indicated that ingestion of oocytes through food and water is the main mode of transmission of *T. gondii* in humans (Asthana et al., 2006). Animals and humans can become infected by ingesting tissue cysts from undercooked meat or soil, or water contaminated with infective oocysts (Dubey, 2008). *T. gondii* tissue cysts are commonly observed in food-producing animals including pigs, chickens, sheep, and goats (Tender et al., 2000). Sheep and goats are more susceptible to *T. gondii* infection. Abortion and neonatal infections in sheep and goats occur as a result of primary infection during pregnancy (Buxton, 1990).

Many studies have been conducted to investigate the seroprevalence of *T. gondii* in small ruminants in different areas of the world (Dubey, 2009). However, toxoplasmosis has not been well-studied among livestock in Mongolia. In a previous study, the seroprevalence of *T. gondii* among sheep in Mongolia was estimated through an indirect enzyme-linked immunosorbent assay

(iELISA) based on recombinant *T. gondii* matrix antigen 1 and a latex agglutination test (LAT). The overall seroprevalence rate of *T. gondii* was 24% (42/175) by iELISA and 16% (29/175) by LAT (Tumurjav et al., 2010). Furthermore, the seroprevalence of *T. gondii* in wild Pallas' cats, which is a small-sized felid species (*Otocolobus manul*), was 13% (2/15) (Brown et al., 2005). In my previous study, the seroprevalence of *T. gondii* among cattle in Mongolia was examined using an iELISA based on recombinant antigen TgGRA7. A total of 1,438 cattle sera from 20 of the 21 provinces of Mongolia and the capital city of Ulaanbaatar were evaluated. According to this study, 18.7% of cattle were seropositive for specific antibodies to *T. gondii* (Pagmadulam, et al., 2018). confirming the presence of *T. gondii* in Mongolia.

In Mongolia, there is only one report documenting the seroprevalence of *T. gondii* in sheep, but none for domestic goats. Information on the seroprevalence of *T. gondii* in small ruminants such as goats and sheep will be helpful in implementing preventive strategies for public health. Therefore, the main objectives of this survey were to investigate the seroprevalence of *T. gondii* in small ruminants (sheep and goats) from different areas in Mongolia and to evaluate the risk factors associated with seropositivity.

2.2. Materials and methods

Ethics statement

This study was performed in strict accordance with 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 protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit number 18-15).

Study area and samples

Blood samples were taken from 1,078 goats and 882 sheep in 17 of 21 provinces of Mongolia during 2016–2018. Mongolia is divided into four major regions comprising 21 provinces: the central region (Tov, Umnogobi, Gobisumber, Dornogobi, Selenge, and Dundgobi), the western region (Gobi-Altai, Khovd, Bayan-Olgii, Uvs, and Zavkhan), the eastern region (Sukhbaatar, Dornod, and Khentii), and the khangai region (Huvsgol, Arkhangai, Uvurkhangai, Bayankhongor, Bulgan, and Orknon).

Sample collection

Approximately 2 ml of blood was taken from each animal (goats and sheep) by venal puncture into glass tubes containing anticoagulant (EDTA). All samples were kept in an icebox and transferred to the Laboratory of Molecular Genetics, at the Institute of Veterinary Medicine, Mongolian University of Life Sciences. Then blood samples were centrifuged to collect the sera and the sera kept frozen at -30°C until analysis.

Recombinant protein expression and purification of soluble protein TgGRA7

Recombinant TgGRA7 (rTgGRA7) was expressed as glutathione S-transferase (GST) fusion protein in *E. coli* DH5α (Takara Bio, Inc., Japan) using previously described methods (Terwaki et al., 2013). The glutathione *S*-transferase (GST)-tagged recombinant protein was separated by thrombin protease in accordance with the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). The expression of rTgGRA7 (29 kDa) was then confirmed by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue R250 staining (MP Biomedicals, Inc., France). The rTgRGA7 protein concentration was subsequently measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA).

iELISA

Fifty microliters of purified antigen at a final concentration of 0.1 µM were added along with coating buffer (carbonate-bicarbonate buffer; Sigma, St. Louis, MO, USA) to the wells of an ELISA plate (Nunc, Roskilde, Denmark) and the plate was incubated overnight at 4°C. After washing with phosphate-buffered saline (PBS: 0.05%, Tween 20), the plate was blocked with 100 µl of blocking solution containing 3% skimmed milk in PBS (PBS-SM) for 1 h at 37°C. After one further wash with washing buffer, goat and sheep sera were diluted 1:200 with 3% skim milk in PBS, and then 50 µl of positive control, negative control, and test serum samples were added to the wells and incubated at 37°C for 1 h. After washing the plates six times with washing buffer, 50 µl of horseradish peroxidase-conjugated anti-sheep IgG (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:5,000 and anti-goat IgG (Bethyl Laboratories) diluted 1:10,000 (with 3% skim milk in PBS) were added to each well and incubated at 37°C for 1 h. After washing the plates six times with washing buffer, 50 µl of substrate solution were quickly added to each well and incubated at room temperature for 1 h in the dark. The absorbance values at 415 nm (A_{415}) of each reaction were determined using an ELISA reader. The readings for recombinant antigens were subtracted from those of GST protein. The cut-off point was determined as the mean A_{415} value for negative goat sera (n = 9 negative sera) and negative sheep sera (n = 9 negative sera) plus three standard deviations. The reference negative sera used for *Toxoplasma* studies were confirmed by

both a commercial latex agglutination test (Toxocheck-MT, Eiken Chemical, Tokyo, Japan) and a TgGRA7-based iELISA.

Statistical analysis

Data analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). The Chi-square test was used to analyze data. Associations were tested using odds ratios (ORs) and 95% confidence intervals (CIs) after adjustment. A *P* value of < 0.05 was considered statistically significant using the VassarStats online tool (http://vassarstats.net/).

2.3. Results

The sera from goats (n=1,078) and sheep (n=882) were collected and tested in 17 of 21 provinces, all regions, and the capital city of Ulaanbaatar in Mongolia. The overall seroprevalence of *T. gondii* in goats was 32% (345/1078) ranging from 0% to 65% (Table 6). Although no correlation was found with sex, age was associated with seroprevalence in goats, with higher seroprevalence being observed in 1–2-year-old (39.7%) and >7-year-old animals (36.7%) (Table 7). Furthermore, geographical location was identified as a risk factor in goats (Table 7). The highest seroprevalence was identified in goats in the eastern and western regions of Mongolia (45.6% and 42.7%, respectively). In particular, higher rates were observed in Uvs and Zavkhan provinces in the western region (61.3% and 65.0%, respectively, Table 6).

The overall seroprevalence of *T. gondii* in sheep was 34.8% (307/882) ranging from 10.5% to 80.5% (Table 8). There was no correlation detected between seroprevalence and the age or sex of animals (Table 9). However, geographical differences were detected with high seroprevalence observed in the eastern region of Mongolia (55.4%) ($P < 0.0001$) (Table 9). The highest seroprevalence was identified in Khentii province, in the eastern region of Mongolia, with a

prevalence of 80.5%, followed by Umnugobi province, in the central region, with a prevalence of 61.3%.

2.4. Discussion

The livestock industry plays a crucial role in the economy of Mongolia. The total livestock population, comprising cattle, sheep, goats, camels, and horses, was 66 million in 2018 (Mongolian Statistical office, 2018). Among them, cattle, sheep, and goats are the main sources of food for Mongolians and meat products derived from sheep and goats are a daily part of many peoples' diet. Recently, the goat and sheep populations have reached 41.6%–45.4% of the total livestock. The most consumed meats are mutton (31.5%), chevon (27.7%), and beef (21.3%) in Mongolia (Mongolian Statistical office, 2017).

The Food and Agriculture Organization of the United Nations and the World Health Organization (WHO and FAO) have recently shown that toxoplasmosis is a foodborne disease of global concern, with the greatest burden of all parasitic infections (FAO/WHO, 2014). Due to increased awareness to food safety, monitoring of pathogen infection in livestock needs to be implemented in Mongolia. Therefore, I used a TgGRA7-based iELISA to examine the seroprevalence of *T. gondii* antibodies in Mongolia. Previous research demonstrated that iELISA with TgGRA7 is an effective diagnostic method for the detection of IgG in acute and chronic infection with higher potency, specificity, and sensitivity than other serological tests (Terkawi et al., 2013, Ichikawa-Seki et al., 2015, Fereig et al., 2016). The present study showed the relatively high seroprevalence of *T. gondii* in sheep (34.8%) and goats (32%) in Mongolia. Sheep and goats are highly susceptible to *T. gondii infection,* which plays a major role in the transmission of toxoplasmosis into humans (Tzanidakis et al., 2010). Raw or undercooked meat from these

animals is potentially hazardous if ingested by humans or other animals. Thus, the prevalence of *T. gondii* infection in goats and sheep may be a source of infection for humans and animals in Mongolia. These results imply the high prevalence of *T. gondii* in Mongolia, which may present a risk of human infection. Consuming contaminated water, unwashed vegetables, and fruits, as well as direct environmental contamination can cause *T. gondii* infection in humans (Hill and Dubey 2016). Mongolians do not typically consume high risk foods such as undercooked, raw meat, or unpasteurized milk from livestock. Aditionally, *T. gondii* DNA was detected in the milk from one sheep and eight Bactrian camels in Mongolia (Iacobucci et al., 2019). Therefore, the consumption of contaminated water, unpasteurized milk, soil, and unwashed, raw vegetables and fruits might be potential risk factors for *T. gondii* prevalence among Mongolians.

China and the Russian Federation are the only neighboring countries of Mongolia. In China, the seroprevalence of *T. gondii* in sheep ranged from 0.8% to 39.3% in different areas (Hide, 2016), but information on the prevalence in goats is limited (Dong et al., 2018). In the Russian Federation, antibodies to *T. gondii* were detected in goats (43.9%), cats (39.9%), and humans (30.9%) (Shuralev, et al., 2018). The overall seroprevalence of *T. gondii* in sheep (34.8%) and goats (32%) in Mongolia was similar that of neighboring countries.

Previous studies have shown an association between *T. gondii* seropositivity and identified risk factors in ruminants including animal age, environmental conditions, breed, the presence of cats, and rodent control (Jones et al., 2011, Gazzonis et al., 2015, Magalhaes et al., 2016).

In the present study, the risk factors of sex, age, and location were investigated in terms of the seroprevalence of *T. gondii*. In the present study, only geographical location had a significant association with *T. gondii* seropositivity for both sheep and goats. The higher seroprevalence in the present study might be related to the location and differences in the environmental conditions

in the sampling area. Mongolia has an extreme climate that is highly variable between regions. The country has four seasons with large temperature fluctuations and low precipitation. A previous study reported the differing prevalence of *T. gondii* in areas with varying altitudes. In the case of school children in Panama, lower prevalence was seen in the areas of highest altitude, while higher prevalence was observed in areas near the sea (Walton, 1966). In the present study, significantly higher seroprevalence of *T. gondii* in goats was identified in the Eastern region (45.5%) which can be attributed to the lower altitude of the Eastern region of Mongolia. Moreover, the highest seroprevalence of *T. gondii* in sheep was observed in the Eastern region of Mongolia (55.4%).

