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Serodiagnosis of ovine toxoplasmosis in Mongolia by an enzyme-linked immunosorbent assay with recombinant *Toxoplasma gondii* matrix antigen 1

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Abstract

Toxoplasma gondii matrix antigen 1 (TgMAG1), known as the 65-kDa protein, which is abundantly expressed in both bradyzoites and tachyzoites, was evaluated as a candidate for the development of a diagnostic reagent for ovine toxoplasmosis. The TgMAG1 gene was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST), and the recombinant TgMAG1 (rTgMAG1) was tested in an enzyme-linked immunosorbent assay (ELISA). The ELISA with rTgMAG1 showed a highly specific reaction with sera from mice experimentally infected with *T. gondii* but not with the closely related *Neospora caninum*. The antibodies to TgMAG1 were detectable from the acute to the chronic infectious stages in a mouse model. A total of 175 serum samples collected from sheep in 7 provinces of Mongolia were examined for the serodiagnosis of *T. gondii* infection by the ELISA with rTgMAG1, and the results were compared with those from the commercialized latex agglutination test (LAT). Of 175 serum samples analyzed, 42 (24.00%) and 29 (16.57%) samples were positive by the ELISA and LAT, respectively. Of 29 LAT-positive samples, 27 (93.10%) were positive by the ELISA. These results suggest that rTgMAG1 could be used as a reliable antigen for the detection of *T. gondii* infection in sheep.

Key words: ELISA, Mongolia, sheep, TgMAG1, *Toxoplasma gondii*

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Introduction

Toxoplasma gondii is an obligate intracellular parasite that infects a variety of mammals, including humans and domestic animals. Infection occurs through ingestion of undercooked meat, exposure to and ingestion of oocysts excreted in feces of infected cats, and transplacental transmission from an acutely infected woman to her fetus^{6,27}. Infection generally produces mild or asymptomatic diseases in healthy adults but can cause serious illness in immunocompromised patients⁶. Toxoplasmosis causes heavy economic loss within the sheep industry, an important source of food for humans worldwide^{5,7}. Indeed, the infection not only results in significant reproductive (and hence economic) loss but also has implications for public health and zoonotic transmission because of the consumption of infected meat or milk^{7,8}. The life cycle of *T. gondii* has two phases: the sexual phase, which takes place only in members of the Felidae family (domestic and wild cats), and the asexual phase, which takes place in any warm-blooded animal⁶. The clinical ovine toxoplasmosis occurs when the oocysts excyst in the small intestine and release sporozoites, which differentiate into tachyzoites by the fourth day and multiply in the mesenteric lymph nodes; during this time, the parasite may be found circulating in the blood. At this stage, sheep develop a fever that may last until 10 days after infection and may be accompanied with early embryonic death and abortion. Later, the tachyzoites change into bradyzoites and tissue cysts that persist mainly within the muscles and brain^{4,7}.

Laboratory diagnosis of *T. gondii* infection is based on the detection of the parasite by means of polymerase chain reaction (PCR), mouse inoculation, or cell culture assays and on serological tests that detect specific immunoglobulin antibodies^{6,27}. Although all these tests have shortcomings, serological tests, particularly the enzyme-linked immunosorbent

assay (ELISA), seem to be the most practical and economical. Although the detection of specific antibodies by ELISA using crude *T. gondii* antigens requires mass production of the parasite either from the peritoneal fluids of infected mice or from tissue cultures, recombinant proteins would be alternative sources of antigens^{1,13,18,19}. However, it is necessary to develop a reliable, sensitive, and specific diagnostic test using parasite-specific antigens.

A *T. gondii* matrix antigen 1 (TgMAG1), known as the 65-kDa protein, originally identified on cysts and later found to be expressed on both bradyzoites and tachyzoites^{10,16,17}, was documented to be immunogenic during infection with *T. gondii* in a mouse model and found to be a promising tool for the serodiagnosis of human toxoplasmosis^{12,14,16}. However, its usefulness has not yet been confirmed in animal toxoplasmosis. In this study, we evaluated the usefulness of TgMAG1 as a serodiagnostic antigen for the epidemiological investigation of ovine toxoplasmosis in Mongolia.

