Pathological and epidemiological studies on equine glanders in Mongolia

（モンゴル国における馬鼻疽の病理学的および疫学的研究）

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ABBREVIATIONS

Ag:	Antigen
B. mallei:	*Burkholderia mallei*
CFT:	Complement fixation test
ELISA:	Enzyme-linked immunosorbent assay
hr:	Hour
HE:	Hematoxylin and Eosin
IHC:	Immunohistochemistry
M:	mol/L
min:	Minute
NaCl:	Sodium chloride
OIE:	World Organization for Animal Health
PBS:	Phosphate-buffered saline
PPD:	Purified protein derivative
PTAH:	Phosphotungstic Acid-Hematoxylin
RBT:	Rose Bengal plate agglutination test
RT:	Room temperature
SAB:	Streptavidin-biotin technique
TBS:	Tris-HCl buffered saline

NOTES

Contents of the present thesis were published in the following articles.

1. **Ochbayar E, Baatarjargal P, Khurtsbaatar O, Altanchimeg A, Batbaatar V, Aoshima K, Kobayashi A, Kimura T.** 2020. Pathological and immunohistochemical analyses of naturally occurring equine glanders using an anti-BpaB antibody. *Veterinary Pathology.* Vet Pathol. 1-5, © The Author(s) 2020. doi: 10.1177/0300985820953422.

2. **Ochbayar E, Khurtsbaatar O, Ulziisaikhan G, Batbold TS, Baatarjargal P, Batbaatar V, Aoshima K, Kobayashi A, Kimura T.** 2020. Seroprevalence of equine glanders in horses in the central and eastern parts of Mongolia. J Vet Med Sci. 82(9):1247-1252, ©2020 The Japanese Society of Veterinary Science

GENERAL INTRODUCTION

Glanders, caused by the Gram-negative bacterium *Burkholderia mallei*, is a highly contagious and often fatal zoonotic disease of solipeds causing severe disease in animals and humans.

Glanders is classified both as a category B infection by the Center for Disease Control and Prevention in the United State (U.S.) and as a Tier 1 biological select agents and toxins by the U.S. Department of Health and Human Services and the U.S. Department of Agriculture. In fact, it was used as a biological weapon during the American Civil War, World War I and II, and the Russian invasion of Afghanistan [15, 39, 45]. Glanders has been eradicated from Great Britain, United States of America, Canada and Western Europe [35]. However, glanderous horses are still reported sporadically in Asia, the Middle East, Africa, and South America [5, 23, 24, 26, 37, 49]. Additionally, outbreaks of glanders have also been found in camels [46] and lions in zoos [14]. Therefore, it has regained the status of a re-emerging infectious disease in part of the world.

No vaccine is commercially available for both humans and animals [33, 44, 48]. Therefore, control measures of glanders are mainly based on disease surveillance and clinical symptoms, followed by elimination of *B. mallei*-infected animals from the herds. Ingestion of contaminated food or water is the major route of infection in glanders [33].

Complement fixation test (CFT) is the prescribed by World Organization for Animal Health (OIE) for the control of the disease in international trade of horses.

CFT is used worldwide for the serodiagnosis of glanders and is also recommended for surveillance investigations [4, 16, 22]. Enzyme-linked immunosorbent assay (ELISA) [4, 22, 11, 42, 43] and Western blotting [6, 10, 47] using recombinant *B. mallei* antibodies are considered to be reliable due to their high sensitivities; however, these tests are too expensive for routine diagnosis of glanders in the rural areas of developing countries. Mallein test (allergic hypersensitivity test) is not generally recommended in the present days due to the animal welfare [33]. However, this test is useful in the field diagnosis of endemic areas. Rose Bengal plate agglutination test (RBT) is simpler, rapid to perform, and is commercially available; however, it has only been validated in Russia [12, 31, 33].

Mongolian Ministry of Food, Agriculture and Light Industry has reported that the total number of animals in Mongolia reached 70.9 million heads of livestock last year, including 4.2 million horses, 4.7 million cattle, 0.4 million camels, 32.2 million sheep, and 29.2 million goats. Mongolians have been using horses broadly for riding and daily life of nomads from ancient time. Horses are the closest animal for Mongolians so that equine glanders can endanger human health, food safety, economy (export and import) and national security.

Up to the 1940s in Mongolia, glanders was one of the most common infectious diseases in animals. From the mid-1960s to the mid-1970s, diagnostic, therapeutic, and preventive projects were carried out against glanders, which nearly eliminated the disease [18, 32]. Controlling measures included double intradermal mallein tests and CFTs followed by a culling program [32]. The mallein test has been conventionally used in Mongolia for the screening of equine glanders

because it is inexpensive and easy to apply. Glanders was also included in a project to combat several chronic infectious diseases, carried out from 2003-2007 and 2012 by the government of Mongolia [29, 32].

Identification of glanderous horses is made initially by detecting clinical symptoms such as nasal discharge and skin nodules, which is followed by mallein test or other serological diagnostic tests. However, little is known about the pathology of equine glanders in Mongolia. Although the histopathology of equine glanders has been reported previously from other countries, little information is available about the *in-situ* localization of *B. mallei* in naturally glanderous horses. Additionally, the disease surveillance and monitoring are still required in Mongolia.

This study was performed to investigate the pathology and epidemiology of naturally occurring equine glanders in Mongolia. Chapter I aimed to investigate the histopathology and immunohistochemical localization of *B. mallei* in natural cases of equine glanders. Chapter II aimed to investigate the prevalence of equine glanders in Mongolia using serological diagnostic methods.

CHAPTER I

Pathological and immunohistochemical analyses of naturally occurring equine glanders using an anti-BpaB antibody

INTRODUCTION

Glanders is a fatal zoonotic disease caused by the gram-negative, immotile, aerobic rod-shaped bacterium *B. mallei*, a facultative intracellular bacterium. Natural *B. mallei* infections occur predominantly in donkeys, mules, and horses. it is still endemic in the Middle East, Asia, Africa, and South America. Additionally, outbreaks of glanders have recently been reported in Turkey [2], Brazil [5], Iran [14], India [24], Pakistan [31], Bahrain [46], and Iraq [49].

Donkeys and mules infected with *B. mallei* develop the acute form of the disease [33, 37, 40]. In horses, the chronic or latent form is more common [1, 2, 15, 33, 49]. The clinical signs of chronic cases are sticky purulent nasal discharge mixed with blood and multiple skin nodules especially in the hindlimbs [8, 9, 23, 24]. Pathologically, glanders are divided into skin, nasal, and pulmonary forms [1, 13]. The gross and histologic lesions of equine glanders have been reported previously from many countries. However, limited information is available on the *in-situ* localization of *B. mallei* in naturally occurring glanders in horses.

Nomadic Mongolians have been using horses extensively for riding and daily life since ancient time. Mongolia has more than 4 million horses; equine population outnumbers the country's human population. Horses are the most coveted animals for Mongolians, even for people living in urban areas. Considering the close relationship between Mongolian people and horses, controlling glanders in an urgent public health issue.

The present study aimed to investigate the histopathological changes and

immunohistochemical localization of *B. mallei* in natural cases of equine glanders in Mongolia.

MATERIALS AND METHODS

Animals

The 4 cases of glanders analyzed in this study occurring in Arabian horses in 2018 in Mongolia. Cases 1 and 2 were from a small farm in the Ulziit district, and cases 3 and 4 were from a small farm in the Nalaikh distruct of Ulaanbaatar city. Cases 1 and 4 were female and cases 2 and 3 were male. The cases had nasal discharge and multiple cutaneous nodules on the hindlimbs and abdomen. Horse owners detected nasal discharge (cases 2, 3, and 4) and skin nodules (cases 1 and 2) for approximately 5 months before euthanasia. These horses had been imported from Russia three years ago before at the age of 2-5 years. Other native Mongolian horses in the herds did not show any clinical signs. The four glanderous horses tested positive for *B. mallei* in CFT, RBT and mallein tests. The infected horses were euthanized and necropsied at the Institute of Veterinary Medicine, Mongolian University of Life Science, in Ulaanbaatar, Mongolia.

Histopathology

Tissue samples were collected from the mucous membranes of the nasal cavity (septum and conchae), lung, liver, spleen, kidney, inguinal lymph nodes, axillary lymph nodes, mediastinal lymph nodes, heart, and skin. These were fixed in 10% neutral-buffered formalin and then embedded in paraffin. Three-micrometer-thick sections were stained with Hematoxylin and Eosin (HE) for histopathological examinations. Serial sections from the skin, nasal cartilage,

mediastinal lymph nodes, and lungs were stained with Phosphotungstic Acid-Hematoxylin (PTAH) to demonstrate fibrin. The slides were reviewed by two veterinary pathologists.

Immunohistochemistry

Indirect immunohistochemical staining was performed using the labeled streptavidin-biotin (SAB) technique (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan). The sections were dewaxed and heated for 15 min with 0.01 M citric acid buffer (pH6.0) in a microwave for antigen retrieval. Subsequently, they were treated with 0.3% hydrogen peroxide in methanol and blocked with 10% normal rabbit serum (Nichirei biosciences, Tokyo, Japan). Anti-*B. mallei* BpaB antibody [clone BpaB#4; Kerafast, diluted 1:100 in phosphate-buffered saline (PBS)] was added, and the sections were incubated for 18 hours at 4°C. These sections were washed with PBS and incubated with biotinylated rabbit anti-mouse immunoglobulin (Histofine SAB-PO Kit, Nichirei) for 20 minutes at room temperature (RT). After further washing with PBS, the sections were incubated with streptavidin-conjugated peroxidase for 10 minutes at RT. The bound peroxidase was detected with 3,3′-diaminobenzidine [200 μg in Tris-HCl buffer (TBS), pH7.6]. The sections were counterstained with hematoxylin. As the negative control, sections were incubated without primary antibody.