The climate and weather characteristics of the eastern region may explain the higher *T. gondii* seroprevalence found in the region. The eastern region in Mongolia characterized by steppes with annual precipitation relatively higher (180-280 mm) (National Agency Meteorology and the Environmental monitoring, 2019). This high level of precipitation may make the spread of oocysts easier, which may contribute to the higher seroprevalence of *T. gondii* in the eastern region.

Moreover, I found higher seroprevalence of *T. gondii* in sheep and goats of 1–2 years of age compared with 3–4 and 5–6-year-old animals, and this difference was statistically significant for goats. These results indicate that sheep and goats are exposed to *T. gondii* at a young age. Because winter conditions, particularly heavy snow and/or low temperatures, cause serious damage to livestock in Mongolia, *T. gondii* infection might decrease the survival of young animals. Therefore, the seroprevalence in animals over 3 years of age might be lower than in 1–2-year-old animals.

Some species of wild cats such as *Felis silvestris* (wild cat), *Lynx lynx* (Euasian lynx), *O. manul* (Pallas' cat), and *Uncia uncia* (snow leopard) are distributed across Mongolia. Among them,

O. manul (Pallas' cat) and *U. uncia* (snow leopard) inhabit the Huvsgul mountain range and the Mongol Altai mountain range, which are located in the western region of Mongolia (Clark et al., 2006). These wild cat species might be another source of infection. This distribution is consistent with the significantly higher seroprevalence of *T. gondii* observed in goats in the western regions of Mongolia (42.6%). Domestic cat populations are not widespread across whole regions in the country and Mongolian herders do not regularly keep cats as domestic pets, lowering the chance of contact with infected cat feces. However, some domestic cats are kept and even shared among households to control rodents. In this study, I found similar seropositive rates of *T. gondii* in goats (32%) and sheep (34.8%). This may be attributed to the fact that all livestock are fed by grazing on open pastures and river water, and several animals are herded together. Thus, soil, grass, and river water contaminated with *Toxoplasma* oocysts from wild cats might be sources of infection in goats and sheep in Mongolia.

Statistics have been reported on the number of livestock that died of disease in 2017: sheep 53%, goats 26.8%, cattle 16.6%, horses 3.5%, and camels 0.1% (Mongolian Statistical office, 2018). However, it is not clear which types of diseases caused death among these livestock. The occurrence of abortion among livestock might indicate *T. gondii* seroprevalence. The Mongolian National Statistical Office reported that the rate of abortion among all livestock was 44.6% in the khangai region, 25.7% in the central region, 14.7% in the western region, and 14.0% in the eastern region in 2017 (Report of national statistics office of Mongolia, 2018). Such reported cases of abortion might have pathogenic effects on livestock. In my study, a higher prevalence of *T. gondii* was confirmed in goats and sheep, indicating that they are susceptible hosts of *T. gondii* infection. This higher seroprevalence of *T. gondii* might affect livestock mortality rates.

In conclusion, my data may be useful in developing and improving prevention and control strategies for the management of toxoplasmosis in livestock in Mongolia. This data may also be helpful in determining whether domestic and wild cats shed oocysts in provinces that show higher seroprevalence. Further studies to assess the impact of *T. gondii* infection in humans are required in Mongolia.

2.5. Summary

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii*. Consumption of raw or undercooked meat is the main risk factor for acquiring *T. gondii* infection in humans. Meat and meat products derived from goats and sheep are mainly consumed in Mongolia; however, there is limited epidemiological information on *T. gondii* infection in small ruminants in this country. The main objective of the present study was to investigate the seroprevalence of *T. gondii* in sheep and goats in Mongolia. The seroprevalence of *T. gondii* IgG antibodies was determined by an indirect enzyme-linked immunosorbent assay based on the recombinant antigens of dense granule protein 7 of *T. gondii*. A total of 1,078 goat and 882 sheep blood samples were collected from 17 of 21 provinces and the capital city of Mongolia. Overall, the seroprevalence of *T. gondii* among the sheep and goat samples was 34.8% and 32%, respectively. The seroprevalence among goat samples was significantly higher in western (42.7%) and eastern (45.6%) regions compared with other regions (24%). Additionally, the seroprevalence among sheep was significantly higher in eastern regions (55.4%) compared with other regions (26%–33%). Age, but not sex, was considered a risk factor for *T. gondii* seropositivity in goats, whereas no statistically significant differences were observed in sheep for age or sex. In conclusion, the present study demonstrates

the high seroprevalence of *T. gondii* in small ruminants in Mongolia. My results highlight that country wide control measures are required to minimize infections in livestock.

Table 6: Seroprevalence of *T. gondii* in goats in different provinces of Mongolia

Regions	Provinces	No.samples	No.Positive	Seroprevalence (%)	95% CI
Central region	Tuv	16	3	18.8	4.9-46.3
	Umnugobi	48	0	0.0	0-9.2
	Dornogobi	81	25	30.9	21.3-42.2
	Selenge	142	35	24.6	17.9-32.7
	Dundgobi	41	10	24.4	12.9-40.6
	Ulaanbaatar	30	13	43.3	25.9-62.3
Western region	Gobi-Altai	60	14	23.3	13.7-36.3
	Khovd	60	13	21.7	12.4-34.5
	Bayan-Ulgii	40	22	55.0	38.6-70.4
	Uvs	80	49	61.3	49.6-71.7
	Zavkhan	20	13	65.0	40.9-83.6
Eastern region	Sukhbaatar	69	26	37.7	26.5-50.2
	Dornod	59	31	52.5	39.2-65.5
	Khentii	41	20	48.8	33.1-64.6
Khangai region	Bulgan	100	25	25.0	17.1-34.8
	Huvsgul	140	34	24.3	17.6-32.4
	Uvurkhangai	11	0	0.0	0.0-32.1
	Bayankhongor	40	12	30.0	15.1-44.1
Total		1078	345	32.0	29.2-34.8

CI, confidence interval calculated by http://vassarstats.net/.

Table 7: Analysis of risk factors associated with *T. gondii* infection in goats

	Risk factor	No. samples	No. positive	No. negative	Seroprevalence (%)	OR (95%CI)	*P*-value
Sex	Female	703	210	493	29.9		
	male	266	92	174	34.6	0.80 (0.59-1.08)	0.157
	Unknown	109	43	66	39.4	0.65(0.43-0.99)	0.044
Age	1-2 years	131	52	79	39.7		
	3-4 years	290	81	209	27.9	1.69 (1.10-2.62)	0.016
	5-6 years	230	65	165	28.3	1.67 (1.06-2.26)	0.026
	> 7 years	60	22	38	36.7	1.13 (0.60-2.13)	0.689
	Unknown	171	87	84	50.9	0.63 (0.40-1.00)	0.053
Regions	Central	358	86	272	24.0		
	Western	260	111	149	42.7	0.42(0.30-0.59)	<.0001
	Eastren	169	77	92	45.6	0.37(0.25-0.55)	<.0001
	Khangai	291	71	220	24.4	0.97(0.68-1.40)	0.920

OR: odd ratio

CI: confidence interval

- reference group

95% CI and *P*-value calculated by http://vassarstats.net/

Table 8: Seroprevalence of *T. gondi* in sheep in different provinces of Mongolia

Regions	Provinces	No.samples	No.Positive	Seroprevalence (%)	95% CI
Central region	Tuv	47	18	38.30	24.8-53.6
	Umnugobi	31	19	61.29	42.2-77.5
	Dornogobi	81	29	35.80	25.6-47.2
	Selenge	136	24	17.65	11.8-25.3
	Dundgobi	41	19	46.34	30.9-62.3
	Darkhan-Uul	5	1	20	1.05-70.1
Capital city	Ulaanbaatar	30	14	46.67	25.9-62.3
Western region	Gobi-Altai	54	25	46.30	32.8-60.2
	Khovd	78	25	32.05	22.1-43.7
	Bayan-Ulgii	32	11	34.38	19.1-53.2
	Uvs	80	18	22.50	14.2-33.4
	Zavkhan	20	4	20.00	6.6-44.2
Eastern region	Sukhbaatar	19	2	10.53	1.8-34.5
	Dornod	59	31	52.54	39.2-65.5
	Khentii	41	33	80.49	64.6-90.6
Khangai region	Bulgan	60	21	35.00	23.4-48.4
	Huvsgul	40	8	20.00	9.6-36.1
	Uvurkhangai	28	5	17.86	6.7-37.5
Total		882	307	34.81	31.6-38.0

CI, confidence interval calculated by http://vassarstats.net/.

Table 9: Analysis of risk factors associated with *T. gondii* infection in sheep

	Risk factor	No. samples	No. positive	No. negative	Seroprevalence (%)	OR (95%CI)	*P*-value
Sex	Female	522	169	353	32.38		
	Male	255	80	175	31.37	1.04(0.75-1.44)	0.777
	Unknown	105	58	47	55.24	0.38(0.25-0.59)	<.0001
Age	1-2 years	110	40	70	36.36		
	3-4 years	355	112	243	31.55	1.23 (0.79-1.94)	0.348
	5-6 years	293	88	205	30.03	1.33 (0.83-2.11)	0.224
	> 7 years	70	18	52	25.71	1.65 (0.85-3.20)	0.136
	Unknown	250	87	163	34.80	1.07 (0.67-1.70)	0.777
Regions	Central	371	124	247	33.42		
	Western	264	83	181	31.44	1.09(0.78-1.53)	0.597
	Eastern	119	66	53	55.46	0.40(0.26-0.61)	< .0001
	Khangai	128	34	94	26.56	1.38(0.88-2.17)	0.15

OR: odd ratio, CI: confidence interval, - reference group, 95% CI and *P*-value calculated by http://vassarstats.net/

