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Bacteriology

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

Ochbayar ERDEMSURAKH[1,2], Khurtsbaatar OCHIRBAT[3], Ulziisaikhan GOMBOSUREN[3], Batbold TSERENDORJ[3], Baatarjargal PUREVDORJ[4], Batbaatar VANAABAATAR[3], Keisuke AOSHIMA[1], Atsushi KOBAYASHI[1] and Takashi KIMURA[1]*

[1]Laboratory of Comparative Pathology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
[2]Laboratory of Pathology, School of Veterinary Medicine, Mongolian University of Life Sciences, Khan-Uul district, Zaisan 17042, Ulaanbaatar, Mongolia
[3]Laboratory of Infectious Disease and Immunology, Institute of Veterinary Medicine, Mongolian University of Life Sciences, Khan-Uul district, Zaisan 17042, Ulaanbaatar, Mongolia
[4]Laboratory of Pathology, Institute of Veterinary Medicine, Mongolia University of Life Sciences, Khan-Uul district, Zaisan 17042, Ulaanbaatar, Mongolia

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ABSTRACT. Glanders is a contagious and fatal equine disease caused by the gram-negative bacterium *Burkholderia 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. In this study, we 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). We randomly collected blood samples from horses in central and eastern Mongolia between 2018 and 2019. Of 337 horses, 26 (7.7%) and 28 (8.3%) were seropositive using RBT and CFT, respectively. Interestingly, seropositivity in horses resulting from crossbreeding of Mongolian native horses with thoroughbred horses was higher than that in Mongolian native horses. Our observations suggest that equine glanders are still endemic to Mongolia.

KEY WORDS: glanders, horse, Mongolia, seroprevalence

Equine glanders are fatal zoonotic bacterial infectious diseases caused by *Burkholderia mallei*. *B. mallei* primarily affects horses [1, 5, 6, 18, 28, 34], donkeys [21, 30], and mules [2]. Glanders has been eradicated in Western Europe, Great Britain, the United States of America and Canada. However, the disease has re-emerged in Asia, the Middle East, Africa, and South America. For instance, outbreaks of glanders have recently been reported in Bahrain [32], Brazil [2, 5, 6, 21], Pakistan [7], India [17, 18, 30], Turkey [1], and Iran [10, 19].

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 [19, 20, 30]. Transmission of *B. mallei* occurs mainly through ingestion of contaminated feed or water, or otherwise through direct skin or mucous membrane contact with excretions from infected animal tissues [25]. 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 [9, 20, 25].

Since glanders are zoonotic diseases caused by *B. mallei*, bacterial infection reportedly occurs in veterinarians, veterinary students, farmers, horse handlers, slaughterhouse workers and laboratory workers, who often come into contact with infected animals [26, 31]. However, animal to human and human to human transmission has rarely been reported [14]. No vaccine is commercially available for human or animals [26] and 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 [9]. The mallein skin test [2, 3, 8, 24] (allergic hypersensitivity test) has been widely used in conventional field tests. However, due to animal welfare concerns, this test is not currently recommended [26]. The complement fixation test (CFT) is considered a suitable screening test for the diagnosis of equine glanders [4, 11, 12, 25, 27, 28, 30]. CFT is mandated by the World Organization for Animal Health (OIE) as a confirmatory test for international trade [26]. Rose Bengal

*Correspondence to: Kimura, T.: tkimura@vetmed.hokudai.ac.jp

plate agglutination test [8, 25] (RBT) is approved in Russia but, due to its relatively low sensitivity, it is not commonly used in other countries. Other serological tests, including immunoblot assay [28, 33] and enzyme-linked immunosorbent assay (ELISA) [4, 15, 16, 29], 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 the gold standard diagnostic methods for equine glanders, but these processes are time-consuming [26].

Mongolia is located in central Asia, between Russia and China. Geographically, Mongolia is divided into four different zones: gobi-desert (South), mountain (West), forest (North), and steppe-field (East).

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 [23]. 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 [13, 14]. This project involved 16 provinces and using CFT, found that 24,760 out of 126,960 (19.5%) horses were seropositive. Using the mallein test they also detected 241,157 (4.8%) of 5,046,070 horses and 380 (0.1%) of 332,684 camels to be positive for *B. mallei* [13, 14]. 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 [22]. 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) [22].

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 parts 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 m*l*) 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 (Fig. 1). 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 18–25°C RT (room temperature) by centrifugation at 2,500 rpm for 10 min and then transferred into new tubes, labeled, and stored at −20°C until use.