Materials and Methods

Parasite culture and purification: *T. gondii* RH strain (genotype 1)²², PLK strain (genotype 2)³, and *Neospora caninum* Nc-1 strain⁹ tachyzoites were maintained in African green monkey kidney (Vero) cells cultured in a minimum essential Eagle medium (MEM; Sigma, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and 50 µg/mL kanamycin at 37°C in a 5% CO₂ air environment. For the purification of tachyzoites, the parasites and host cell debris were washed in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and syringed three times with a 27-gauge needle. The parasites were then filtered through a 5.0 µm pore filter (Millipore, USA), washed twice with PBS, and

pelleted at 1,500 rpm for 10 min²⁹).

T. gondii MAG1 isolation and sequencing: Total RNA of purified parasites (RH and PLK strains) was extracted using the TRI reagent (Sigma, USA) and subjected (0.5 µg) to one-step RT-PCR (Takara, Japan) with TgMAG1-specific primers (5'-TTGAATTCATGGATTGCGGACAGTG CAGAA-3' and 5'-TTCTCGAGAATCAAG CTGCCTGTTCCGCTAA-3') designed on the basis of the sequence reported in GenBank (U09029) to amplify the TgMAG1 gene. The RT-PCR products were subcloned into the pGEM[®]-T Easy Vector (Promega Corporation, USA), and the resulting plasmids were transformed into *Escherichia coli* DH5a strain and later cultured in an LB broth medium with ampicillin overnight. The plasmid DNA was then purified, and the cDNA inserts of positive clones were sequenced using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, USA).

Cloning, expression, and purification of recombinant proteins: Full-length TgMAG1 lacking the signal peptide (1-25 aa) was amplified using the primer set (5'-TTG AATTCGAGCCAAAGGGTGCCAGAGCTAC-3' and 5'-TTCTCGAGAATCAAGCTGCCTGTT CCGCTAA-3'). The PCR products were inserted into the pGEX-4T-3 vector (Amersham Pharmacia Biotech, USA) using the *Eco*RI and *Xho*I sites and expressed in the *E. coli* BL21 strain according to the manufacturer's instructions. The recombinant protein was then purified using glutathione-Sepharose 4B beads (Amersham Biosciences, USA).

Production of anti-TgMAG1 sera: Eight-week-old ICR mice (n = 5) were immunized intraperitoneally with 100 µg of purified rTgMAG1 and GST emulsified in 100 µl of Freund's complete adjuvant (Sigma, USA). Two boosters were given intraperitoneally using 50 µg of the same antigens emulsified in Freund's

incomplete adjuvant (Sigma, USA) at 14-day intervals. Sera were collected two weeks after the last booster and checked for specific antibodies by an indirect immunofluorescence assay (IFAT).

SDS-PAGE and Western blot analysis: Protein expression was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue, and the antigenicity of recombinant proteins was tested by Western blot analysis. For the preparation of parasite lysates, *T. gondii* and *N. caninum* tachyzoites were purified as described above, resuspended in PBS, disrupted three times by a freeze-thaw cycle in liquid nitrogen, and then sonicated in ice slurry. The protein concentration of the lysates was determined with a BCA protein assay kit (Pierce, USA), and the lysates were stored at -80°C until use²⁹. Proteins in the extracts were size-separated under reducing conditions by electrophoresis in 12% SDS-PAGE and then electroblotted onto the polyvinylidene difluoride membrane (Immobilon-P; Millipore, USA). The membrane was blocked with phosphate-buffered saline Tween 20 (PBST) with 3% skimmed milk and probed with specific primary antibodies. After washing with PBST, a secondary antibody, horseradish peroxidase-conjugated IgG (Bethyl, USA), was applied. Finally, bands were visualized by using a solution contained 3-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Japan) and H₂O₂.