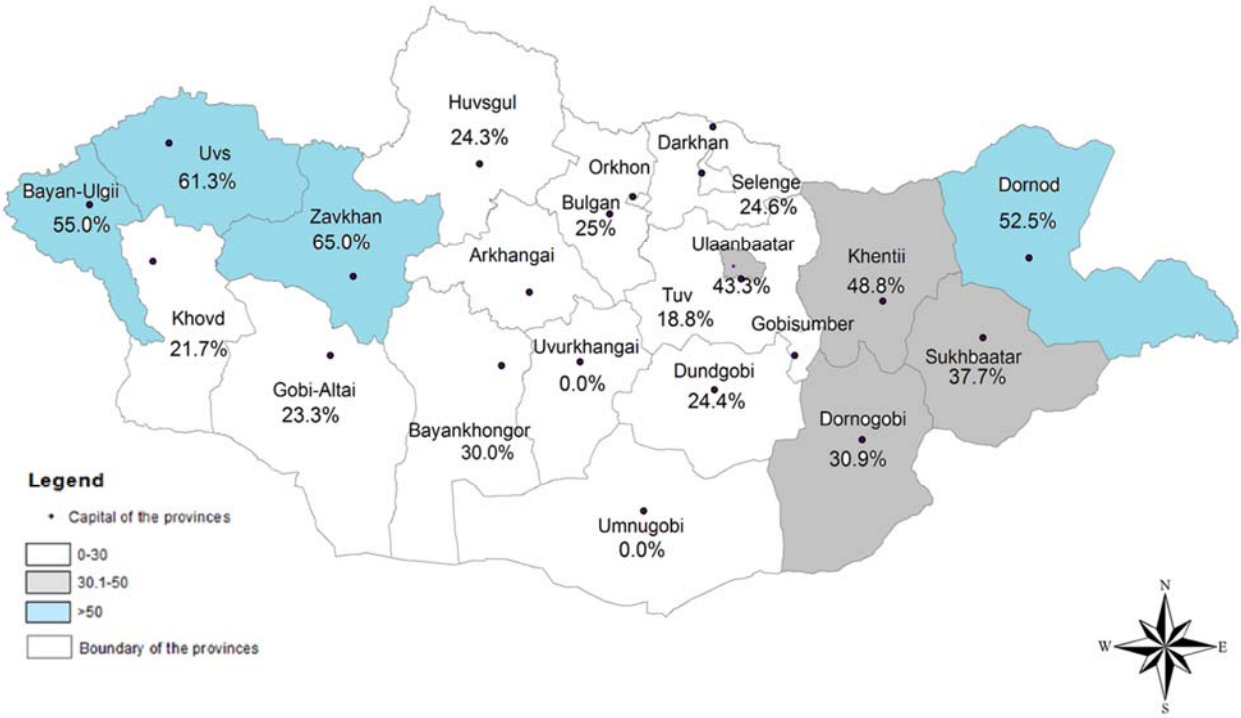


Fig. 3. Geographical distribution of *T. gondii* in Mongolian goat used in this study. Infection rates are indicated as follows: White 0-30%, grey 30.1-50% and light blue >50%.

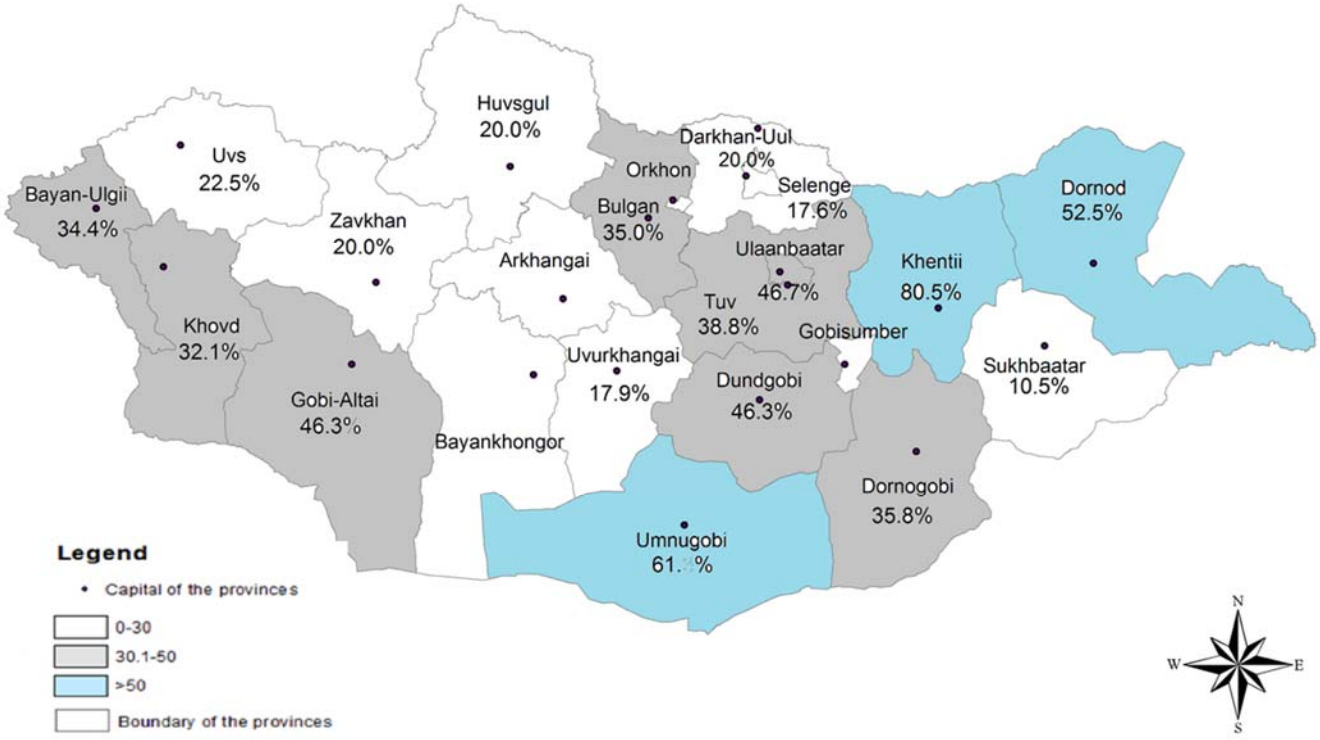


Fig. 4. Geographical distribution of *T. gondii* in Mongolian sheep used in this study. Infection rates are indicated as follows: White 0-30%, grey 30.1-50% and light blue >50%.

CHAPTER 3

Isolation and characterization of antiprotozoal compound-producing *Streptomyces* species from Mongolian soils

3.1. Introduction

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii*, an intracellular protozoan parasite. This common protozoan infection, which has a worldwide occurrence, can infect humans and all other warm-blooded animals (Hill et al., 2002). The recommended therapies against toxoplasmosis are sulfadiazine, pyrimethamine, and spiramycin. However, their ability to control toxoplasmosis is limited and significant side effects have been reported with standard treatments based on them (Peterson et al., 2003; Doliwa et al., 2013). This situation necessitates the discovery and development of new, effective drugs against toxoplasmosis.

In contrast, *Plasmodium,* another intracellular protozoan parasite and the causal agent of malaria, is restricted to tropical and subtropical regions of the globe. Malaria is a major life-threatening disease and was responsible for over 216 million cases and over 445,000 deaths worldwide in 2016 (World Malaria report, 2017). Millions of people are infected by malaria parasites annually, and many of them, especially children, die from these infections. A vaccine against malaria is not yet available, and chloroquine, sulfadoxine, pyrimethamine and artemisinin-based combination drugs are the currently available antimalarial agents. However, drug resistance has been documented for all antimalarial drug classes (Ashley et al., 2014).

Natural products derived from plants and microorganisms are recognized as an essential source of antibiotics and bioactive compounds. Among the microorganisms, actinomycetes are a

vital group of naturally occurring antibiotic producers (Barka et al., 2016). They are recognized for their wide range of biological activities and have been shown to have antibacterial (Heinemann et al., 1953; Kominek, 1972), antifungal (Hwang et al., 2001; Hohmann et al., 2009), antiparasitic (Burg et al., 1979), antiviral, antitumor, immunostimulatory, immunosuppressive, herbicidal (Omura et al., 1979) and enzyme inhibitory properties (Ganesan et al., 2011).

Actinomycetes characterized by a complex life cycle belonging to the phylum *Actinobacteria*, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria (Barka et al., 2016).

The *Streptomyces* species are filamentous gram-positive bacteria and belonging to the class of *Actinomycetes* and phylum of *Actinobacteria,* more than 450 *Streptomyces* species have been described (Anderson et al, 2001). They are widely distributed in terrestrial and aquatic ecosystems and are abundant in soil. This bacterial group is well known for their ability to produce important bioactive compounds. Indeed, approximately 7,630 bioactive metabolites are known to be produced by *Streptomyces* species (Berdy, 2005).

Mongolia is one of the largest landlocked countries in the world and its extreme and variable climate has preserved ecosystems with rich microbial biodiversity (Baljinova, 2015). Recently, *Streptomyces* species have been found in soil and potential sources of bioactive secondary metabolites explored by researchers in Mongolia (Norovsuren et al., 2007; Ara et al., 2013). In the present study, Mongolian soil-isolated actinomycetes isolates were assessed for their anti-*Toxoplasma* and antimalarial activities.

3.2. Material and methods

Isolation of actinomycetes

Soil samples were collected from Khentii and Uvurkhangai provinces and from around Ulaanbaatar, Mongolia's capital city. The samples were obtained by removing a 5 to 10 cm depth of the loose surface litter layer. The samples were dried, and appropriate aliquots of the diluted samples were prepared in saline solution and then spread on Gauze's synthetic no. 1 media (Soluble starch: 20 g, KNO_3: 1 g, NaCl: 0.5 g, $MgSO_4 \cdot 7\ H_2O$: 0.5 g, K_2HPO_4: 0.5 g, $FeSO4 \cdot 7\ H_2O$: 0.01 g, Agar: 15 g, Distilled water: 1L, pH 7.4). Single actinomycetes colonies were purified by re-streaking onto agar plates containing the same media, after which they were stored as slants in vials at 4 ˚C. The Microbial Synthesis Laboratory of the Institute of General and Experimental Biology, Mongolian Academy of Sciences screened the actinomycetes from the soil samples for bioactive compounds, and four isolates (N6, N12, N18, N25) were selected for evaluation in this study.

Phylogenetic analysis

Genomic DNA was extracted using the QIAamp DNA blood mini kit *(Qiagen, Hilden, Germany)* according to the manufacturer's instructions. The 16*S* rRNA gene from *Streptomyces* sp. was polymerase chain reaction (PCR)-amplified using 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 1492R (5′-TAC GGC TAC CTT GTT ACG ACT T-3′), and 330F (5′-CGG CCC AGA CTC CTA CGG GAG GCA GCA-3′) universal primers (Lane et al., 1991). The PCR products from the isolates were purified using a PCR product purification kit (Qiagen, Hilden, Germany). The PCR amplicons were confirmed as authentic 16*S* rRNA products via sequencing on the 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The resultant sequences were

analyzed by utilizing the Molecular Evolutionary Genetics Analysis 10.0 package with computing platform software (MEGA X) (Kumar et al., 2018). The sequences from the isolates were compared with the sequences in the GenBank database (www.ncbi.nlm.nih.gov). A phylogenetic tree was then constructed using the maximum-likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

Fermentation and compound extraction

In this study, the strains were grown on ISP 2 (International Streptomyces project-2) broth at 28°C for 7 days as a production media for obtaining crude extracts. The fermentation was carried out with 250 ml of ISP 2 media in a 500 ml Erlenmeyer flask at 28°C for 7 days with shaking (200 rpm). The supernatant and mycelia from each sample was separated by centrifugation (2,236 × g, 10 min). Each supernatant was extracted with an equal volume of ethyl acetate and evaporated using the Ren-1000 rotary evaporator at 50°C (IWAKI, Shizuoka, Japan). Crude extracts were obtained from four *Streptomyces* strains for antiprotozoal testing.