RESULTS

Gross findings

At necropsy, all four horses were severely emaciated. They had moderate bilateral nasal discharge (purulent or hemorrhagic) with multiple 0.5- to 1.0-cm-diameter round ulcerated nodules on the nasal mucosa. Two horses (cases 1 and 2) had multiple small cutaneous lesions on the stifle, thigh, gaskin, and hock of the medial hindlimbs as well as the ventral abdomen, which occasionally erupted to form 1-2-cm diameter yellowish nodules (Fig. 1). Numerous gray, hard, approximately 0.3 cm diameter miliary nodules were present on the surface of the cranial, caudal, and accessory lobes of the lungs in all horses. Additionally, multifocal to coalescing, yellowish nodules surrounded by tan-white fibrous tissues (Fig. 2) were frequently found in the parenchyma of the lungs of four horses.

Histopathological findings

Histologically, multiple pyogranulomas (Fig. 3a) and small abscesses (Fig. 4a) were frequently found in the lungs in all cases. In case 3, severe infiltration of neutrophils, macrophages and lymphocytes in the lumen and lamina propria of bronchi and bronchioles was observed multifocally (Fig. 5). In the other three horses, however, the bronchi and bronchioles were spared.

Pyogranulomas and small abscesses were sometimes observed in the liver (cases 1 and 2) and mediastinal lymph nodes (case 4), but their frequency was lower than that in the lung.

Skin nodules in cases 1 and 2 consisted of dermal and subcutaneous abscesses (Fig. 6); they were also occasionally ulcerated. Severe diffuse necrosis with multifocal ulceration was observed in the nasal mucosa of cases 2 and 4, associated with submucosal abscesses and cartilage destruction (Fig. 7).
Fibrin exudation was frequently observed around the pyogranuloma in the lungs (Figs. 8a and 8b), mediastinal lymph nodes, nasal mucosa, and skin, but not in the liver.

No gross or histologic lesions were observed in the spleen, kidney, inguinal lymph nodes, axillary lymph nodes, or heart.

Immunohistochemistry assays

Immunohistochemistry (Table 1) revealed *B. mallei* BpaB antigen in the cytoplasm of neutrophils at the center of the pyogranuloma (Fig. 3b) and in small abscesses (Fig. 4b) in the lung of all horses, in the liver of two horses (cases 1 and 2), and in the mediastinal lymph nodes of one horse (case 4). Macrophages, epithelioid macrophages, and multinucleated giant cells within the pyogranuloma also contained the antigen (Fig. 9). Further, *B. mallei* antigen was detected in the alveolar cells, morphologically suggestive of type II pneumocytes; these were distinguished from hemosiderin-laden macrophages by Berlin blue staining (Fig. 10).

Punctate positive signals were occasionally detected in the cytoplasm of bronchiolar epithelial cells (Figs. 11a and 11b). In case 3, infiltration of *B. mallei* antigen-positive neutrophils and macrophages was observed in the lumen of

bronchi and the bronchioles. In case 4, intravascular monocytes and neutrophils located in the pulmonary blood vessels were also positive for *B. mallei* antigen (Fig. 12a and 12b).

The *B. mallei* BpaB antigen was also detected in the cytoplasm of neutrophils in the dermal and subcutaneous abscesses in the skin (cases 1 and 2) and in the submucosal abscesses in the nasal mucosa (cases 2 and 4). BpaB antigen positivity was not detected in the spleen, kidney, inguinal lymph nodes, axillary lymph nodes, and heart in all cases.

DISCUSSION

Current studies have described the histopathological and immunohistochemical findings observed in four cases of naturally occurring equine glanders in Mongolia. Clinical, gross, and histological findings in the four glanderous horses were similar to those previously reported in chronic cases of equine glanders [1, 2, 5, 28], except for marked fibrin exudation surrounding the pyogranulomas and abscesses. Relatively active bacterial proliferation might correlate with increased vascular permeability and the resultant fibrin exudation and severe neutrophilic infiltration. Few studies have been published using immunohistochemistry for examining bacterial localization in natural cases of equine glanders. We used monoclonal antibody against the BpaB antigen, which is expressed on the surface of *B. mallei* bacteria [44]. Consistent with immunohistochemistry for BpaB in *B. mallei*-infected mice and marmosets [48], BpaB was detected within macrophages, neutrophils, and respiratory epithelial cells of naturally infected glanderous horses. Localization of BpaB antigen-positive neutrophils and macrophages was well correlated with the localization of pyogranulomas and abscesses in the tissues.

Although the mode of *in vivo* dissemination of *B. mallei* is not fully understood, it is assumed that the bacteria could traverse the pharyngeal mucosa, and perhaps the intestinal mucosa after oral infection [3, 38, 45]. Subsequently, the bacteria would be seeded into the internal organs, particularly the lung, where lesions almost always occur [3, 48]. In the present study, one of the four horses

(case 3) showed bronchitis and bronchiolitis. These characteristics of the infiltration of BpaB-positive neutrophils and macrophages in the airway lumen would indicate bacterial spread through the airway within the lung. However, transbronchial spread does not appear to be necessary for bacterial dissemination. The other three cases did not show bronchitis or bronchiolitis. The localization of pyogranulomas and abscesses did not coincide with that of bronchi or bronchiole. In contrast, the pulmonary artery close to the pyogranuloma or abscess rarely contained BpaB-positive neutrophils and macrophages, indicating the possibility of hematogenous bacterial dissemination by those cells.

In conclusion, the results demonstrate that glanders occurs naturally in horses in Mongolia and *B. mallei* infects phagocytic cells and pulmonary epithelial cells in naturally infected horses. To the best of our knowledge, this is first report showing immunohistochemical localization of *B. mallei* in infected horses.

Table 1. Localization of *Burkholderia mallei* BpaB antigen using immunohistochemistry in horses with glanders.

Case No.	Tissues							
	Liver	Spleen	Kidney	Heart	Lung	Mediastinal lymph node	Nasal mucosa	Skin
1	+	-	-	-	+	-	-	+
2	+	-	-	-	+	-	+	+
3	-	-	-	-	+	-	-	-
4	-	-	-	-	+	+	+	-

Abbreviations: +: bacterial antigen positive

-: bacterial antigen negative

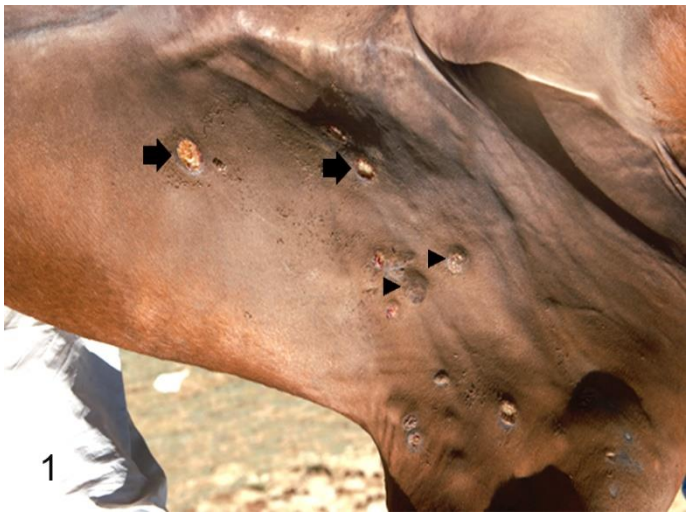

Figure 1. Glanders, skin, horse, case 1.

Multiple cutaneous nodules (arrowheads) and erupting lesions (arrows) on the medial hindlimbs.

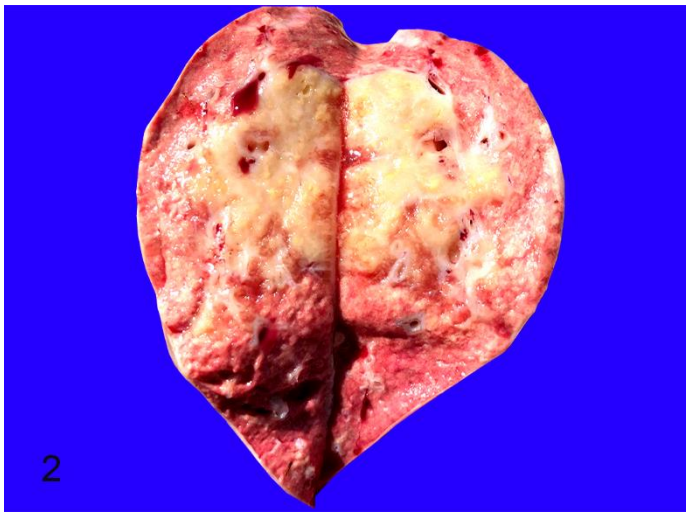

Figure 2. Glanders, lung, horse, case 4.

Multiple to coalescing yellowish nodules surrounded by tan-white fibrous tissues on the cut surface of lung.

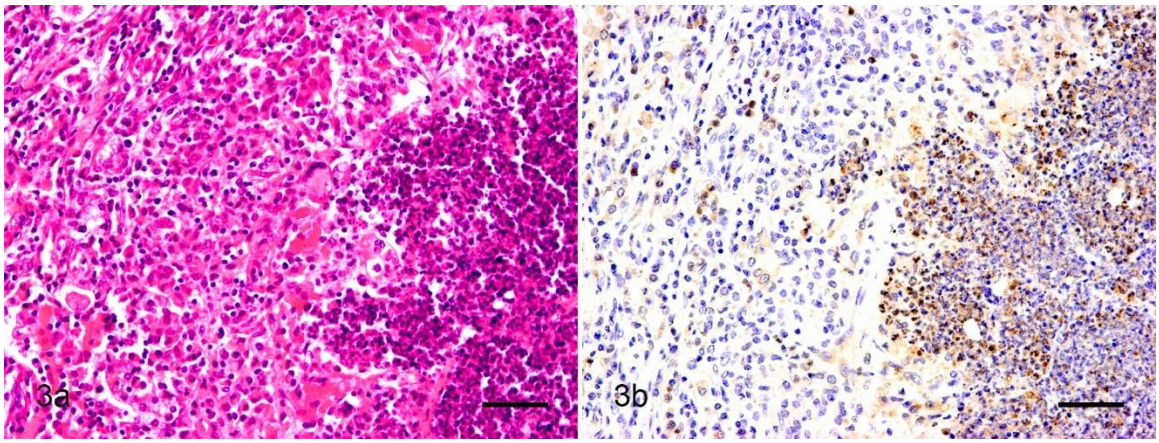

Figure 3. Histopathological changes of the lung in horse infected with glanders, case 1.