Immunofluorescence microscopy analysis: Infected cells with *T. gondii* and *N. caninum* were coated on IFAT slides, dried, and fixed with 4% paraformaldehyde for 10 min. Antiserum raised against the rTgMAG1 was applied as the first antibody (1:100) on the fixed smears and incubated at 37°C for 1 hr in a moist chamber. After washing with PBST three times, Alexa-Fluor[®] 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes,

USA) was applied as a secondary antibody (1:300), and incubation was then conducted at 37°C for 30 min. After appropriate washing with PBS, the glass slides were mounted by adding 6 µl of a 50% glycerol-PBS (v/v) solution and covered with a glass cover slip. Fluorescent signals were observed by confocal laser scanning microscopy (TCS NT; Leica, Germany).

ELISA: Ninety-six-well plates (Nunc, Denmark) were coated with rTgMAG1 (1 µg/ml) and GST (1 µg/ml) diluted in the antigen coating buffer (0.05 M carbonate buffer, pH 9.6) at 4°C overnight. The plates were washed with washing solution (PBS containing 0.05% Tween 20) and then the wells were blocked with blocking solution (PBS containing 3% skim milk) at 37°C for 1 hr. After discarding the blocking solution the wells were incubated with the test sera (1:100) at 37°C for 1 hr. After washing, the wells were incubated with horseradish peroxidase-conjugated anti-sheep IgG antibody (Bethyl, USA; 1:4000) at 37°C for 1 hr. After washing, the wells were incubated with substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H₂O₂, 0.5 mg of 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) per ml] at room temperature for 1 hr. The optical density (OD) was measured with the MTP-500 microplate reader (Corona Electric, Japan) at 415 nm. The positive cut-off value was calculated as the mean OD value of the 30 serum samples from IFAT-negative animals plus 3-fold of their standard deviation.

Latex agglutination test: The latex agglutination test (LAT) was performed according to the manufacturer's instructions (Toxocheck-MT; Eiken Chemical, Japan). It was considered positive when agglutination was observed at dilutions of 1:32 and greater.

Experimental infection of mice: Eight-week-old female mice (ICR strain; n = 10/group) were intraperitoneally inoculated with either *T. gondii*

PLK strain (400 tachyzoites/mouse) or *N. caninum* Nc-1 strain (40,000 tachyzoites/mouse) with 0.2 ml of MEM. The blood samples were serially collected once a week from tail bleeds using capillary hematocrit tubes (Hirschmann Laborgerate, Germany). The mice were housed in a P2 level facility, and the experiments were conducted in accordance with the Stipulated Regulations for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Serum samples: A total of 175 serum samples were collected from sheep in Selenge (n = 25), Tuv (n = 25), Hovd (n = 25), Uvs (n = 25), Dornod (n = 25), Bulgan (n = 25), and Sukhbaatar (n = 25) provinces in Mongolia.

Results

The full lengths of TgMAG1 genes were cloned from the RH strain (genotype 1)²²⁾ and the PLK strain (genotype 2)³⁾, and their sequences were compared with those from the previously reported ME49 strain (genotype 2)¹⁷⁾. The amino acid similarity was > 99% among the 3 strains (data not shown), indicating that the TgMAG1 gene is very well conserved among different strains. The TgMAG1 gene without a signal peptide was then amplified, cloned into the pGEX-4T-3 plasmid, and expressed in *E. coli* with the molecular mass estimated by SDS-PAGE to be 91 kDa, including an additional 26 kDa from the GST tag (Fig. 1A). The rTgMAG1 was reacted with sera from mice experimentally infected with *T. gondii*, but not with *N. caninum* (strong antibody responses to *N. caninum* was confirmed by an ELISA with rNcSAG1²⁾) by Western blot analysis (Fig. 1B). The purified rTgMAG1 contained 3 lower-molecular-sized bands that are believed as the degradation products of rTgMAG1 (Fig. 1A and 1B). On the other hand, anti-rTgMAG1 serum produced in mice recognized the native TgMAG1