Fractionation and compound identification

Fractionation of the compounds was carried out on thin layer chromatography (TLC) plates (Silica gel 60, $F_{254+366}$, 2 mm, EMD Millipore, Billerica, MA, USA). Chloroform and methanol (9:1 ratio) were used as the solvent system to fractionate the crude extracts. The fractions were scraped from each TLC plate, and ethyl acetate was added to the scrapings. The silica was then removed by centrifugation. The purified fractions were further subjected to liquid chromatography-high resolution mass spectrometry (LC-HRMS) to identify candidate compounds.

LC-HRMS analysis

LC-HRMS was performed using the following conditions: high-performance liquid chromatography (HPLC) was carried out with the UltiMate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) using a SPELCO Ascentis Express C18 column (2.1 mm i.d. × 50 mm, Sigma-Aldrich, St Louis, MO, USA) at 40°C. The mobile phase consisted of 0.1% HCOOH aq. (A) and 0.1% HCOOH-CH_3CN (B), with a gradient of 95% A to 0% A (0 to 5 min) and 0% A (5 to 8.5 min). The flow rate was 0.4 ml min^{-1}. MS data acquisition was performed on the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using positive electrospray ionization with a spray voltage of 4 kV and a capillary temperature of 350°C. The MS range was scanned from m/z 100 to 2000 (resolution 15,000), and the MS data analyses were performed by Xcalibur 2.2 (Thermo Fisher Scientific).

Chemicals

Resistomycin (BioViotica, Dransfeld, Germany), amphomycin (Bioaustralis, Smithfield NSW, Australia), bafilomycin A1 (Sigma-Aldrich) and tylosin phosphate (LKT Laboratories, St. Paul, MN, USA) were prepared in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution, followed by storage at −30°C until use. Phenazine-1-carboxylic acid (PCA) was prepared from a 10 mg/ml stock solution (Toronto Research Chemicals, North York, Canada) in DMSO. All the crude extracts and fractions were also prepared in DMSO.

In vitro anti-Toxoplasma activity

Human foreskin fibroblast (HFF) cells were grown in 96-well plates (100 µl cell suspensions containing 1×10^5 cells/ml) in Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Nichirei Bioscience, Tokyo, Japan). The HFF cells were incubated at 37°C in a 5% CO_2 atmosphere for 48 h before they were infected with 100 µl of the green fluorescent protein-expressing *T. gondii* RH strain (RH-GFP) (Nishikawa et al., 2003) at 5×10^4 tachyzoites per well, with incubation for 4 h. The extracellular parasites were aspirated and new media (100 µl/well) was added before adding the extracts and compounds to the 96-well plates. The crude extracts and/or compounds (100 µl/well) were added to the infected HFF cells after 4 h infection and then incubated for a further 72 h. The fluorescence intensity of RH-GFP was measured by a microplate reader (SH-900, Corona, Ibaraki, Japan). The percentage inhibition of parasite growth (RH-GFP) was calculated by utilizing the following formula: [(average fluorescence intensity of GFP with medium alone) − (the fluorescence intensity of GFP treated with either of the extracts or compounds) / (average fluorescence intensity of GFP with medium alone)] × 100 (Leesombun et al., 2016).

In vitro antimalarial activity

P. falciparum (3D7 strain) cultures were maintained using human erythrocytes (1% hematocrit) in RPMI 1640 medium (Sigma–Aldrich). *P. falciparum* was synchronized to the ring stage by sorbitol lysis to >90% purity. The crude extracts and compounds with the desired concentrations were then added to the 96-well plates (50 µl per well). The infected RBCs (50 µl per well, parasitemia: 0.5%, hematocrit: 2%) were added and incubated at 37°C for 24 h (Kimura et al., 2012). Parasite growth was determined by adding 100 µl of lysis buffer with SYBR Green I stain (SYBR® Green I Nucleic Acid Stain 10,000×, Lonza Rockland, Rockland, ME, USA). The

relative fluorescence inhibition values of the wells were determined using a fluorescence plate reader (Fluoroskan Ascent, Thermo Fisher Scientific, Vantaa, Finland) at 485–518 nm excitation and emission wavelengths. Parasite growth inhibition percentages were calculated using the following formula: [(average excitation and emission wavelengths for *P. falciparum* with media) − (average excitation and emission wavelengths for *P. falciparum* with either of the extracts or compounds) / (average excitation and emission wavelengths for *P. falciparum* with media)] × 100, as described above. The uninfected erythrocytes and background fluorescence signals from the drugs were subtracted (Smilkstein et al., 2004).

In vitro cytotoxicity assay

HFF cells in DMEM supplemented with 10% FBS were added to 96-well plates (cell suspensions, 1×10^5 cells/ml) and incubated at 37°C with 5% CO_2 for 48 h. The compounds or the crude extracts were prepared in DMSO and tested at 100 µg per ml, the highest final concentration. Cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added to the culture after 72 h of incubation and the cell viability was measured at 450 nm using the MTP-120 microplate reader (Corona, Ibaraki, Japan). HFF cell viability (%) was expressed as [(the absorbance of cells treated with the extracts or compounds / (the absorbance of cells cultured with medium alone) × 100] (Leesombun et al., 2016).

Hemolysis assay

Each of drugs were prepared in PBS as designed concentration and then added 3% of erythrocyte suspension in PBS. All the sample tubes incubated at 37ºC for 3 h. And then the mixtures were centrifuged at 1500 rpm for 5 min. 100 µl of supernatant of all samples transferred

into 96-well plate. The absorbance values of supernatants were determined with microplate reader at 540 nm. The hemolysis rate of RBCs was calculated by using the following formula.

$$\text{Hemolysis rate} = (A_{\text{sample}} - A_{\text{negative control}})/(B_{\text{positive control}} - B_{\text{negative control}}) \times 100\%$$

Statistical analysis

GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was used for analysis of the IC_{50} values from the percentage inhibition of the parasites. The final mean IC_{50} values were calculated based on at least three independent experiments, and the data represents the mean ± SD.

3.3. Results

Isolation and identification of actinomycetes

In the present study, four actinomycetes (N6, N12, N18, N25) were isolated from four different soil samples in Mongolia. The 16S rDNA gene sequences for these actinomycetes and related organisms were retrieved from the GenBank database (www.ncbi.nlm.nih.gov). Comparisons of the GenBank gene sequences with those from the four isolates revealed that the isolates belong to the *Streptomyces* genus. The phylogenetic analysis revealed the following: isolate N25 (1,490 bp, GenBank accession number: MK509920) shares the highest nucleotide sequence identity (99%) with *Streptomyces canus* (NR_043347.1), strain N6 (1,489 bp, GenBank accession number: MK509917) shares 100% nucleotide sequence identity with *S. cirratus* (KR085952.1), strain N12 (1,492 bp, GenBank accession number: MK509918) shares 100% nucleotide sequence identity with *S. peucetius* (NR_024763.1), and strain N18 (1,492 bp, GenBank accession number: MK509919) shares 99% nucleotide sequence identity with *S. bacillaris* (NR_041146.1) (Fig. 5).

In vitro anti-Toxoplasma and antimalarial activities of antibiotics derived from Streptomyces spp.

Some well-known antibiotics derived from various *Streptomyces* species were tested *in vitro* against *T. gondii* and *P. falciparum* (Table 10). Kanamycin and resistomycin did not inhibit the growth of *T. gondii* or *P. falciparum*. However, amphomycin and tylosin phosphate inhibited *P. falciparum* growth with IC_{50} values of 9.3 µM and 182.7 µM, respectively. Bafilomycin A1 was also active against *P. falciparum* (IC_{50}: 34.1 nM), but its selectivity index (SI) was quite low (SI: 1.0). Amphomycin, tylosin phosphate and Bafilomycin A1 did not inhibit the growth of *T. gondii*.

In vitro anti-Toxoplasma and antimalarial activities of the crude extracts

To identify novel compounds with anti-*Toxoplasma* and antimalarial activities in the *Streptomyces* species present in our isolates, crude extracts from these isolates were tested (Table 11). The *S. bacillaris* (N18) and *S. canus* (N25) crude extracts both exhibited significant antiprotozoal activities against *T. gondii* (RH-GFP) and *P. falciparum* (3D7), respectively. However, the crude extracts from *S. cirratus* (N6) and *S. peucetius* (N12) were less effective in this regard. N18 inhibited *P. falciparum* (3D7) growth (IC_{50} of 156.9 ng/ml; SI: 2.01) and inhibited *T. gondii* growth (IC_{50} of 91.8 ng/ml; SI: 3.4), but cytotoxicity was observed against the HFF host cells (IC_{50} of 316 ng/ml). While the IC_{50} value of the N25 extract against *P. falciparum* was 4.9 µg per ml, the extract was also active against *T. gondii* (IC_{50} 19.2 µg/ml), with no apparent cytotoxicity noted in the HFF host cell line.

In vitro anti-Toxoplasma and antimalarial activities of TLC fractions from S. canus (N25)

Analysis of the *S. canus* (N25) crude extract revealed ten frations that produced single bands on the TLC plate (Fig. 9). After purifying the compounds, their cytotoxicities and their anti-*Toxoplasma* and anti-malarial activities were evaluated. Two of the ten fractions (Fractions 8 and 9) possessed antiprotozoal properties against *T. gondii* and *P. falciparum*, respectively (Table 3). Fraction 8 possessed antiprotozoal activity against *T. gondii* (IC_{50}: 2.6 µg/ml; SI: 53.4) and *P. falciparum* (IC_{50}: 4.8 µg/ml; SI: 28.9), but fraction 9 possessed the highest activity against *T. gondii* (IC_{50}:122.2 ng/ml, SI: 239.7) and *P. falciparum* (IC_{50}:163.8 ng/ml, SI: 178.8).

Identifying the chemical components in the fractions displaying antiprotozoal activity

The crude extract (90 mg) from *S. canus* (N25) was run on preparative silica gel TLC plates and developed with $CHCl_3$: MeOH (9:1) as the mobile phase to produce ten fractions (F1 to F10). The active fractions, F8 and F9, had a retention factor (Rf) of 0.76 and 0.91 to 0.86, and was extracted with a mixed solution of $CHCl_3$: MeOH (2:1) to yield 2 mg and 4 mg of the active compound, respectively. The active compound was showed single peak on silica gel TLC (hexane:ethyl acetate=1:9, Rf 0.9) and HPLC (Rt: 2.95 min). From LC-HRMS analysis; 225.0661 $[M+H]^+$ (calculated for $C_{13}H_9N_2O_2$: 225.0659) and 239.0820 $[M+H]^+$ (calculated for $C_{14}H_{11}N_2O_2$: 239.0815), PDA-UV spectrum and compared with standard compound, the active compounds were considered to be 1-phenazinecarboxylic acid (PCA) (Fig. 6) and its metabolite PCA methyl ester, respectively (Fig. 7).

In vitro anti-Toxoplasma and antimalarial activity of PCA

Commercially available PCA ($C_{13}H_8N_2O_2$: 224.21) was tested against *T. gondii* (IC_{50}: 55.5 µg/ml, 247.3 µM) and *P. falciparum* (IC_{50}: 6.4 µg/ml, 28.6 µM) *in vitro.* No cytotoxic effects of the candidate compound were observed in the HFF host cells (Fig. 8).