(a) Pyogranuloma. Karyorrhectic neutrophils were surrounded by macrophages, epithelioid cells, multinucleated giant cells, lymphocytes and fibroblasts. Hematoxylin and eosin stain (HE). (b) *B. mallei* immunolabeling was present in the cytoplasm of neutrophils and macrophages within pulmonary pyogranuloma. Immunohistochemistry (IHC) for *B. mallei* BpaB antigen (Ag). Bars = 60 µm.

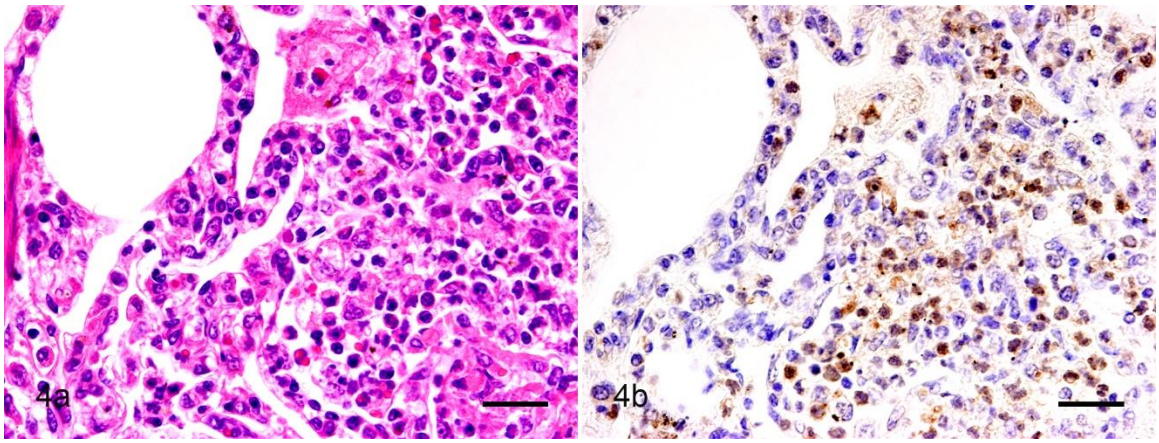

Figure 4. Histopathological changes of the lung in horse infected with glanders, case 2.

(a) Small abscesses containing degenerated neutrophils and macrophages. HE. (b) *B. mallei* antigen was detected in the cytoplasm of neutrophils and macrophages cells. IHC for *B. mallei* BpaB Ag. Bars = 30 μm.

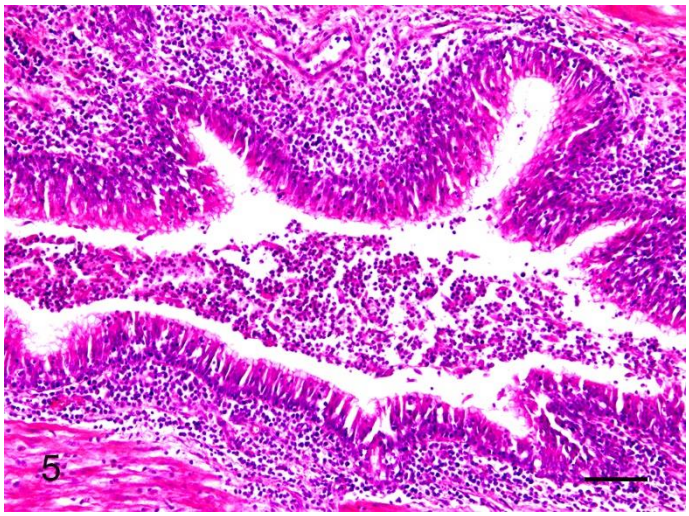

Figure 5. Histopathological changes of the lung in horse infected with glanders, case 3.

Severe infiltration of neutrophils, macrophages and lymphocytes in the lumen and lamina propria of bronchiole. HE. Bars = 300 μm.

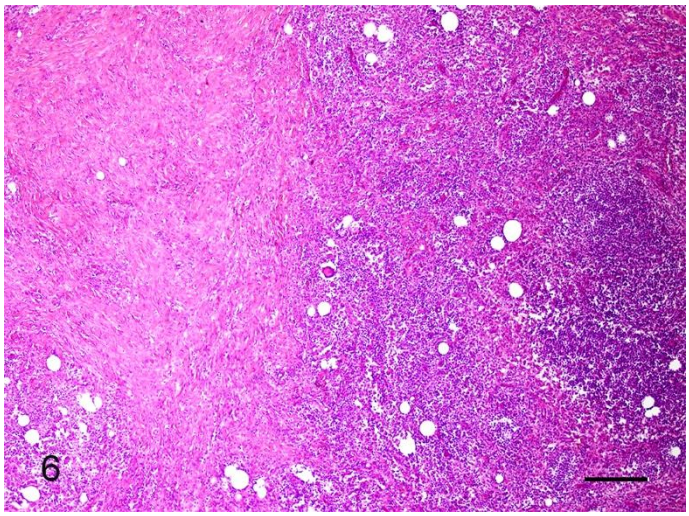

Figure 6. Histopathological changes of the skin in horse infected with glanders, case 2.

Subcutaneous lesion of skin nodule. Severe multifocal abscesses composed of neutrophils, macrophages, and multinucleated giant cells were associated with severe fibrosis. HE. Bars = 300 μm.

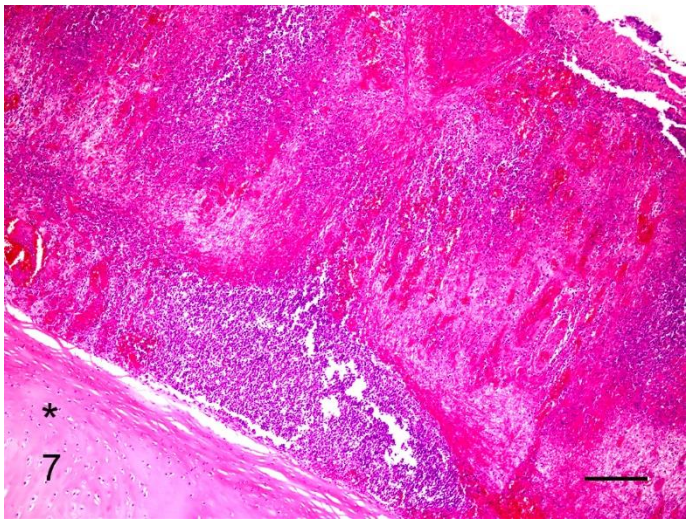

Figure 7. Histopathological changes of the nasal mucosa in horse infected with glanders, case 2.

Severe diffuse necrosis of nasal mucosa with submucosal abscesses (asterisk: cartilage). HE. Bars = 300 µm.

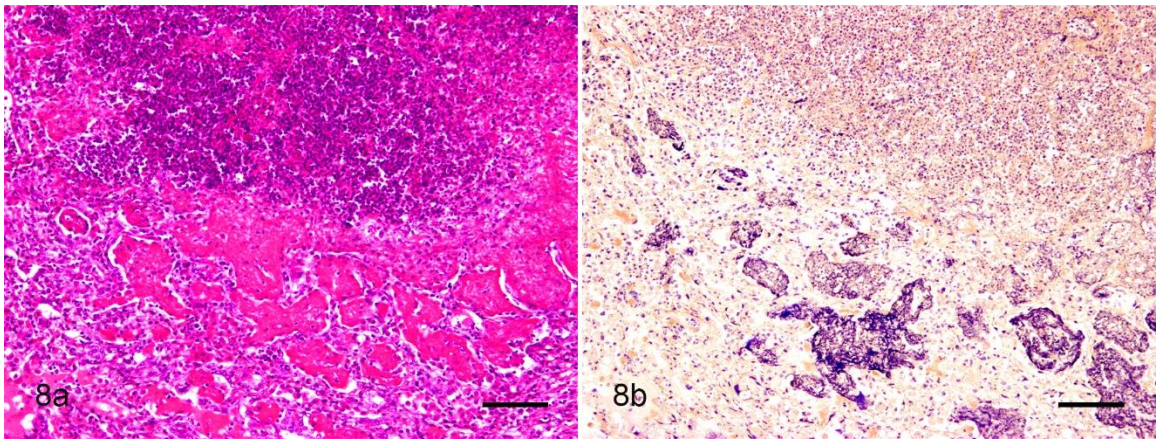

Figure 8. Fibrin exudation in the lung, case 1.

(a) Pyogranuloma with peripheral fibrin exudation. HE. (b) Phosphotungstic acid hematoxylin stain indicates fibrin exudation around pyogranuloma. Bars = 120 μm.

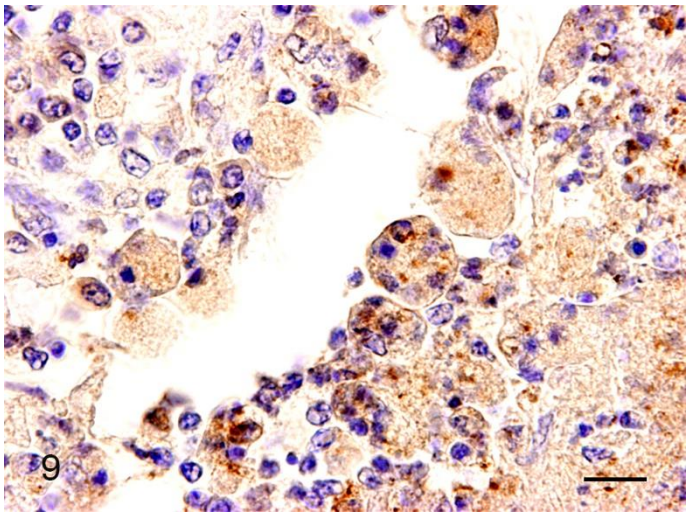

Figure 9. Immunohistochemical analysis using anti-*B. mallei* BpaB monoclonal antibody in lung tissues from horses infected with glanders, case 4.