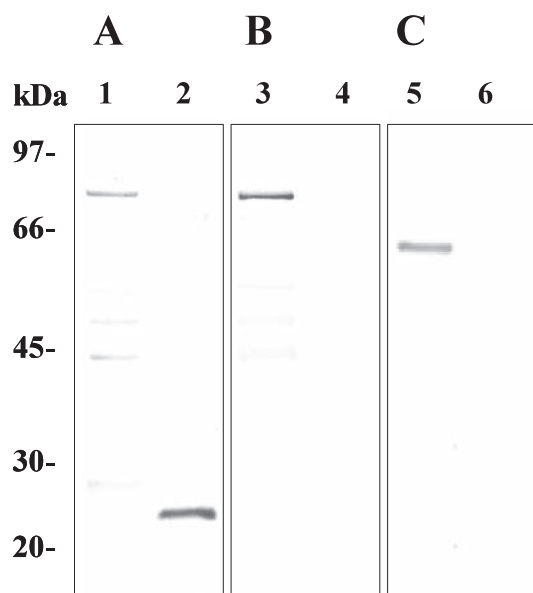


Fig. 1. Analysis of rTgMAG1 expressed in *E. coli*. A) 12% SDS-polyacrylamide gel (SDS-PAGE) stained with Coomassie blue. Lane 1, rTgMAG1; lane 2, GST. B) Western blot analysis of rTgMAG1 with *T. gondii*-infected mouse serum. Lane 3, rTgMAG1; lane 4, GST. C) Western blot analysis of native TgMAG1 with anti-rTgMAG1 serum produced in mouse. Lane 5, *T. gondii* lysate; lane 6, *N. caninum* lysate.

with a molecular mass of 65 kDa on the *T. gondii* lysate but not on the *N. caninum* lysate (Fig. 1C). Likewise, the same antisera were tested by IFAT, and a strong reaction was observed only with *T. gondii* but not with *N. caninum* tachyzoites (data not shown). Furthermore, to evaluate whether the recombinant protein can be used as a diagnostic antigen, rTgMAG1 was tested in an ELISA using sera from mice experimentally infected with *T. gondii* and *N. caninum*. As shown in Fig. 2A, all sera from 10 mice infected with *T. gondii* (2 months post-infection) were positive (optical density, > 0.1), whereas all sera from 10 mice infected with *N. caninum* (2 months post-infection; strong antibody responses to *N. caninum* was confirmed by an ELISA with rNcSAG1²⁾) and all sera from uninfected 10 mice were negative (optical density, < 0.1). In addition, serial serum samples collected from 10 mice experimentally infected with *T. gondii* were tested by the ELISA. As shown in Fig. 2B, the

antibody response to TgMAG1 was detected in serial serum samples from the second week post-infection and maintained at high level until 6 months post-infection.

A total of 175 samples collected from sheep in different provinces in Mongolia (Fig. 3; 2 provinces in western, northern and eastern parts, respectively, and 1 province in central part) were tested for the detection of antibodies to *T. gondii* infection by the ELISA with rTgMAG1, and the results were compared with those from the commercialized latex agglutination test (LAT). As shown in Table 1, of 175 serum samples analyzed, 42 (24.00%) and 29 (16.57%) samples were positive by the ELISA and LAT, respectively. Of 29 LAT-positive samples, 27 (93.10%) were positive by the ELISA. On the other hand, the positive rates of ELISA among 7 provinces ranged from 12.00% (Hovd province) to 36% (Selenge province). The positive rates of ELISA among different aged groups are ranged from 8.7% (1-2 years) to 26.1% (> 6 year).

Discussion

Toxoplasmosis is a world-wide zoonosis of increasing concern in human and veterinary medicine. Since 1950s, *T. gondii* has been recognized to be responsible for major economic losses in livestock through abortions, still births, and neonatal losses, especially in sheep^{4,6,7)}. Because sheep are an important source of meat and milk to human beings and the ingestion of contaminated meat poses risks to public health, the development of a reliable and sensitive test for the diagnosis of ovine toxoplasmosis is required. Therefore, the aim of this study was to develop a serodiagnostic test using a recombinant antigen for the detection of *T. gondii* infection in sheep.