3.4. Discussion

Natural products play a vital role in the discovery and development of new and effective drugs against various human infections (Lahlou et al., 2007). Most *Streptomyces* species have the ability to produce various bioactive secondary metabolites with antibacterial, antitumor, antifungal, and anti-parasitic properties, as well as producing enzyme inhibitors. Widely distributed across ecosystems and gram-positive bacteria, Streptomycetes are important sources of natural antibiotics (Berdy, 2005).

Previous studies have reported on compounds isolated from *Streptomyces* spp. with potential antimalarial activities such as gancidin W (Zin et al., 2017), trioxacarcins (Maskey et al., 2004), metacycloprodigiosin (Isaka et al., 2002), and munumbicins (Castillo et al., 2002). Other antiparasitic compounds isolated from *Streptomyces* spp. (i.e., valinomycin, staurosporine and butenolide) are reportedly active against *Leishmania major*, *Trypanosoma brucei* and other *Trypanosoma* spp. (Pimentel-Elardo et al., 2010). Furthermore, spiramycin, a macrolide antibiotic produced by *S. ambofaciens* (Omura et al., 1979), was reported to be effective against acute toxoplasmosis and was able to prevent vertical transmission of the parasite (Serranti et al., 2011). In the present study, we isolated and identified four *Streptomyces* species from Mongolian soil samples. Based on their molecular characterizations, the isolates were identified as *S. canus* (N25), *S. cirratus* (N6), *S. bacillaris* (N18) and *S. peucetius* (N12). We also evaluated the efficacies of some well-known antibiotics (e.g., amphomycin, kanamycin, and resistomycin) against *T. gondii*

and *P. falciparum in vitro*, which are derived from the identified strains. *S. canus* produces several antibiotics including telomycin (Fu et al., 2015), amphomycin (Heinemann et al., 1953), amphomycin analogues (Yang et al., 2014), kanamycin (Abou-Zeid., 1971), and resistomycin and tetracenomycin D, which are antifungal metabolites (Zhang et al., 2013). Here, the antibiotic amphomycin was found to exhibit antimalarial activity against *P. falciparum* (IC_{50}: 9.3 μM), without any general cytotoxicity being observed. This is the first report on the antimalarial activity of amphomycin. The bafilomycin analogs, which were obtained from a marine-derived *S. bacillaris* actinomycetes bacterium, have been shown to have autophagy inhibitory activities (Carr et al., 2010; Hu et al., 2012). Moreover, an IC_{50} value of 0.041 ± 0.010 μg/ml was reported in a previous study exploring the *in vitro* antimalarial activity of bafilomycin A against *P. falciparum* (K1 strain) (Isaka et al., 2002). In the present study, bafilomycin A1 also exhibited *in vitro* antimalarial activity against *P. falciparum*, this time against the 3D7 strain, with an IC_{50} value of 34.1 nM (Table 1). However, cytotoxicity was observed in the host mammalian HFF cells with an IC_{50} value of 33.1 nM. In contrast, anti-*Toxoplasma* activity was not observed with these antibiotics (> 100 μM).

The crude extracts from all the strains (N6, N12, N18, N25) were then evaluated to identify novel compounds with antiprotozoal activities. The individual crude extracts from N12 and N6 did not inhibit parasite growth *in vitro* (>100 μg/ml). The crude extract from N18 was able to inhibit parasite growth (IC_{50}: 91.8 ng/ml for *T. gondii*, IC_{50}: 156.9 ng/ml for *P. falciparum* IC_{50}: 316 ng/ml for HFF cells). However, the cytotoxic effect of the N18 extract on the host cells prohibited further assessment of its antiprotozoal activity (SI: 3.4 for *T. gondii*, SI: 2.0 for *P. falciparum*) (Table 2). Consequently, because of its lower cytotoxicity (>100 μg/ml), only the N25

extract (IC_{50}: 19.2 µg/ml for *T. gondii*, IC_{50}: 4.9 µg/ml for *P. falciparum*) was fully evaluated chemically.

LC-HRMS is a powerful tool used for the identification of bioactive molecules (Nielsen et al., 2011). In my study, LC-HRMS was utilized to confirm the identity of the compound in the N25 extract as PCA and its derivative, PCA methyl ester. PCA and its derivatives comprise a large group of heterocyclic nitrogen-containing compounds produced by bacteria such as *Pseudomonas* spp. and *Streptomyces* spp. (Pierson and Pierson, 2010). PCAs display a broad-spectrum of biological properties including antifungal (Morohoshi et al., 2013), anticancer (Cimmino et al., 2013) and antimalarial activities (Hussain et al., 2011; De Souza et al., 2014). PCA, a precursor of other phenazine derivatives, is a well-known antibiotic with a broad spectrum of biological properties, and is also known as tubermycin B or shenqinmycin. Tubermycin B, an antibiotic produced by *P. aeruginosa*, possesses antifungal activity (Rane et al., 2007). Shenqinmycin is also an antifungal compound (Zhao et al. 2018). Previous studies have reported on a wide range of activities for PCA including antifungal (Abraham et al., 2015; Simionato et al., 2017), anti-bacterial, and anti-cancer (Karuppiah et al., 2016). PCA has previously been found to be a product of *S. kebangsaanensis* (Sarmin et al., 2013) and *S. anulatus* (Gebhardt et al., 2002). The present study is the first to report on PCA and its metabolite being identified in *S. canus* to date.

Hemolytic activities of crude extract N25, crude extract N18, Amphomycin, Bafilomycin A1, fractions, 8, 9,10 and PCA were investigated on human erythrocytes (RBCs). All the tested compounds have showed low hemolytic effect at a high concentration which is considering safe (Table 13).

Another study reported that PCA can react with O_2 to generate cell-damaging reactive oxygen species (Xu et al., 2015). To the best of our knowledge, antiprotozoal activities against toxoplasmosis and malaria have not been reported yet for PCA. Furthermore, the present study has shown that PCA derived from *S. canus* exhibited antiprotozoal activities against *T. gondii* (IC_{50}: 55.5 µg/ml) and *P. falciparum* (IC_{50}: 6.4 µg/ml) *in vitro*. This result indicates that *S. canus* (N25) is a potential antibiotic producer with anti-protozoan properties.

My study has reported on the antiprotozoal activities of *S. canus* (N25) derived from Mongolian soil samples against *T. gondii* and *P. falciparum*. That two previously known compounds, PCA and amphomycin, are produced by *S. canus* and also possess anti-*Toxoplasma* and antimalarial properties *in vitro*, is a new finding. The present results suggest that *S. canus* isolated from Mongolian soil may be a potential source of new treatments for human parasitic protozoan infections.

3.5. Summary

Natural resources are recognized as important sources of potential drugs for treating various infections, and microorganisms are a rich natural source of diverse compounds. Among the world's microorganisms, actinomycetes, which are abundant in soil and marine, are the well-known producers of a wide range of bioactive secondary metabolites and antibiotics. In the present study, four actinomycetes (samples N25, N6, N18, and N12) were isolated from soil samples in Mongolia. Phylogenetic analysis of these isolates revealed that they share the highest similarity with *Streptomyces canus* (N25), *S. cirratus* (N6), *S. bacillaris* (N18) and *S. peucetius* (N12), based on 16S rRNA gene sequencing. Crude extracts were obtained from them using ethyl acetate, and the crude fractions were separated by thin layer chromatography. The fractions were then

evaluated for their cytotoxicities and their anti-*Toxoplasma* and antimalarial activities *in vitro*. The *S. canus* (N25) crude extract was selected for further chemical characterization based on its antiprotozoal activities. Using liquid chromatography-high resolution mass spectrometry, phenazine-1-carboxylic acid (PCA) was detected and identified in the active fractions of the metabolites from strain N25. I next confirmed that commercially available PCA possesses antiprotozoal activity against *T. gondii* (IC_{50}: 55.5 µg/ml) and *Plasmodium falciparum* (IC_{50}: 6.4 µg/ml) *in vitro.* The results of this study reveal that soil actinomycetes are potential sources of antiprotozoal compounds, and that PCA merits further investigation as an anti-protozoal agent.