B. mallei immunolabeling was present in the cytoplasm of epithelioid macrophages and multinucleated giant cells. IHC for *B. mallei* BpaB Ag. Bars = 30 µm.

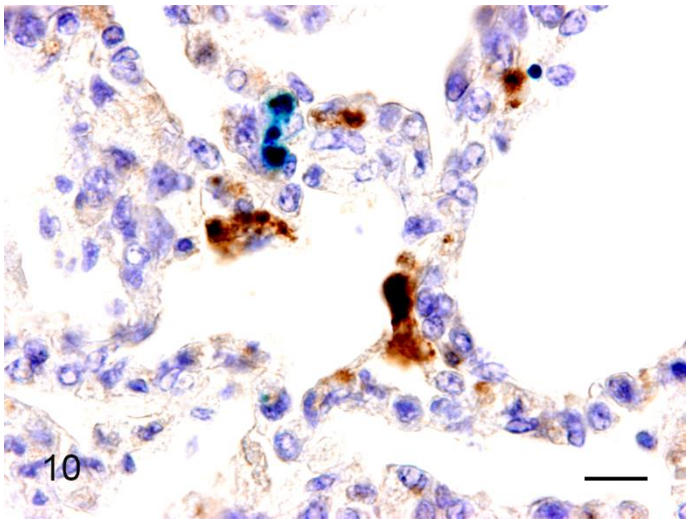

Figure 10. Immunohistochemical analysis using anti-*B. mallei* BpaB monoclonal antibody in lung tissues from horses infected with glanders, case 2.

B. mallei immunolabeling was present in the type II pneumocytes. IHC for *B. mallei* BpaB Ag. Prussian Blue stain indicated the hemosiderin (Blue color). Bars = 10 µm.

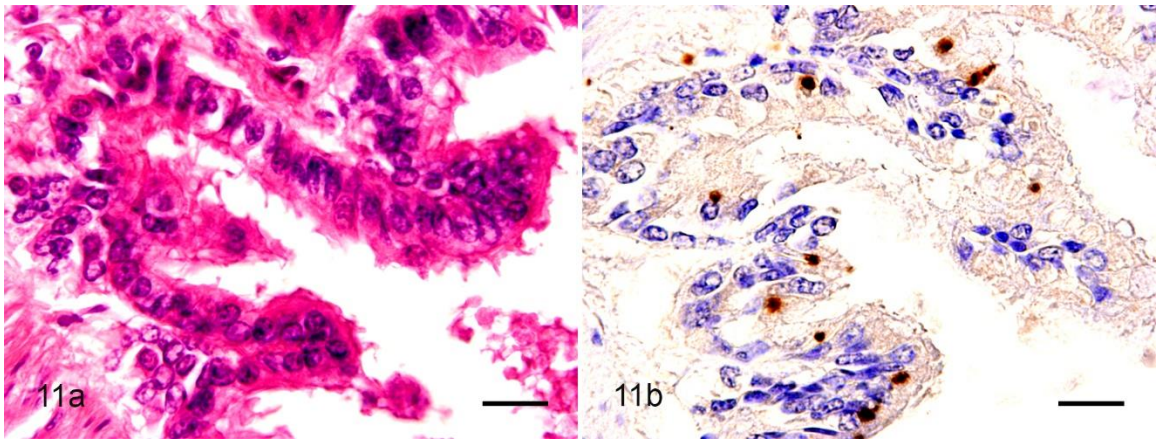

Figure 11. Histological changes in the bronchioles in case 1.

(a) Bronchiolar epithelial cells. HE. (b) *B. mallei* immunolabeling was present in the cytoplasm of bronchiolar epithelial cells. IHC for *B. mallei* BpaB Ag. Bars = 10 μm.

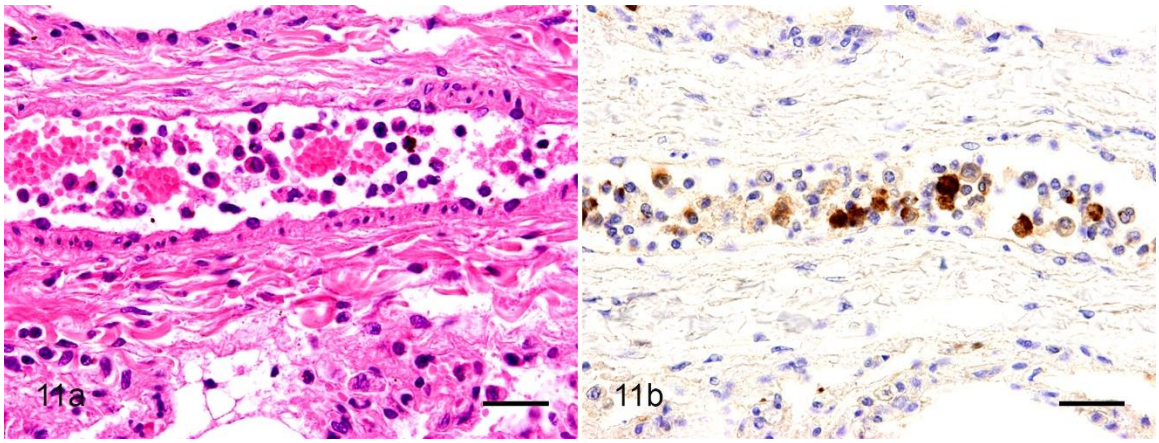

Figure 12. Pulmonary arteries in case 4.

(a) Pulmonary artery contained monocytes and a few lymphocytes and neutrophils. HE. (b) *B. mallei* immunolabeling was present in the cytoplasm of monocytes and neutrophils. IHC for anti-*B. mallei* BpaB Ag. Bars = 30 μm.

SUMMARY

Glanders is caused by the gram-negative bacterium *B. mallei*. In this study, histopathology and immunohistochemical localization of *B. mallei* in natural cases of equine glanders were investigated. Four horses showing clinical signs of nasal discharge and multiple cutaneous nodules or papulae in the hindlimbs and abdomen were reported in Mongolia. They tested positive for *B. mallei* infection on CFT, RBT, and mallein tests. Gross and histological lesions observed in these cases were similar to those previously reported in equine glanders. Immunohistochemistry using a monoclonal antibody to *B. mallei* BpaB showed localization of the bacterial antigen in the cytoplasm of neutrophils, macrophages, epithelioid cells, and multinucleated giant cells in the pyogranulomas and abscesses in target organs. Some alveolar type II cells and bronchiolar epithelial cells also contained the antigen. The results demonstrate that glanders occurs naturally in horses in Mongolia and *B. mallei* infects phagocytic cells and pulmonary epithelial cells in naturally infected horses.

CHAPTER II

Seroprevalence of equine glanders in horses in the central and eastern parts of Mongolia

INTRODUCTION

Equine glanders is fatal zoonotic bacterial infectious diseases caused by *B. mallei*. *B. mallei* primarily affects horses [2, 5, 7, 24, 36, 49], donkeys [37, 42], and mules [40]. Glanders has been eradicated in western Europe, Great Britain, the United States of America and Canada. However, this disease has re-emerged in Asia, the Middle East, Africa, and South America. For instance, outbreaks of glanders have recently been reported in Bahrain [46], Brazil [5, 7, 37, 40], Pakistan [10], India [24, 25, 42], Turkey [2], and Iran [14, 15].

Typical clinical symptoms of glanders in horses are nasal discharge, ulcerations of the nasal mucosa, and multiple skin nodules, especially in the hindlimbs and abdomen [26, 28, 42]. Transmission of *B. mallei* occurs mainly through ingestion of contaminated food or water, or otherwise through direct skin or mucous membrane contact with excretions from infected animal tissues [34]. In contrast to horses, which commonly develop chronic or latent (i.e., clinically inapparent) forms of infection, donkeys and mules frequently develop acute lethal forms [13, 28, 34].

Since glanders is a zoonotic disease, *B. mallei* infection in human reportedly occurs in veterinarians, veterinary students, farmers, horse handlers, slaughterhouse workers and laboratory workers, who often come into contact with infected animals [20, 33], although animal to human and human to human transmission have rarely been reported [20]. No vaccine is commercially available for human or animals [33]. Thus, control measures for glanders are mainly based

on disease surveillance and elimination of *B. mallei*-infected animals from the herds.

The diagnosis of glanders in horses is problematic, especially during the early stages of infection, in which infected horses do not show outward symptoms [13]. The mallein skin test (allergic hypersensitivity test) [12, 27, 31, 40] has been widely used in conventional field tests. However, due to animal welfare concerns, this test is not currently recommended [33]. CFT is considered as a suitable screening test for the diagnosis of equine glanders [4, 16, 17, 33, 34, 36, 42]. CFT is mandated by OIE as a confirmatory test for international trade [33]. RBT [12, 34] is approved in Russia, but to due to its relatively low sensitivity, it is not commonly used in other countries. Other serological tests, including western blot assay [36, 47] and ELISA [4, 21, 22, 41], have also been reported as useful diagnostic methods for glanders; however, these assays have not been validated as standard diagnostic methods to date. Isolation and identification of *B. mallei* from clinical samples, including cutaneous lesions and nasal exudates, are considered as the gold standard diagnostic methods for equine glanders, but these processes are time-consuming [33].