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 [25] were added to each well, and the plates were incubated for 45 min at 37°C and centrifuged for 5 min at 600 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 (in 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 18–25°C RT (room temperature) for 3 min, and any visible agglutination was considered positive.

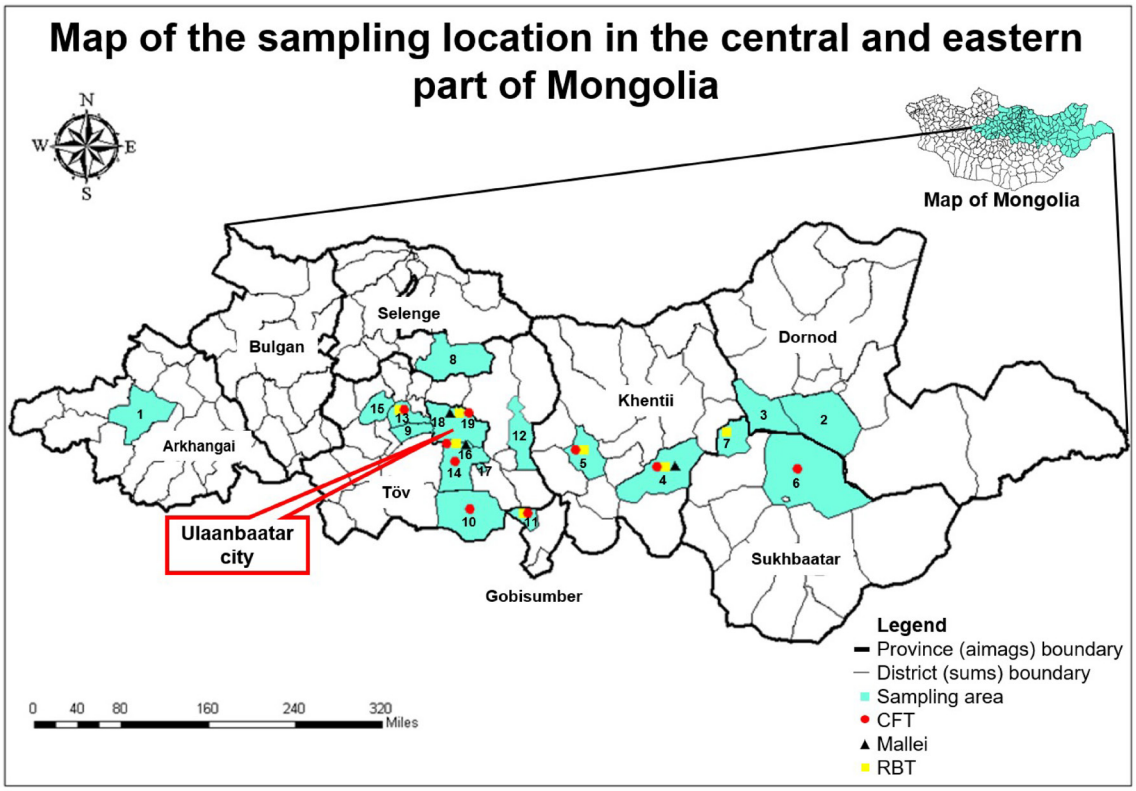

Fig. 1. Map of Mongolia showing the areas in which horse sera were collected. The samples were taken from Ondor-Ulaan[1], Bulgan[2], Holonbuir[3], Bayankhutag[4], Jargaltkhaan[5], Sukhbaatar[6], Tumentsogt[7], Mandal[8], Argalant[9], Bayantsagaan[10], Bayantal[11], Bayandelger[12], Bayantsogt[13], Sergelen[14],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

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 m*l* (0.95–1.05 mg/m*l*) 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 positive.

Statistical analysis

The statistical analyses were performed using Fisher's exact test. $P<0.05$ was considered 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 1). 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 1 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 2). 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 3), 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 years or older.

Table 1. 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) Number indicates the location of each districts in Fig. 1. b) Number of positive cases. CFT, complement fixation test; RBT, Rose Bengal plate agglutination test.

Table 2. 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 3. 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) Number of positive cases by complement fixation test (CFT). b) Number of tested cases by CFT.

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 1). 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 1 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. 2). 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 (Fig. 1). 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

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

Our data suggest 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 another. This result was inconsistent with the previous report [24], 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 difference of 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.

Crossbreeding of Mongolian native horses with imported thoroughbred horses has been recently done in Mongolia to produce high-performance racing horses for the traditional Mongolian Naadam festival. Our results suggest that thoroughbred horses and crossbreed horses are 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 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 [18] and pose a hidden risk to humans [31]. 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. Our 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.

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