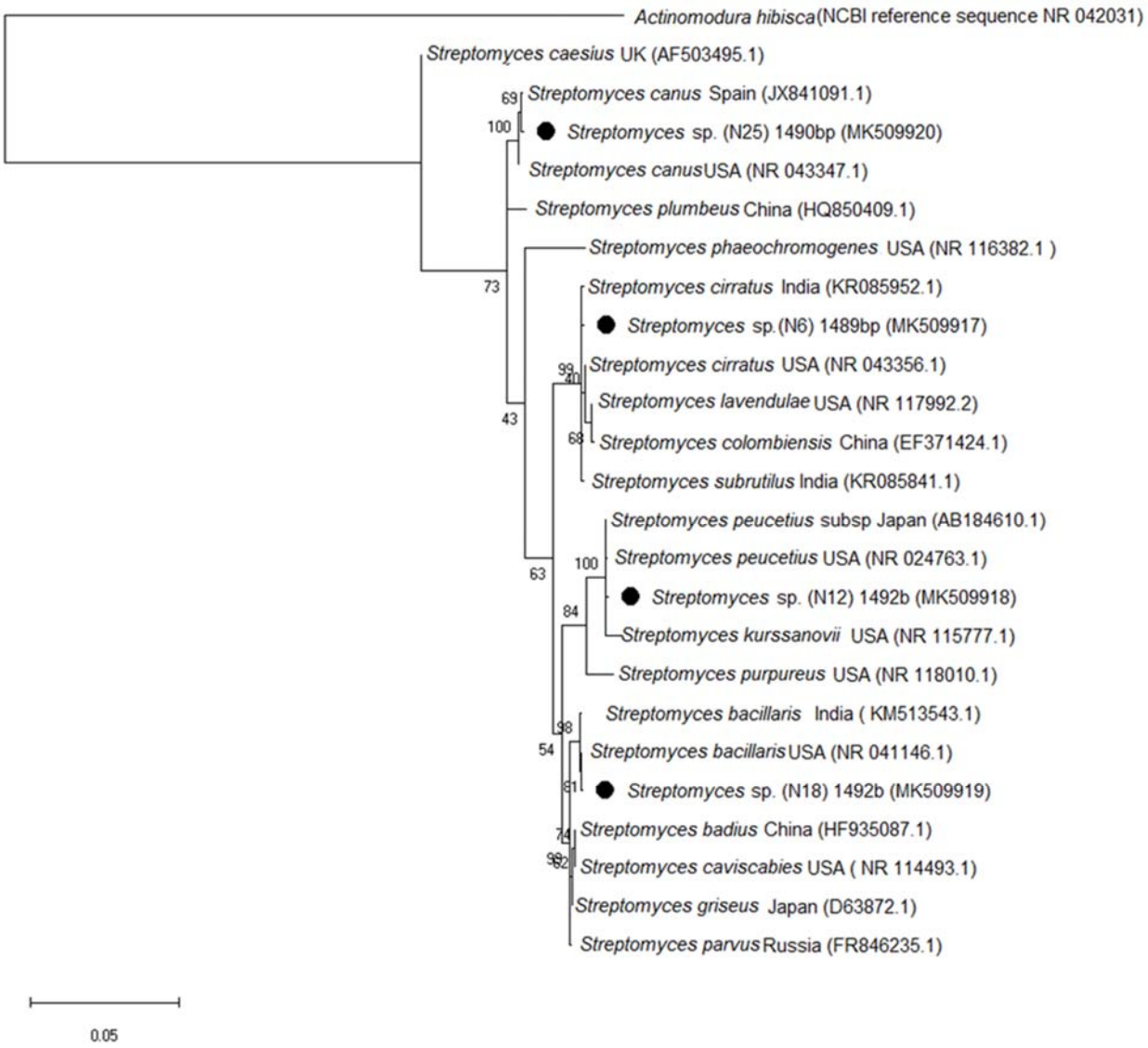
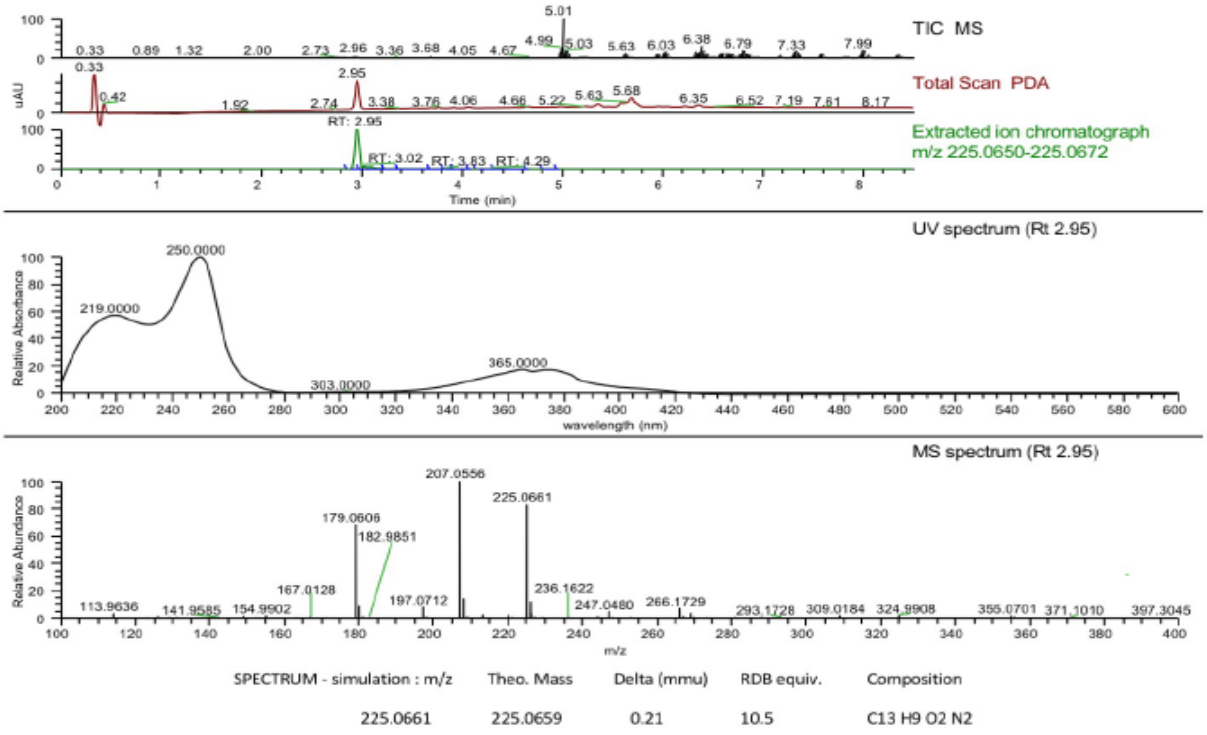


Fig. 5. 16S rRNA gene nucleotide sequences was subjected to a phylogenetic analysis for isolates.

A.



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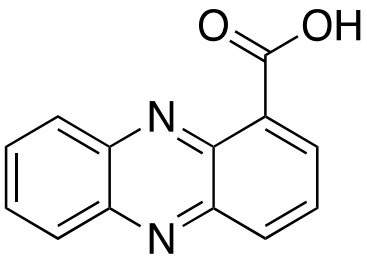
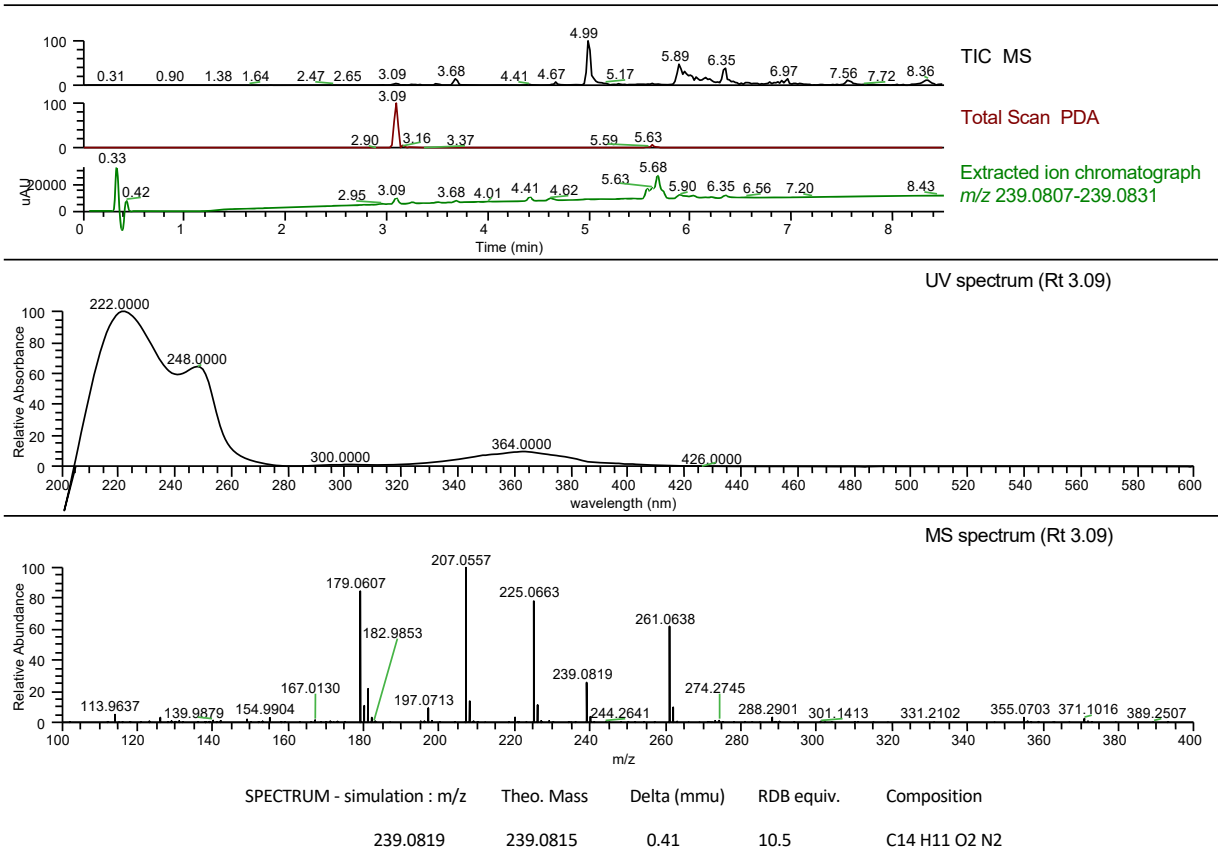


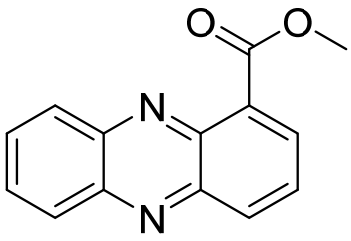
Phenazine-1-carboxylic acid

Fig. 6. A. LC-HRMS analysis, an ion with *m/z* 225.0661 $[M+H]^+$ was observed (the molecular formula was calculated as $C_{13}H_9N_2O_2$: 225.0659 atomic mass). **Fig. 6.B**. Its photodiode-array-ultra violet spectrum was compared with that of the standard compound, and the active compound was considered to be phenazine-1-carboxylic acid.

A.



B.



1-phenazinecarboxylic acid-methyl ester

Fig. 7. A. From LC-HRMS analysis calculated for $C_{14}H_{11}N_2O_2$: 239.0815.

Fig. 7. B. The compound was considered to be PCA methyl ester.

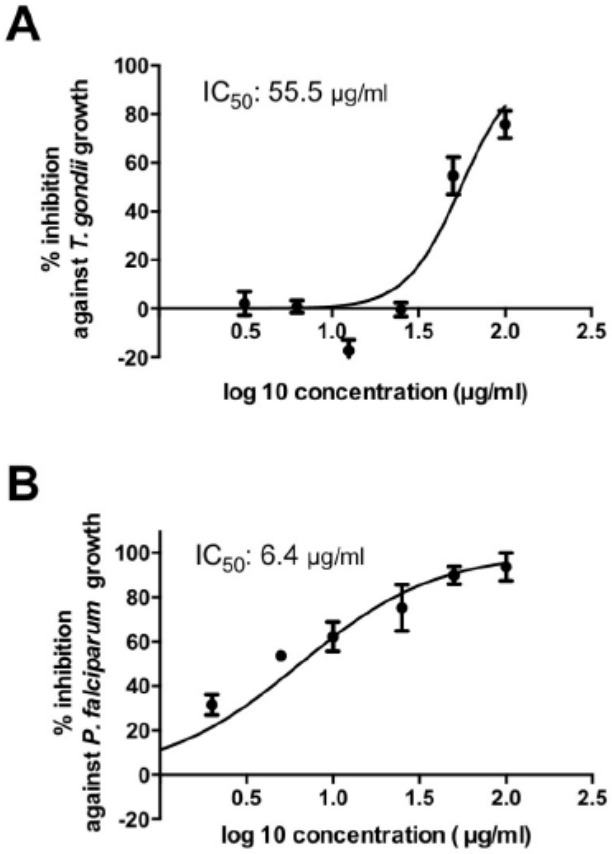


Fig. 8: *In vitro* anti-*Toxoplasma* and antimalarial activity of PCA

All IC_{50} values were calculated based on three independent experiments.

Table 10: Anti-*Toxoplasma* and animalarial activities of antibiotics derived from *Streptomyces*

Antibiotics	Actinomycetes	IC_{50} for *T. gondii*	IC_{50} for *P. falciparum* (Selectivity index)	IC_{50} for HFF cells
Amphomycin (Heinemann et al., 1953)	*Streptomyces canus*	>100 µM	9.3 µM	>100 µM
Kanamycin (Abou-Zeid et al., 1971)	*Streptomyces canus*	>100 µM	>100 µM	11.5 µM
Resistomycin (Zang et al., 2013)	*Streptomyces canus*	>100 µM	>100 µM	1.2 µM
Tylosine phosphate [7]	*Streptomyces cirratus*	>100 µM	182.7 µM	>100 µM
Bafilomycin A1 [20]	*Streptomyces bacillaris*	>100 µM	34.1 nM (1.0)	33.1 nM

All IC_{50} values were calculated based on three independent experiments.