Horses are one of the most important livestock in Mongolia, and more than 4 million horses were registered in 2020 according to the National Statistical Office of Mongolia [30]. Horses have an important role in the daily work of the nomadic people in the countryside, and horse racing is the most popular sport in Mongolia. Like other livestock in nomadic style, horse herds graze in the fields and move from one place to another on pasture areas throughout Mongolia.

From 1966 to 1968, a project named “Veterinary expeditions of Central and Eastern European countries against brucellosis, tuberculosis, and glanders in Mongolia” successfully eliminated the disease in this country [18, 19]. This project involved 16 provinces and found that 24,760 out of 126,960 (19.5%) horses were seropositive in CFT. Using the mallein test, veterinarians also detected 241,157 (4.8%) out of 5,046,070 horses and 380 (0.1%) out of 332,684 camels to be positive for *B. mallei* [18, 19]. Another government-led country-wide surveillance for *B. mallei* in Mongolia was performed in 2011, in which 43,937 horse serum samples collected from 21 provinces were tested using CFT [29]. The seroprevalence of equine glanders was found in 7 provinces, including Bayan-Ulgii (4 positive in 2,242 examined, 0.18% positivity), Bulgan (23 in 2,254, 1.02% positivity), Dorongovi (24 in 1,960, 1.2% positivity), Orkhon (7 in 280, 2.5% positivity), Uvurkhangia (19 in 2,660, 0.71% positivity), Sukhbaatar (6 in 1,640, 0.37% positivity), and Khuvsugul (1 in 3,306, 0.03% positivity) [29].

Government-led surveillance for glanders has not been carried out in Mongolia since 2012; however, local veterinarians have continuously reported the occurrence of sporadic glanderous cases. Therefore, proper disease surveillance and monitoring are still required in Mongolia. The aim of this study was to investigate the prevalence of equine glanders in the central and eastern part of Mongolia using serological diagnostic methods (i.e., CFT, RBT).

MATERIALS AND METHODS

Ethics statement

For this study, no ethical approvals were required in Mongolia. All blood samples were routinely collected for glanders diagnostic purposes and research studies.

Blood samplings

Blood samples (approximately 5-7 ml) were collected into vacutainers from the jugular vein of horses in the central and eastern parts of Mongolia during the summers of 2018 and 2019 (Figure 12). Of 337 horses, 272 Mongolian native horses, 35 thoroughbreds, and 30 crossbreeds were randomly involved in this study. Of these horses, three Mongolian native horses belonging to a single herd in the Bayankhutag district of Khentii province, and four thoroughbred horses belonging to two herds in Khan-Uul and Nalaikh district of Ulaanbaatar showed clinical symptoms suggestive of glanders, such as bilateral nasal discharge (purulent and hemorrhagic) with ulcerated nodules on the nasal mucosa and multiple small, erupting skin nodules on the medial or lateral limbs and ventral abdomen. Another 269 Mongolian native, 31 thoroughbreds, and 30 crossbreeds did not show any clinical signs of glanders and looked healthy. The age of the horses ranged from 2 to 19-years-old. Sera were separated from blood samples after coagulation at RT (18-25°C) by centrifugation at 2,500 rpm for 10 min and then transferred into new tubes, labeled, and stored at -20°C until used.

CFT

All 337 serum samples were analyzed by CFT Antigen of diagnosis for GLANDERS (*Biocombinat,* Ulaanbaatar, Mongolia) according to the manufacturer's instructions. Briefly, the serum of each horse was diluted 1:5 in 0.9% sodium chloride (NaCl) solution. Diluted sera were inactivated for 30 min at 56°C, and then 50 µl was added to the wells of 96-well-round-bottom microwell plates in quadruplicate. Serum was mixed with complement (1:40 diluted guinea-pig complement, 50 µl in each well) and antigen (1:30 diluted anti-*Burkholderia mallei* serum, 50 µl in each well) on the plates. The plates were covered and incubated at 4°C for 12 hr overnight. One hundred microliters of 2% suspension of sensitized sheep red blood cells [34] were added to each well, and the plates were incubated for 45 min at 37°C and centrifuged for 5 min at 600 x g. Samples with 100% (4 wells) hemolysis were negative, those with 25-75% (2 or 3 wells) hemolysis were classified as suspicious and samples showing no hemolysis (4 wells) were classified as positive.

RBT

RBT was performed for glanders using the color strip reaction of agglutination (Kursk *Biofactory*, Kursk, Russia) according to the manufacturer's instructions. Briefly, 30 µl of serum was mixed thoroughly with an equal volume of Rose Bengal antigen on a white porcelain plate using a stick. The plate was then shaken in a slow rotation manner at RT (18-25°C) for 3 min, and any visible

agglutination was considered positive.

Mallein test

This test was performed on 7 glanders-suspected horses and 8 randomly selected healthy horses kept together in three herds in Bayankhutag, Khan-Uul, and Nalaikh districts. They were injected intradermally with 0.2 ml (0.95-1.05 mg/ml) of concentrated mallein purified protein derivative (PPD) (*Biocombinat,* Ulaanbaatar, Mongolia) on the middle of the vertical side of the horse's neck. The reaction to PPD injection was examined at 24 hr, 48 hr and 72 hr on the horse's neck. If the skin showed marked firm painful swelling of about 6 mm or more in diameter after 24 hr and 48 hr on the injection site, the test was considered to be positive.

Statistical analysis

The statistical analyses were performed using Fisher's exact test. $P<0.05$ was considered to be statistically significant.

RESULTS

CFT

Seropositivity of *B. mallei* was detected in 28 (8.3%) of 337 equine samples. Seropositive horses belonged to Bayankhutag, Jargaltkhaan, Sukhbaatar, Bayantsagaan, Bayantal, Bayantsogt, Sergelen, Khan-Uul, and Nalaikh districts (Table 2). Bayantal showed the highest positive rate, although the sample size was too small (50%, 1 in 2). It was followed by Bayankhutag (40%, 10 in 25), Khan-Uul (27.3%, 6 in 22), Nalaikh (16.7%, 2 in 12), Sergelen (12.9%, 4 in 31), Sukhbaatar (9.5%, 2 in 21), Bayantsagaan (7.7% 1 in 13), Bayantsog (6.3%, 1 in 16), and Jargaltkhaan (1.7%, 1 in 59). Figure 12 shows the distribution of sampling sites and locations where animals were found to be positive.

Mongolian native horses showed lower seropositivity for *B. mallei* than crossbreed horses (5.1% versus 26.6%, $P<0.01$) and thoroughbred horses (5.1% versus 17.1%, $P<0.05$) (Table 3). No statistically significant difference was observed in seropositivity between crossbreed and thoroughbred horses. Seven horses that showed clinical signs suggestive of glanders were seropositive for *B. mallei*.

No seropositivity was detected among horses aged 2 years (Table 4), even though the young horses were not kept separated from the older horses. However, there is no statistically significant difference in seropositivity between two-year-old horses and horses aged 3 year or older.

RBT

Seropositivity of *B. mallei* was detected in 26 (7.7%) of the 337 equine samples. Seropositive horses belonged to Bayankhutag, Jargaltkhaan, Tumentsogt, Bayantal, Bayantsogt, Khan-Uul, and Nalaikh districts (Table 2). Bayankhutag showed the highest positive rate (52%, 13 out of 25), followed by Tumentsogt (50%, 1 in 2), Bayantal (50%, 1 in 2), Bayantsogt (31.3%, 5 in 16), Nalaikh (16.7%, 2 in 12), Khan-Uul (13.6%, 3 in 22), and Jargaltkhaan (1.7%, 1 in 59). Figure 12 shows the distribution of sampling sites and locations where animals were found to be positive.

Mongolian native horses showed lower seropositivity for *B. mallei* than crossbreed horses (4.0% versus 33.3%, $P<0.01$) and thoroughbred horses (4.0% versus 14.2%, $P<0.05$) (Table. 3). No statistically significant difference was observed in seropositivity between crossbreed and thoroughbred horses. Seven horses that showed clinical signs suggestive of glanders were seropositive for *B. mallei*.

Although the number of *B. mallei*-seropositive horses identified by RBT were comparable to those identified by CFT (26 and 28, respectively), the number of horses showing double positive for RBT and CFT was only 17. Among 26 horses that were positive for RBT, 9 were negative for CFT. In addition, among 28 horses that were positive for CFT, 11 were negative for RBT.

Mallein test

Among the 15 horses screened by the mallein test, 8 (53.3%) were positive for the skin hypersensitivity reaction for *B. mallei*. These horses were also positive for CFT and RBT. The positive rates of glanders were 36.4% in Bayankhutag (4 in 11 tested), 100% in Khan-Uul (2 in 2 tested), and 100% in Nalaikh districts (2 in 2 tested). The locations of the mallein-positive horses are shown in the map (Figure. 12). Of the 8 horses which were positive with the mallein test, seven horses showed clinical signs suggestive of glanders. However, one crossbreed horse positive for mallein test did not show any clinical signs and looked healthy.

DISCUSSION

The current study described the prevalence of equine glanders in the central and eastern parts of Mongolia using serological diagnostic methods. Although the majority of examined horses were healthy and did not have symptoms suggestive of glanders, 7.7% and 8.3% of horses were seropositive using RBT and CFT, respectively.

The data suggested that seropositive rates obtained by RBT were comparable to those obtained using CFT; however, a considerable number of horses showed positive for one test but negative for the other. This result was inconsistent with the previous report [31], in which RBT data showed a high agreement with CFT data. The discrepancy between RBT and CFT found in this study might be due to the different bacterial strains used for antigen preparations. Mongolian isolate M1 was used for CFT, but Russian strain 5584 was used for RBT. Cross-reactivity of RBT has not been investigated for *B. mallei* strains circulating in Mongolia.