In the present study, we evaluated the usefulness of rTgMAG1 expressed in *E. coli* as the GST fusion protein for the detection of ovine

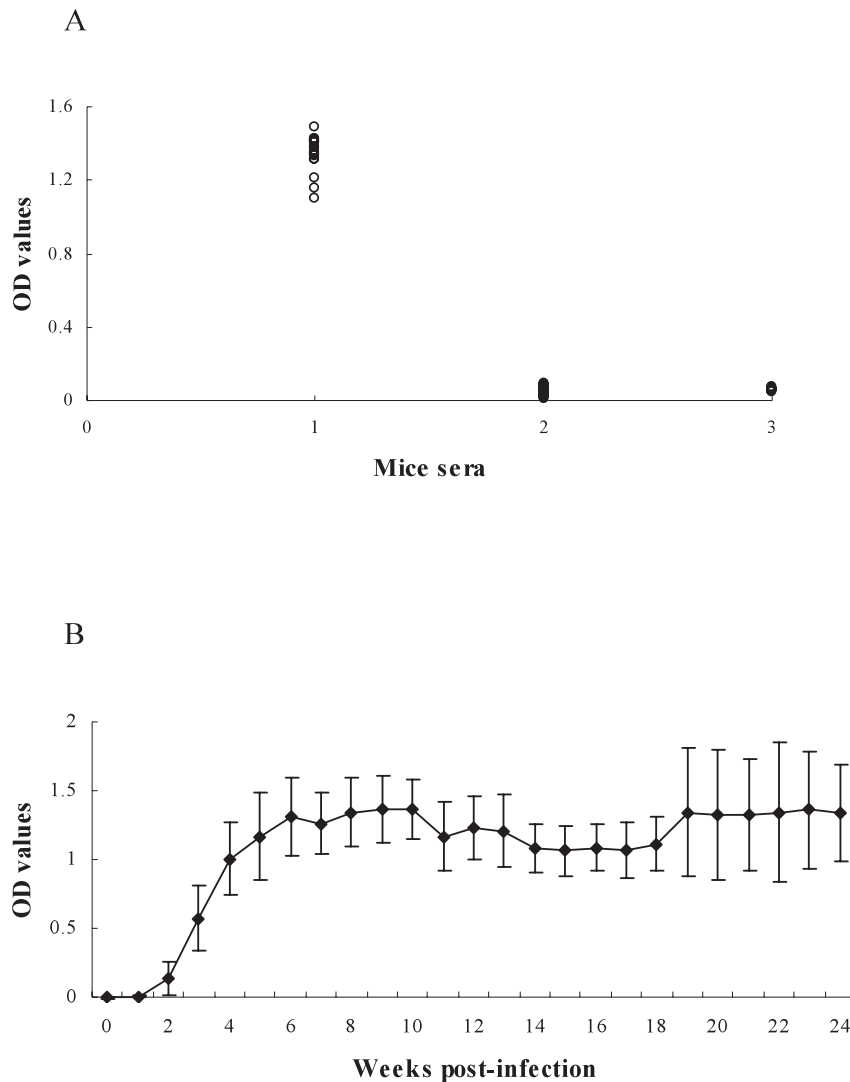


Fig. 2. Detection of antibody to *T. gondii* by the ELISA with rTgMAG1. A) Specificity of the ELISA. Lane 1, sera from mice infected with *T. gondii* (n = 10); lane 2, sera from mice infected with *N. caninum* (n = 10); lane 3, sera from uninfected mice (n = 10). B) Sensitivity of the ELISA. Serial serum samples from mice experimentally infected with *T. gondii* (n = 10).

toxoplasmosis. The rTgMAG1 reacted with sera from mice experimentally infected with *T. gondii*, but not with the closely related parasite *N. caninum*, indicated that rTgMAG1 has good antigenicity for detecting a specific antibody to *T. gondii*. Moreover, mice experimentally infected with *T. gondii* developed detectable antibody responses to TgMAG1 as early as the second week post-infection and maintained these responses until 24 weeks post-infection. These results indicated that the ELISA with rTgMAG1 could detect antibodies to *T. gondii* in both the

acute and chronic stages.