Table 11: Anti-*Toxoplasma* and animalarial activities of crude extracts from four actinomycetes

Actinomycetes	IC_{50} for *T. gondii* (Selectivity index)	IC_{50} for *P. falciparum* (Selectivity index)	IC_{50} for HFF cells
N6	>100 µg/ml	>100 µg/ml	>100 µg/ml
N12	>100 µg/ml	>100 µg/ml	>100 µg/ml
N18	91.8 ng/ml (3.4)	156.9 ng/ml (2.0)	316 ng/ml
N25	19.2 µg/ml	4.9 µg/ml	>100 µg/ml

All IC 50 values were calculated based on three independent experiments.

Table 12: Anti-*Toxoplasma* and animalarial activities of the TLC fractions from *Streptomyces canus* (N25)

Fractions	IC_{50} for *T. gondii* (Selectivity index)	IC_{50} for *P. falciparum* (Selectivity index)	IC_{50} for HFF cells
1	>50 µg/ml	>50 µg/ml	>100 µg/ml
2	>50 µg/ml	>50 µg/ml	>100 µg/ml
3	>50 µg/ml	>50 µg/ml	>100 µg/ml
4	>50 µg/ml	>50 µg/ml	>100 µg/ml
5	>50 µg/ml	>50 µg/ml	>100 µg/ml
6	>50 µg/ml	>50 µg/ml	>100 µg/ml
7	>50 µg/ml	>50 µg/ml	>100 µg/ml
8	2.6 µg/ml (53.4)	4.8 µg/ml (28.9)	138.9 µg/ml
9	122.2 ng/ml (239.7)	163.8 ng/ml (178.8)	29.3 µg/ml
10	>50 µg/ml	18.4 µg/ml	>100 µg/ml

>50 µg/ml: No activity at 50 µg/ml; the highest dose tested.

The IC50 values of fractions 8, 9, and 10 were calculated based on three independent experiments.

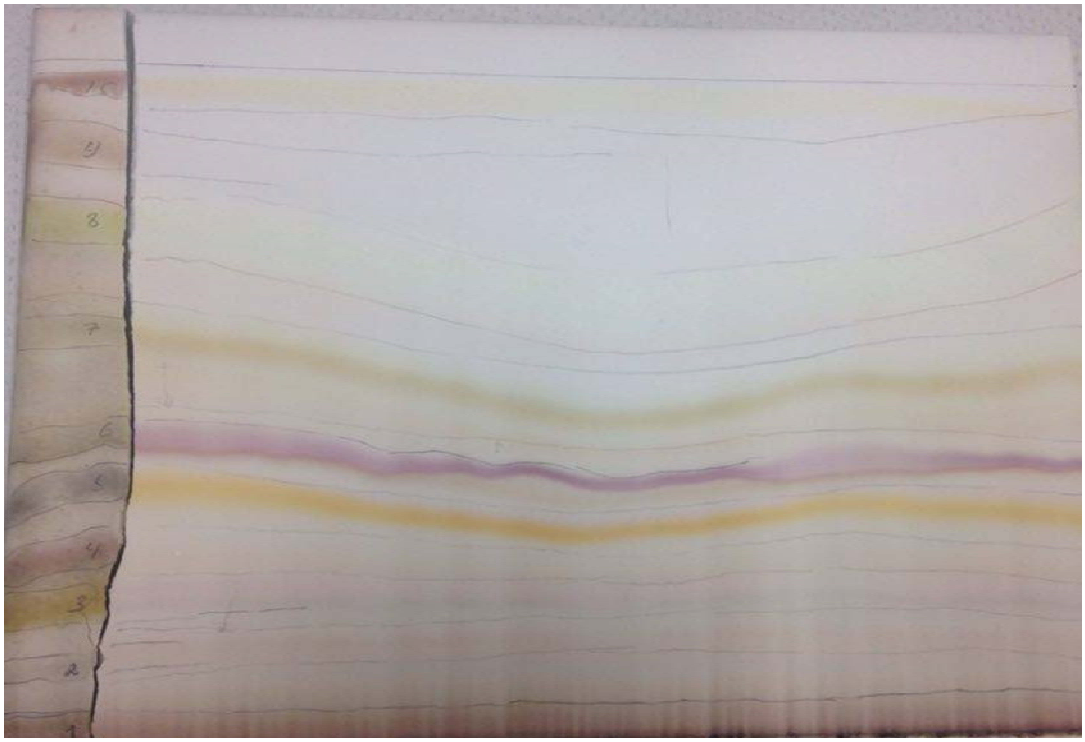


Figure 9. Thin layer chromatography (TLC)

The chloroform and methanol (9:1 ratio) were used as the solvent system to fractionate the crude extracts.

Table 13: Hemolysis rate (%)

Drugs	Concentrations	Hemolysis rate (%)
Amphomycin	100 µM	0.41
Bafilomycin A1	100 nM	7.78
Crude extract N25	10 µM	0.41
Crude extract N18	100 µg/ml	3.01
Fraction 8	100 µg/ml	-1.97
Fraction 9	100 µg/ml	0.10
Fraction 10	100 µg/ml	6.02
PCA	100 µg/ml	-1.35
Tylosine phosphate	100 µM	-4.8

Phosphate buffered saline (PBS) and RBC lysis buffer (0.83% NH_4Cl; 0.01 M Tris-HCl, pH 7.2) are used for negative and positive controls, respectively.

General discussion

Apicomplexan parasites including *Plasmodium falciparum* and *Toxoplasma gondii* are globally distributed protozoan parasites that cause human infectious diseases such as malaria and toxoplasmosis (Hill et al., 2002). Neosporosis is a serious disease in cattle and dogs caused by *Neospora caninum* and major reproductive problems of this infection are abortion and stillbirths in cattle and neurological disorders in dogs (Dubey et al., 2007). The livestock sector plays a crucial economic role in Mongolia because milk and meat products derived from livestock are served as main foods. The Mongolian livestock consist of cattle, sheep, goat, camel and horses, a total population are estimated to 66 million heads in 2018 (Mongolian statistical office, 2018). Generally, *T. gondii* or *N. caninum* infection affects the livestock industry by decreasing productivity due to abortion and stillbirth in livestock and available data of the prevalence of those parasites in Mongolia are limited. Additionally, discovery and development of new effective drugs against those infections from indigenous resources must be required. Therefore, the main objectives of the present study are to perform the country-wide seroprevalence of *T. gondii* in cattle, goat, sheep and *N. caninum* infections in cattle in Mongolia and to screen and characterize antiprotozoal compounds against *T. gondii* and *P. falciparum* from actinobacteria (*streptomyces* sp.) isolated from Mongolian soils.

In chapter 1, seroprevalences of *T. gondii* and *N. caninum* of cattle sera from 20 of 21 provinces of Mongolia were examined. Overall seroprevalences of *T. gondii* and *N. caninum* were 18.7% and 26.2%, respectively. In my study, both *N. caninum* and *T. gondii* prevalence rates in the central region of Mongolia (27.1% and 30.8%, respectively) were significantly higher than the other region, suggesting that climate condition may be one risk factor of such infections,

particularly warmer temperatures. In contrast, the overall lowest *T. gondii* and *N. caninum* prevalence rates (9% and 20.8%, respectively) were observed in the western region of Mongolia. The infections may increase in warmer and humid conditions than colder and dry climates (Tenter et al., 2000). Thus, climate and geographical conditions in Mongolia may affect both *N. caninum* and *T. gondii* seroprevalence rates.

Cattle infected with *T. gondii* may be an infectious source for human. However, human infection of *T. gondii* has not been well studied in Mongolia. Consumption of undercooked and raw meat is not a typical practice among Mongolians. Therefore, intake or consumption of contaminated water, soil, and raw vegetables might be a potential risk factor of *T. gondii* infection in human. Although another source of *T. gondii* infection is cats, members of the family *Felidae*, cats are not common domestic animals in Mongolia. Generally, domestic cats do not widespread in whole regions of Mongolia and herders do not regularly maintain cat as a pet. However, small number of domestic cats are kept in household to control rodents. Thus, the transmission of *T. gondii* infection via oocysts shed by domestic cats and wild felines may facilitate infection in human and livestock.

In the present study, the seroprevalence of *N. caninum* was significantly higher in female cattle (27.5%) compared with male cattle (20.4%), suggesting that sex is an important risk factor. This result may affect reproductive problems such as abortion and stillbirth in cattle in Mongolia. *N. caninum*-specific antibody titers increase during pregnancy and peak within a month after calving in *N. caninum*-infected dams (Ybañez et al., 2013). Therefore, the detection rate of *N. caninum*-specific antibodies in female cattle might be higher than that in male animals.

Moreover, I investigated the mixed infection with *T. gondii* and *N. caninum* in cattle. The seroprevalence rate was 6.8%, indicating that the mixed infection with *T. gondii* and *N. caninum* are not common in Mongolia.

Further studies are urgently needed to determine the infection of *T. gondii* and *N. caninum* in cats and dogs in Mongolia. They would be helpful to understand of infection sources of those diseases. Especially, to our knowledge, there are no reports about the prevalence of *N. caninum* infection in the dogs in Mongolia. Therefore, the relationship between dogs and cattle for *N. caninum* infection in Mongolia should be studied.

In chapter 2, a total 1,078 goats and 882 sheep blood samples were investigated the seroprevalence of *T. gondii* from 17 of 21 provinces and capital city of Mongolia. The present study showed that relatively higher seroprevalence of *T. gondii* was observed among sheep (34.8%) and goat (32%) than cattle (18.7%), respectively. Sheep and goats are highly susceptible to *T. gondii* infection which play a major role in the transmission of toxoplasmosis to humans (Tzanidakis, et al., 2010). In this study, the risk factors of sex, age and location to the seroprevalence were investigated.

Among the risk factors, only the location had a significant association with *T. gondii* seropositivity. The higher seroprevalence might be related to the location and differences of the environmental condition at the sampling area. Mongolia has an extreme climate and highly variable between to the regions. The significantly higher seroprevalence of *T. gondii* in goats was found in the Eastern region (45.5%) which can be attributed to the lower altitude of the Eastern region of Mongolia. Moreover, the highest seroprevalence of *T. gondii* in sheep was observed in the Eastern region of Mongolia (55.4%).

The risk factors of sex and age to the seroprevalence were investigated. No significant differences were observed according to the sex as predisposing factors for *T. gondii* infection in goat and sheep in Mongolia. The highest seroprevalence of *T. gondii* in sheep and goat were observed in young animals than old animals, which probably due to the different numbers of animals available for sampling.

My current data could be useful for Mongolian veterinarians and householders in order to develop and improve prevention and control measure of toxoplasmosis in livestock. Furthermore, it will be important to determine whether domestic and wild cats shed oocysts in provinces with higher seroprevalences. In addition, the impact of *T. gondii* infection in human should be studied based on the issue of food safety and public health.