In this study, the overall seroprevalence rate of equine glanders was highest in the Bayankhutag district of Khentii province in Mongolia. In this district, one herd with approximately 300 horses, seemed to be severely affected because 10 in 15 horses (comprised of 3 glanderous horses and 12 randomly selected clinically normal horses) were seropositive for glanders, as determined by CFT. However, 10 serum samples collected from 3 other herds in the district were seronegative for glanders, indicating an uneven distribution of *B. mallei* carriers among the horse

population.

Recently crossbreeding of Mongolian native horses with imported thoroughbred horses has been popular in Mongolia to produce high-performance racing horses for the traditional Mongolian Naadam festival. The results suggest that thoroughbred horses and crossbreed horses were more susceptible to *B. mallei* infection than Mongolian native horses. Thoroughbreds might be more susceptible to *B. mallei* because most of them are not born in Mongolia but are imported from glanders-free countries. The relatively lower seropositivity in Mongolian native horses might be due to the adaptation of bacteria to their host over time. Because thoroughbred horses and crossbreed horses were kept intermingled with Mongolian native horses in each herd, there was no difference in hygiene status between breeds.

In conclusion, this study suggests that asymptomatic *B. mallei* infection occurs in the horse population in Mongolia. It has been suggested that pre-symptomatic or asymptomatic carriers of *B. mallei* are the potential source of infection for the healthy equine population [15, 24] and pose a hidden risk to humans [20]. Recent epidemiological information of glanders in Mongolia has been obtained solely by a definitive diagnosis of symptomatic horses, and there is no official report of occurrence of glanders after 2012. These findings indicate the necessity for large-scale epidemiological surveys of equine glanders as well as the necessity of establishing control measures that lead to disease eradication. Public awareness of the presence of glanders among horses, with an emphasis on its economic impact and public health implications, is hereby strongly recommended.

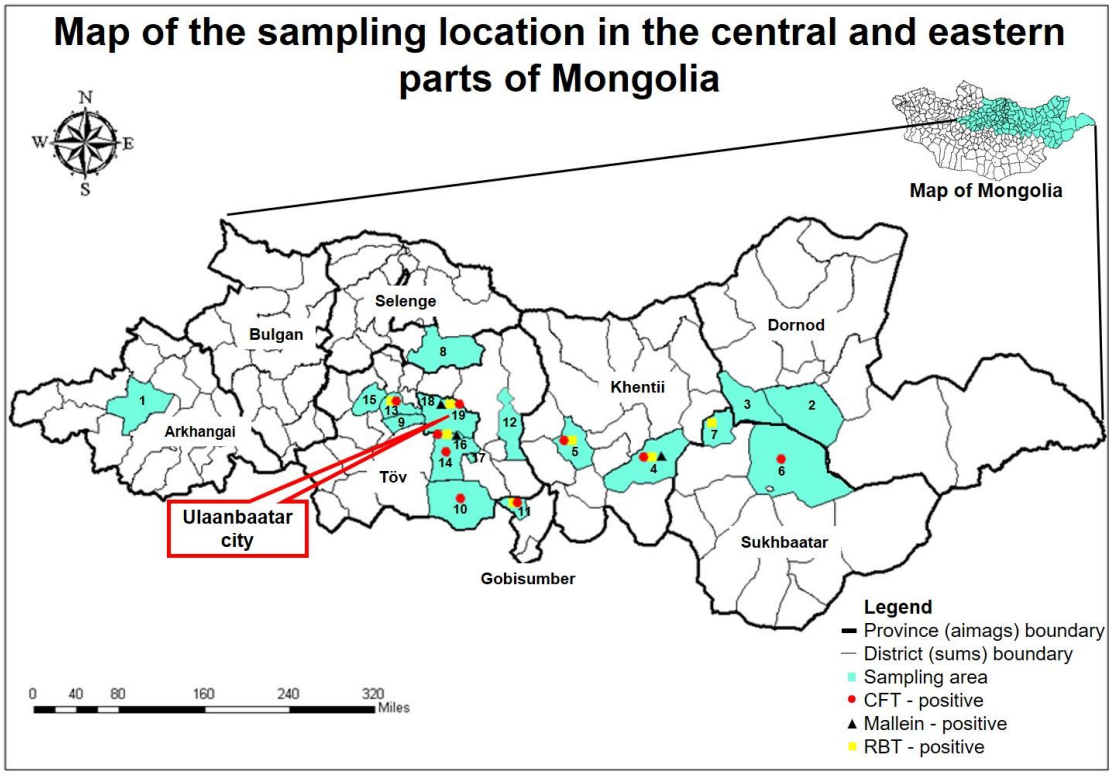

Figure 12. Map of Mongolia showing the areas in which horse sera were collected. The samples were taken from Ondor-Ulaan[1], Bulgan[2], Holonbuir[3], Bayankhutag[4], Jargaltkhaan[5], Sukhbaatar[6], Tumentsogt[7], Mandal[8], Argalant[9], Bayantsagaan[10], Bayantal[11], Bayandelger[12], Bayantsogt[13], Sergelen[14], Ugtaal-tsaidam[15], Khan-Uul[16], Bagakhangai[17], Songinokharikhan[18], and Nalaikh[19] districts. Number indicates the location of each district.

● CFT: Complement fixation test, ▲ Mallein test, ■ RBT: Rose Bengal agglutination test

Table 2. Seroprevalence of *Burkholderia mallei* in horses in randomly selected regions in the central and eastern parts of Mongolia (2018 and 2019).

No.[a)]	Province	District	No. of serum samples tested	CFT analyses	RBT analyses
				Positive[b)] (%)	Positive[b)] (%)
1	Arkhangai	Ondor-Ulaan	65	0 (0)	0 (0)
2	Dornod	Bulgan	5	0 (0)	0 (0)
3		Holonbuir	2	0 (0)	0 (0)
4	Khentii	Bayankhutag	25	10 (40%)	13 (52%)
5		Jargaltkhaan	59	1 (1.7%)	1 (1.7%)
6	Sukhbaatar	Sukhbaatar	21	2 (9.5%)	0 (0)
7		Tumentsogt	2	0 (0)	1 (50%)
8	Selenge	Mandal	2	0 (0)	0 (0)
9	Töv	Argalant	8	0 (0)	0 (0)
10		Bayantsagaan	13	1 (7.7%)	0 (0)
11		Bayantal	2	1 (50%)	1 (50%)
12		Bayandelger	1	0 (0)	0 (0)
13		Bayantsogt	16	1 (6.3%)	5 (31.3%)
14		Sergelen	31	4 (12.9%)	0 (0)
15		Ugtaaltsaidam	9	0 (0)	0 (0)
16	Ulaanbaatar city	Khan-Uul	22	6 (27.3%)	3 (13.6%)
17		Bagakhangai	25	0 (0)	0 (0)
18		Songinokharikhan	17	0 (0)	0 (0)
19		Nalaikh	12	2 (16.7%)	2 (16.7%)
Total			337	28 (8.3%)	26 (7.7%)

(a) Numbers indicates the location of each districts in Fig. 12. (b) Number of positive cases. CFT, complement fixation test; RBT, Rose Bengal plate agglutination test.

Table 3. Seroprevalence of *Burkholderia mallei* in horses in the central and eastern parts of Mongolia (2018 and 2019).

Horse breeds	No. of serum samples tested	CFT analyses	RBT analyses
		Positive[a)] (%)	Positive[a)] (%)
Mongolian native	272	14 (5.1%)	11 (4.0%)
Crossbreed	30	8 (26.6%)	10 (33.3%)
Thoroughbred	35	6 (17.1%)	5 (14.2%)
Total	337	28 (8.3%)	26 (7.7%)

(a) Number of positive cases. CFT, complement fixation test; RBT; Rose Bengal plate agglutination test.

Table 4. Age-dependent difference in seroprevalence of *Burkholderia mallei* in horses.

Breeds	Young (2 years of age)	Adult (3-10 years of age)	Old (11-19 years of age)
	Positive[a)]/No. of tested[b)]	Positive[a)]/No. of tested[b)]	Positive[a)]/No. of tested[b)]
Mongolian native	0/26	11/210	3/36
Crossbreed	0/3	5/23	3/4
Thoroughbred	0/2	6/27	0/6
Total	0/31	22/260	6/46

(a) The number of positive cases by complement fixation test (CFT)

(b)The number of tested cases by CFT

SUMMARY

Glanders is a contagious and fatal equine disease caused by the Gram-negative bacterium *B. mallei. B. mallei* is prevalent among horse populations in Asia, the Middle East, and South America. More than four million horses have been registered in Mongolia in 2020. However, the recent prevalence of glanders has not been well investigated. This study was aimed to investigate the seropositivity of *B. mallei* in horse populations in Mongolia using the complement fixation test (CFT) and Rose Bengal plate agglutination test (RBT). 337 blood samples were randomly collected from horses in central and eastern parts of Mongolia between 2018 and 2019. Of 337 horses, 28 (8.3%) and 26 (7.7%) were seropositive using CFT and RBT, respectively. Interestingly, seropositivity in horses resulting from crossbreeding of Mongolian native horses with thoroughbred horses was higher than that in Mongolian native horses. These observations suggest that equine glanders are still endemic to Mongolia, and also suggest that asymptomatic *B. mallei* infection is occurs in horse populations in Mongolia.

CONCLUSION

B. mallei, the etiological agent of the disease known as glanders, primarily affects horses and is transmitted to humans by direct contact with infected animals. *B. mallei* remains distributed in horse populations in Asia, the Middle East, Africa, and south America. Mongolians have been using horses broadly for riding and daily life of nomads from ancient time. Horses are the closest animal for Mongolians so that equine glanders can endanger human health, food safety, economy (export and import) and national security. In Mongolia, government-let surveillance for glanders has not been performed recently, although sporadic glanderous cases have been reported by local veterinarians in Mongolia. The objective of this study was to perform pathological and epidemiological surveys of glanders in Mongolia.