The high specificity and sensitivity of rTgMAG1 in the detection of infection in a mouse model, combined with its reported diagnostic potential in the detection of human toxoplasmosis, motivated us to use the ELISA with rTgMAG1 for the detection of ovine *T. gondii* infection in Mongolia. Ovine sera collected from different provinces in Mongolia were examined for the presence of a specific antibody to *T. gondii* by the ELISA with rTgMAG1, and the results were compared with those from the

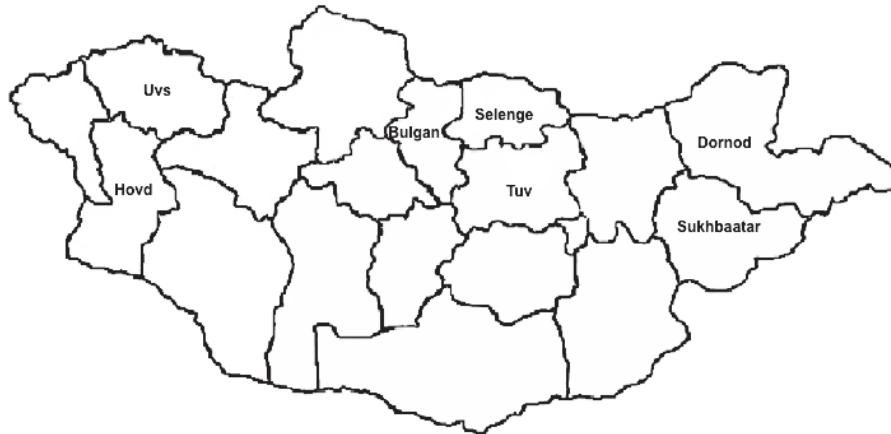


Fig. 3. Map of Mongolia. The 7 provinces sampled are indicated in the figure.

Table 1. Seroprevalence of ovine toxoplasmosis in Mongolia as determined by the ELISA with rTgMAG1 and LAT.

LAT	ELISA		Total
	+	-	
+	27 (15.43%)	2 (1.14%)	29 (16.57%)
-	15 (8.57%)	131 (74.86%)	146 (83.43%)
Total	42 (24.00%)	133 (76.00%)	175 (100 %)

commercialized LAT. Of 175 serum samples analyzed, 42 (24.00%) and 29 (16.57%) samples were positive by the ELISA and LAT, respectively. These results revealed the high sensitivity of the ELISA in the detection of ovine toxoplasmosis. The higher positive proportion detected by the ELISA than by the LAT can be explained by the constant expression of TgMAG1 in tachyzoites and bradyzoites, while only tachyzoite antigens are used in the LAT. On the other hand, 2 LAT-positive samples were negative by the ELISA, which is due to the attributes of the LAT to detect all types of immunoglobulin, unlike the current ELISA, which only detects immunoglobulin G. Further study is needed to evaluate the usefulness of the ELISA with rTgMAG1 for the detection of antibodies to *T. gondii*, including IgG, IgM, and IgA.

The seroprevalence of ovine toxoplasmosis has been reported from many countries: Canada (57%)²⁸⁾, Turkey (55%)²⁴⁾, Poland (53%)¹¹⁾, Bulgaria (48%)²¹⁾, Iran (35%)²⁵⁾, Morocco (27%)²³⁾,

Greece (23%)¹⁵⁾, and Brazil (18%)²⁰⁾. The prevalence of toxoplasmosis across the world is variable depending upon the country's customs, traditions, the lifestyles of the inhabitants, climatic variations, the age of the animals, and the husbandry methods, which are essential elements in epidemiological studies²⁶⁾. In the present study, the prevalence rate of field samples by the ELISA with rTgMAG1 was determined on the basis of different genders, ages, and geographical regions. Notably, no significant differences between females and males were found, while the prevalence was increased in aged animals. The high prevalence in aged animals might be due to longer exposure to infection through the ingestion of infective oocysts from the environment.

In conclusion, the results of the current study demonstrated the usefulness of rTgMAG1 as a diagnostic antigen for *T. gondii* infection as well as the high seroprevalence of ovine toxoplasmosis in Mongolia. Further investigation is required to determine the impact of infection

in the sheep industry and the potential risk of consumption of meat or milk contaminated with *T. gondii* parasites in Mongolia. To our knowledge, this is the first report on a survey for ovine toxoplasmosis in Mongolia.

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