In chapter 3, natural products play a vital role in the discovery and development of new and effective drugs against various human infections (Lahlou et al., 2007). Most *Streptomyces* species have on ability to produce various bioactive secondary metabolites with antibacterial, antitumor, antifungal, and anti-parasitic properties. The streptomyces, which are gram-positive bacteria, widely distributed across ecosystems, and they are important sources of natural antibiotics (Berdy, 2005).

In the present study, I isolated and identified four *Streptomyces* species from Mongolian soil samples and identified as *S. canus* (N25), *S. cirratus* (N6), *S. bacillaris* (N18) and *S. peucetius* (N12). I also evaluated the efficacies of some well-known antibiotics (e.g., amphomycin, kanamycin and resistomycin) against *T. gondii* and *P. falciparum in vitro*, which are derived from the identified strains.

Here, the antibiotic amphomycin was found to exhibit antimalarial activity against *P. falciparum* (IC_{50}: 9.3 μM), without any general cytotoxicity. This is the first report on the antimalarial activity of amphomycin. In the present study, bafilomycin A1 also exhibited *in vitro* antimalarial activity against *P. falciparum* (3D7 strain) (IC_{50}: 34.1 nM). However, cytotoxicity was observed in the mammalian cells, HFF cells (IC_{50}: 33.1 nM). In contrast, anti-*Toxoplasma* activity was not observed with these antibiotics (> 100 μM).

The crude extracts from all the strains (N6, N12, N18 and N25) were then evaluated to identify novel compounds with antiprotozoal activities. Although the crude extracts from N12 and N6 did not inhibit parasite growth *in vitro* (>100 μg/ml), the crude extract from N18 was able to inhibit the growth of *T. gondii* (IC_{50}: 91.8 ng/ml), *P. falciparum* (IC_{50}: 156.9 ng/ml) and HFF cells (IC_{50}: 316 ng/ml). Furthermore, the N25 extract exhibited good antiprotozoal activities against *T. gondii* (IC_{50}: 19.2 μg/ml) and *P. falciparum* (IC_{50}: 4.9 μg/ml), and shows lower cytotoxicity (>100 μg/ml). Therefore, the N25 extract was further evaluated.

In my study, LC-HRMS was utilized to identity the compound in the N25 extract. Then, I successfully identify Phenazine-1-carboxylic acid (PCA) from the N25 extract. PCA and its derivatives comprise a large group of heterocyclic nitrogen-containing compounds produced by bacteria such as *Pseudomonas* spp. and *Streptomyces* spp. (Pierson and Pierson, 2010). The present study is the first to report that PCA was identified in *S. canus*.

Wide range activities of PCA including antifungal (Abraham et al., 2015; Simionato et al., 2017), anti-bacterial, and anti-cancer (Karuppiah et al., 2016) have been reported. However, the activities against *T. gondii* and malaria parasites of PCA have not been confirmed yet. This study shows that PCA exhibited antiprotozoal activities against *T. gondii* (IC_{50}: 55.5 μg/ml) and *P. falciparum* (IC_{50}: 6.4 μg/ml) *in vitro*

My present study demonstrates seroprevalences of *T. gondii* and *N. caninum* in Mongolia that provides valuable new data for development of control measures against these infections in Mongolia. Additionally, the study reveals soil actinobacteria have anti-*Toxoplasma* and antimalarial properties and that funding suggest Mongolian natural resources could be a potential source of treatments for human infections.

General summary

Toxoplasmosis and neosporosis affect the livestock industry by decreasing productivity due to abortion and stillbirth of livestock. Those infections may be a hazard to the livestock industry in Mongolia as a main sector of the economy in the country. Because of the limited information of *T. gondii* and *N. caninum* infections, my present study proposed to determine large-scale seroprevalence of *T. gondii* and *N. caninum* and identify major risk factors in livestock in Mongolia. In addition, antiprotozoal compounds from soil bacteria in Mongolia were characterized in order to develop effective chemotherapeutic agents against toxoplasmosis and Malaria.

In chapter 1, a total of 1,438 cattle sera from 20 of 21 provinces of Mongolia and the capital city of Ulaanbaatar were tested. Overall, 18.7% and 26.2% of cattle were positive for specific antibodies to *T. gondii* and *N. caninum*, respectively. Prevalence rates were higher (*T. gondii* infection: $P < 0.0001$, *N. caninum* infection: $P = 0.002$) in the central region of Mongolia (*T. gondii* infection: 27.1%, *N. caninum* infection: 30.8 %) compared with western region, suggesting that prevalence rates might be influenced by geographical condition, particularly warmer temperatures around this area in Mongolia. The lowest prevalence rates were observed in the western region of Mongolia (*T. gondii*: 9%, *N. caninum*: 20.8%). In addition, the seroprevalence of *N. caninum* in female animals (27.5%) was significantly higher than that in male animals (20.4%) ($P = 0.018$), suggesting an important risk factor of abortion and stillbirth in cattle. The present results showed that *T. gondii* and *N. caninum* infections might be a risk for public health and economy of the livestock industry in Mongolia.

In chapter 2, a total of 1,078 goat and 882 sheep blood samples were collected from 17 of 21 provinces and the capital city of Mongolia. Overall, the seroprevalence of *T. gondii* among the

goat and sheep samples was 32% and 34.8%, respectively. The seroprevalence among goat samples was significantly higher in western (42.7%) and eastern (45.6%) regions compared with other regions (24%). Additionally, the seroprevalence among sheep was significantly higher in eastern regions (55.4%) compared with other regions (26%–33%). Age, but not sex, was considered a risk factor for *T. gondii* seropositivity in goats, whereas no statistically significant differences were observed in sheep for age or sex. In conclusion, the present study demonstrates the high seroprevalence of *T. gondii* in small ruminants in Mongolia.

In chapter 3, four species of actinomycetes were isolated from soil samples in Mongolia. Phylogenetic analysis of these isolates revealed that they shared the highest similarity with *Streptomyces canus* (N25), *S. cirratus* (N6), *S. bacillaris* (N18) and *S. peucetius* (N12), based on 16S rRNA gene sequencing. Crude extracts obtained using ethyl acetate were separated by thin layer chromatography. The fractions were then evaluated for their cytotoxicities and their anti-*Toxoplasma* and antimalarial activities *in vitro*. The *S. canus* (N25) crude extract was selected for further chemical characterization based on its antiprotozoal activities. Using liquid chromatography-high resolution mass spectrometry, phenazine-1-carboxylic acid (PCA) was detected and identified in the active fractions of the metabolites from strain N25. I next confirmed that commercially available PCA possesses antiprotozoal activity against *T. gondii* (IC_{50}: 55.5 μg/ml) and *Plasmodium falciparum* (IC_{50}: 6.4 μg/ml) *in vitro.* The results of this study reveal that soil actinomycetes are potential sources of antiprotozoal compounds, and that PCA merits further investigation as an anti-protozoal agent.

My current study demonstrated high seroprevalences of *T. gondii* and *N. caninum* in livestock in Mongolia which provides new data for development of control measures against these

infections. Additionally, my results suggested that *S. canus* isolated from Mongolian soil may be a potential source of new drugs for parasitic infections.

和文要約

トキソプラズマ症とネオスポラ症は流産や死産により家畜動物の生産性を低下させるため畜産業に悪影響を与える。これらの原虫感染症はモンゴルの主要産業である牧畜業にとっても危険となる。モンゴルにおけるこれら原虫感染症の情報は限られているため、本研究ではモンゴルにおけるトキソプラズマとネオスポラの大規模血清疫学調査を実施し、主な感染リスク要因を見出すことを目的とした。加えて、トキソプラズマ症やマラリアなどの原虫感染症に対する効果的な治療薬を開発するために、モンゴルの土壌細菌由来抗原虫化合物の解析を行った。

第一章では、首都ウランバートルとモンゴル 21 県のうち 20 県から採材した牛血清 1,438 検体を用いて抗体検査を実施した。全体として、トキソプラズマ抗体陽性率は 18.7%、ネオスポラ抗体陽性率は 26.2%であった。モンゴル中央部の抗体陽性率は西部よりも優位に高く（トキソプラズマ抗体陽性率：27.1%、ネオスポラ抗体陽性率：30.8%）、地理的条件、特に他の地域に比べて中央部での温暖な気候が影響していると示唆された。モンゴル西部では最も低い抗体陽性率が確認された（トキソプラズマ抗体陽性率：9%、ネオスポラ抗体陽性率：20.8%）。加えて、雄牛と比較して、雌牛のネオスポラの抗体陽性率が優位に高いことが認められ（雄牛：20.4%、雌牛：27.5%）、流産・死産の重要なリスク要因になることが考えられた。本研究により、モンゴルでのトキソプラズマ及びネオスポラの感染は公衆衛生上の問題と農牧業の経済にとってリスク要因になることが示唆された。

第二章では、首都ウランバートルとモンゴル 21 県のうち 17 県から採材したヤギ血清 1,078 検体とヒツジ血清 882 検体を用いてトキソプラズマ抗体検査を実施した。全体として、トキソプラズマ抗体陽性率はヤギで 32%、ヒツジで 34.8%であった。ヤギにおける抗体陽性率は西部（42.7%）と東部（45.6%）で優位に高く、ヒツジにおける抗体抗体陽性率は東部（55.4%）で優位に高い結果となった。ヤギにおける抗体陽性率は年齢がリスク要因になることが推測されたが、ヒツジにおける抗体陽性率は年齢や性別はリスク要因とはならなかった。本研究により、モンゴルの小型反芻獣にトキソプラズマが広範囲に蔓延していることが明らかとなった。

第三章では、モンゴルの土壌サンプルから分離された 4 種類の放線菌を用いて、抗原虫活性の解析を行った。16S rRNA 配列を用いてこれら分離株の系統解析を実施し、*Streptomyces canus*（N25）、*S. cirratus*（N6）、*S. bacillaris*（N18）、*S. peucetius*（N12）と類似性が高いことが明らかとなった。酢酸エチルで調整した粗抽出サンプルを薄層クロマトグラフィーで分離し、それぞれの画分について細胞毒性、抗トキソプラズマ活性、抗マラリア活性を *in vitro* で評価した。その結果、*S. canus*（N25）の粗抽出サンプルに抗トキソプラズマ活性と抗マラリア活性があることが明らかとなった。高分解能 LC/MS を用いた解析により、活性画分から phenazine-1-carboxylic acid（PCA）を同定した。市販の PCA を用いて抗原虫効果を評価したところ、トキソプラズマ（IC_{50}: 55.5 μg/ml）および熱帯熱マラリア原虫（IC_{50}: 6.4 μg/ml）に対する増殖阻害効果を示した。本研究により、土壌放線菌は抗原虫薬の有望な生物資源であることが示唆され、PCA の今後の詳細な解析が期待された。

本研究により、モンゴルの家畜動物におけるトキソプラズマとネオスポラの蔓延が明らかとなり、これら原虫感染症対策に資する重要なデータを提供することができた。さらに、モンゴルの土壌から分離された *Streptomyces canus* は新しい寄生虫症治療薬の有望な生物資源になると考えられた。

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