In chapter I, histopathology of the lesions and immunohistochemical localizations of *B. mallei* in glanderous horses were investigated. Four horses that had been imported from Russia three years before located at two small farms in Nalaikh and Ulziit districts of Ulaanbaatar showed nasal discharge and multiple cutaneous nodules on the hindlimbs and abdomen. Clinical, gross and histological lesions observed in the four glanderous horses were similar to those in previously reported equine glanders. These results suggest that equine glanders occurs sporadically in Mongolia. The anti-*B. mallei* BpaB monoclonal antibody detected localization of the bacterial antigens in the cytoplasm of phagocytic cells in the pyogranulomas and abscesses in target tissues. In addition, some bronchiolar

epithelial cells and alveolar type II cells contained the antigen. These results demonstrate that glanders occurs naturally in horses in Mongolia and *B. mallei* infects phagocytic cells and pulmonary epithelial cells in naturally infected horses.

In chapter II, blood samples used for seroepidemiological studies of glanders were collected from 272 Mongolian native horses, 35 thoroughbred and 30 crossbreeds in central and eastern part of Mongolia. Of these horses, three Mongolian native and four thoroughbred horses showed clinical symptoms suggestive of glanders. All serum samples were examined by CFT and RBT to detect *B. mallei* antibodies. Of 337 horses, 28 (8.3%) and 26 (7.7%) were seropositive by CFT and RBT, respectively. Interestingly, seropositivity in crossbred horses of Mongolian native horses and thoroughbred horses was higher than that in Mongolian native horses. This study suggests that asymptomatic *B. mallei* infection is occurs in horse population in Mongolia.

These studies suggest that equine glanders is still endemic in Mongolian horse populations. Therefore, large-scale epidemiological surveys as well as establishment of control measures against equine glanders are needed to eradicate this disease from Mongolia.

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REFERENCES

1. Anicic, M., Kukolj, V. and Marinkovic, D. 2017. Morphological characteristic of three classic forms of natural equine glanders-a disease with high zoonotic significance. *Acta Vet. Brno.* **67**: 572-577.
2. Arun, S., Neubauer, H., Gurel, A., Awildiz, G., Kussu, B., Yesildere, T., Meyer, H., Hermanns, W., Gurel, D. A., Ayyildiz, G. and Kusqu, B. 1999. Equine glanders in Turkey. *Vet. Rec.* **144**: 255-258.
3. Caswell, J. L. 2015. Respiratory system. p. 573. *In: Jubb, Kennedy, and Palmer's Pathology of Domestic Animals.,* 6th edition ed., (Maxie MG eds.) Elsevier, Inc, 6th ed. Ontario, Canada:
4. Elschner, M. C., Karine, L., Harisankar, S., Tripathi, B.N., Saqib, M., Gardner, I., Saini, S., Kumar, S., EI-Adawy, H., Melzer, F., Khan, I., Malik, P., Sauter-Louis, C. and Neubauer, H. 2019. Evaluation of the comparative accuracy of the complement fixation test, Western blot and five enzyme-linked immunosorbent assays for serodiagnosis of glanders. *PLOS ONE.* **14**: 1-12.
5. Elschner, M. C., Klaus, C.U., Liebler-Tenorio, E., Schmoock, G., Wohlsein., Tinschmann, O., Lange, E., Kaden, V., Klopfleisch, R., Melzer, F., Rassback, A. and Neubauer, H. 2009. Burkholderia mallei infection in a horse imported from Brazil. *Equine Vet. Educ.* **21**: 147-150.
6. Elschner, M. C., Scholz, H. C., Melzer, F., Saqib, M., Marten, P., Rassbach, A., Dietzsch, M., Schmoock, G., Santana, V. L. d. A., Souza, M. M. d.,

Wernery, R., Wernery, U. and Neubauer, H. 2011. Use of a Western blot technique for the serodiagnosis of glanders. *BMC Vet. Res.* **7**: 2-6.

7. Falcão, M. V. D., Silveira, P. P. M., Santana, V. L. A., Rocha, L. O. d., Chaves, K. P. and Mota, R. A. 2019. First record of Burkholderia mallei Turkey 10 strain originating from glanderous horses from Brazil. *Brazilian J. Microbiol.* **2**: 1-3.
8. Galyov, E. E., Brett, P. J. and DeShazer, D. 2010. Molecular Insights into Burkholderia pseudomallei and Burkholderia mallei Pathogenesis, *Annu. Rev. Microbiol.* **64**: 495-517.
9. Ghori, M. T., Khan, M. S., Khan, J. A., Rabbani, M., Chaudhary, M. H., Shabbir, M. Z., Ahmed, R., Chaudhry, H. R. and Muhammad, J. 2018. Molecular detection of Burkholderia mallei in nasal swabs from draught horses with signs of respiratory tract infection. *J. Anim. Plant Sci.* **28**: 1717-1724.
10. Ghoria, M. T., Khana, M. S., Khana, J. A., Rabbania, M., Shabbira, M. Z., Chaudhrya, H. R., Alia, M. A., Muhammada, J., Elschnerb, M. C. and Jayarao, B. M. 2017. Seroprevalence and risk factors of glanders in working equine – Findings of a cross-sectional study in Punjab province of Pakistan. *Acta Trop.* **176**: 134-139.
11. Hussein, Z. S. 2018. Detection of Glanders in horses of eight Iraqi provinces by ELISA. *J. Vet. Sci.* **11**: 21-25.
12. Karimi, A. and Mosavari, N. 2019. Development of Rose Bengal test against mallein test for rapid diagnosis of equine glanders. *Trop. Anim.*

Health Prod. **51**: 1969-1974.

13. Kettle, A. N. B. and Wernery, U. 2016. Glanders and the risk for its introduction through the international movement of horses. *Equine Vet. J.* 48: 654-658.

14. Khaki, P., Mosavari, N., Khajeh, N. S., Emam, M., Ahouran, M., Hashemi, S., Mohammad, T, M., Jahanpeyma, D. and Nikkhah, S. 2012. Glanders outbreak at Tehran Zoo, Iran. *Iran. J. Microbiol. **4**: 3-7.*

15. Khan, I., Wieler, L. H., Melzer, F., Elschner, M. C., Muhammad, G., Ali, S., Sprague, L. D., Neubauer, H. and Saqib, M. 2013. Glanders in Animals: A Review on Epidemiology, Clinical Presentation, Diagnosis and Countermeasures. *Transbound. Emerg. Dis.* **60**: 204-221.

16. Khan, I., Wieler, L. H., Melzer, F., Gwida, M., Santana, V. L. de. A., Souza, M. M. A. de, Saqib, M., Elschner, M. C. and Neubauer H. 2011. Comparative evaluation of three commercially available complement fixation test antigens for the diagnosis of glanders. *Vet. Rec.* **169:** 1-4.

17. Khan, I., Wieler, L. H., Saqib, M., Melzer, F., Santana, V. L. D. A., Neubauer, H. and Elschner, M. C. 2014. Effect of incubation temperature on the diagnostic sensitivity of the glanders complement fixation test. *Rev. sci. tech. Off. Int. Epiz.* **33**: 869-875.

18. Kouba, V. 2010. Veterinary expeditions of Central and Eastern European countries against brucellosis, tuberculosis and glanders in Mongolia: a historical report. Centaur global network. http:/centaur.vri.cz/docs/files/Kouba_Mongolia.pdf. [accessed on April 17,

2020].

19. Kouba, V. 2017. Large Country Screening to Discover all Domestic Animal Herds Affected by Selected Zoonoses. *Agric. Trop. Subtrop.* **1**: 5-11.

20. Kristopher, E. V. Z., Marek , T. G. and Carl, H. G. 2013. Glanders: an overview of infection in humans. *Orphanet J. Rare Dis.* **8**: 2-7.

21. Kritsiriwuthinan, K., Wajanaroganab, S., Choosanga, K., Homsiana, J. and Rerkthanoma, S. 2018. Production and evaluation of recombinant Burkholderia pseudomallei GroEL and OmpA proteins for serodiagnosis of melioidosis. *Acta Trop.* **178**: 333-339.

22. Kumar, S., Malik, P., Verma, S. K., Pal, V., Gautam, V., Mukhopadhyay, C. and Rai, G. P. 2011. Use of a Recombinant Burkholderia Intracellular Motility A Protein for Immunodiagnosis of Glanders. *Clin. Vaccine Immunol.* **18**: 1456-1461.

23. Malik, P., Khurana, S. K., Singh, B. K. and Dwivedi, S. K. 2009. Recent outbreak of glanders in India. *Indian J. Anim. Sci.* **79**: 1015-1917.

24. Malik, P., Singha, H., Goyal, S. K., Khurana, S. K., Tripathi, B. N., Dutt, A., Singh, D., Sharma, N. and Jain, S. 2015. Incidence of Burkholderia mallei infection among indigenous equine in India. *Vet. Rec. Open.* **2**: 1-7.

25. Malik, P., Singha, H., Khurana, S. K., Kumar, R., Kumar, S., Raut, A. A., Riyesh, T., Vaid, R. K., Virmani, N., Singh, B. K., Pathak, S. V., Parkale, D. D., Singh, B., Pandey, S. B., Sharma, T. R., Chauhan, B. C., Awasthi, V., Jain, S. and Singh R. K. 2012. Emergence and re-emergence of glanders in India: a description of outbreaks from 2006 to 2011. *Vet. Ital.* **48**: 167-178.

26. Mardani, M. and Kamali, M. 2011. Re-emergence of Glanders in Iran. *Iran. J. Clin. Infect. Dis.* **6**: 1-4.

27. Maurício, B. d. C. F., Rodrigo, M. R., Antônio, A. F. J., Lívia, d. L. O., Mariana, L. S., Vania, L. d. A. S., Marcilia, M. A. d. S., Evandro, d. R. M., Paulo, R. L. F., Rômulo, C. L. and Jenner, K. P. d. R. 2012. Development and validation of a method for purification of mallein for the diagnosis of glanders in equines. *BMC Vet. Res.* **8**: 2-9.

28. Muhammad, G., Khan, M. Z and Athar, M. 1998. Clinico-Microbiological and Therapeutic Aspects of glanders in Equines. *J. Equine Sci.* **9**: 93-96.

29. Nansalmaa, M., Serchmaa, Ts., Tuya, N., Odonchimeg, M., Dagvadorj, Ya., Batsuren, B., Batchuluun, D. and Sugar, S. 2012. The results to establish the prevalence, infectious level of the brucellosis and other infectious diseases. *SCVL proceeding.* **6**: 46-57 (in Mongolia).

30. National Statistics Office of Mongolia (2020). https://www.en.nso.mn/. [accessed on April 17, 2020].

31. Naureen, A., Saqib, M., Muhammad, G., Hussain, M. H. and Asi, M. N. 2007. Comparative evaluation of Rose Bengal plate agglutination test, mallein test, and some conventional serological tests for diagnosis of equine glanders. *J. Vet. Diagnostic Investig.* **19**: 363-367.

32. Odontsetseg, N., Mweene, A. S. and Kida, H. 2005. Viral and bacterial diseases in livestock in Mongolia. *Jpn. J. Vet. Res.* **52**: 151-162.

33. OIE. 2012. World Organisation for Animal Health. Manual of Diagnostic Test and Vaccines for Terrestrial Animals (mammals, brid, and bees),

Glanders. Chapter 2.5.11., Paris.

34. OIE. 2018. World Organisation for Animal Health. Manual of Diagnostic Test and Vaccines for Terrestrial Animals (mammals, brid, and bees), Glanders and Melioidosis. Chapter 2.5.11. Paris.

35. Pawaiya, R. V. S. and Chauhan, R. S. 2008. A review on glanders-a re-emerging zoonosis in India. *Indian J. Vet. Pathol.* **32**: 1-14.

36. Rahman, Md. S., Bhattacharjee, P. K., Sarker, R. R., Parvin, Mst. S., Tasnin, S., Sarker, M.A.S., Neubauer, H., Khatun, F., Wares, Md. A., Nishidate, I. and Elschner, M. C. 2018. Glanders in horses in some selected areas of Bangladesh and comparison between CFT and Immunoblot used for the screening of glanders. *Indian J. Anim. Res.* **B-976**: 1-4.

37. Rinaldo, A. M., Andréa, A. d. F. O., José, W. P. J., Leonildo, B. G. d. S., Marilene, d. F. B. and Silvana, S. A. R. 2010. Glanders in donkey (Equus Asinus) in the state of Pernambuco, Brazil: A case report. *Brazilian J. Microbiol.* 41: 146-149.

38. Rooney, J. R. Respiratory system. In: Robertson, J. L. ed. *Equine Pathology.* 1st ed. Ames, USA: lowa State University Press, Ames; 1996: 43.

39. Scholz, H. C., Pearson, T., Hornstra, H., Projahn, M., Terzioglu, R., Wernery, R., Georgi, E., Riehm, J. M., Wagner, D. M., Keim, P. S., Joseph, M., Johnson, B., Kinne, Joerg., Jose, S., Hepp, C. M., Witte, A. and Wernery, U. 2014. Genotyping of Burkholderia malleifrom an Outbreak of Glanders in Bahrain Suggests Multiple Introduction Events. *PloS Negl. Trop. Dis.* **8**: 1-

4.

40. Silva, K. P. C. d., Takaki, G. M. d. C., Silva, L. B. G. d., Saukas, T. N., Santos, A. S. and Mota, R. A. 2013. Assessment of the effectiveness of the PPD-mallein produced in Brazil for diagnosing glanders in mules. *Brazilian J. Microbiol.* **44**: 179-188.

41. Singha, H., Malik, P., Goyal, S. K., Khurana, S. K., Mukhopadhyay, C., Eshwara, V. K. and Singh, R. K. 2014. Optimization and Validation of Indirect ELISA Using Truncated TssB Protein for the Serodiagnosis of Glanders amongst Equines. *Sci. World J.* **2014**: 1-6.

42. Singha, H., Shanmugasundaram, K., Tripathi, B. N., Saini, S., Khurana, S. K., Kanani, A., Shah, N., Mital, A., Kanwar, P., Bhatt, L., Limaye, V., Khasa, V., Arora, R., Gupta, S., Sangha, S., Sharma, H., Agarwal, S. K., Tapase, J., Parnam, S., Dubey, P., Baalasundaram, S. K., Mandal, B. N., Virmani, N., Gulati, B. R. and Malik, P. 2020. Serological surveillance and clinical investigation of glanders among indigenous equine in India from 2015 to 2018. *Transbound. Emerg. Dis.* **0**: 1-13.

43. Sprague, L. D., Zachariah, R., Neubauer, H., Wernery, R., Joseph, M., Scholz, H. C. and Wernery, U. 2009. Prevalence-dependent use of serological tests for diagnosing glanders in horses. *BMC Vet. Res.* **5**: 1-6.

44. Tomislav, J., Shawn, M. Z., Stephen, B. H., Daniel, G. M., Teresa, L. S., D. Mark, E., Frank, M., Frederick, D. Q., Robert, J. H. and Eric, R. L. 2015. Use of the Common Marmoset to Study Burkholderia mallei Infection. *PLoS One.* **10**: 1-21.

45. Verma, A. K., Saminathan, M., Neha., Tiwari, R., Dhama, K. and Singh, S. V. 2014. Glanders-A Re-emerging Zoonotic Disease: A Review. *J. Biol. Sci.* **14**: 38-51.

46. Wernery, U., Wernery, R., Joseph, M., Al-Salloom, F., Johnson, B., Kinne, J., Jose, S., Jose, S., Tappendorf, B., Hornstra, H. and Scholz, H. C. Natural Burkholderia mallei Infection in Dormadary, Bahrain. *Emerg. Infect. Dis.* **17**: 1277-1279.

47. Yazdansetad, S., Mosavari, N., Tadayon, K. and Mehregan, I. 2019. Development of an immunoblotting assay for serodiagnosis of Burkholderia mallei infection: the whole-cell proteome-based paradigm. *Iran. J. Microbiol.* **11**: 232-238.

48. Zimmerman, Shawn M., Long, M. E., Dyke, J. S., Jelesijevic, T. P., Michel, F., Lafontaine, E. R. and Hogan, R. J. 2018. Use of Immunohistochemistry to Demonstrate In Vivo Expression of the Burkholderia mallei Virulence Factor BpaB During Experimental Glanders. *Vet. Pathol.* **55**: 285-267.

49. Zubaidy, A. J. and Al-Ani, F. K. 1978. Pathology of Glanders in Horses in Iraq. *Vet. Pathol.* 15: 566-568.

SUMMARY IN JAPANESE

鼻疽菌（*Burkholderia mallei*）はウマ科の動物に鼻疽を引き起こすが、感染動物との直接接触によってヒトに伝播することが知られている。馬の鼻疽はアジア、中東、アフリカ、南米において発生が認められる。モンゴルでは馬は古より重要な動物で、現在でも乗馬に広く使われており、また遊牧民の日常生活において欠かせないパートナーである。このようにモンゴル人にとって馬は最も身近な動物であることから、馬の鼻疽はヒトの健康にとっての脅威であり、さらに食品衛生、家畜・畜産加工物の輸出入や国防にとっても問題となりうる疾病である。モンゴルでは、地方の獣医師によって鼻疽様の症状を示す馬の存在が散発的に報告されているにもかかわらず、政府主導の鼻疽サーベイランスは近年行われていない。本研究は、モンゴルにおいて発生する鼻疽の病理学的解析と疫学調査を目的として行われた。

第 1 章では、鼻疽を発症した馬の病理組織学的変化を明らかにし、鼻疽菌の体内分布を免疫組織学的に解析した。ウランバートル近郊のナライク郡とウルツィット郡に位置する小規模の農場 2 軒において、3 年前にロシアから輸入した計 4 頭の馬に、鼻汁の排出と両後肢、腹壁に多発する皮膚結節が認められた。これら

4 頭の臨床症状、病理解剖時の肉眼所見および病理組織学的所見は、過去に報告された馬の鼻疽で認められるものと同様であった。従って、モンゴルにおいて馬の鼻疽が散発していることが確認された。鼻疽菌のオートトランスポーターである BpaB 蛋白に対するモノクローナル抗体を用いて免疫染色を行ったところ、化膿性肉芽腫ないし膿瘍を形成する食細胞の細胞質に BpaB 抗原が検出された。さらに、少数の細気管支上皮細胞とⅡ型肺胞上皮細胞にも抗原が認められた。以上の結果から、モンゴルにおいて馬に鼻疽が自然発生していることが確認され、鼻疽罹患馬において鼻疽菌が食細胞と呼吸上皮に感染することが示された。

第 2 章では、モンゴル中央部および東部において採材した 337 頭の馬血清（モンゴル馬 272 頭、サラブレッド 35 頭、モンゴル馬とサラブレッドの混血 30 頭）を使用して抗体疫学調査を行った。337 頭のうち、モンゴル馬 3 頭とサラブレッド 4 頭に鼻疽を示唆する臨床症状が認められたが、他の馬は無症状であった。血清サンプル中の抗鼻疽菌抗体の検出には補体結合反応とローズベンガル平板凝集反応を使用した。その結果、補体結合反応では 337 頭中 28 頭（8.3%）、ローズベンガル平板凝集反応では 337 頭中 26 頭（7.7%）が陽性を示した。興味深いことに、モンゴル馬とサラブレッドの混血馬において、モンゴル馬より高い抗体陽性

率が認められた。本研究の結果から、モンゴルでは馬に鼻疽菌の無症候感染が生じていることが示唆された。

本研究により、モンゴルでは馬に鼻疽が恒常的に流行していることが示唆された。従って、より大規模な疫学調査と鼻疽対策の確立が、モンゴルからの鼻疽の根絶に向けて必要であると考えられた。