Bearing the brunt: Mongolian khulan (*Equus hemionus hemionus*) are exposed to multiple influenza A strains

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Abstract

The majority of influenza A virus strains are hosted in nature by avian species in the orders of Anseriformes and Charadriformes. A minority of strains have been able to cross species boundaries and establish themselves in novel non-avian hosts. Influenza viruses of horses, donkeys, and mules represent such successful events of avian to mammal influenza virus adaptation. Mongolia has over 3 million domestic horses and is home to two wild equids, the Asiatic wild ass or khulan (*Equus hemionus hemionus*), and Przewalski's horse (*Equus ferus przewalskii)*. Domestic and wild equids are sympatric across most of their range in Mongolia. Epizootic influenza A virus outbreaks among Mongolian domestic horses have been frequently recorded. However, the exposure, circulation and relation to domestic horse influenza A virus outbreaks among wild equids is unknown. We evaluated serum samples of Asiatic wild asses in Mongolia for antibodies against influenza A viruses, using modified protein microarray technique. We detected antibodies against hemagglutinin (H) H1, H3, H5, H7, H8 and H10 influenza A viruses. Asiatic wild asses may represent a previously unidentified influenza A virus reservoir in an ecosystem shared with populations of domestic horses in which influenza strains circulate.

Keywords: Influenza A, virus, serology, Asiatic Wild Asses, exposure, antibodies

Introduction

Historically, two major strains of Equine Influenza virus (EIV) have caused influenza virus outbreaks in domestic equids. The first identified EIV, influenza A/H7N7 or equine-1, was isolated from horses in 1956 [1]. Influenza A/H3N8 or equine-2 was subsequently reported and

remains the major cause of equine influenza [2]. While H7N7 EIV is thought to be equine-specific with limited but unique variation in the HA gene [3], H3N8 EIV appear to bind to avian-like receptors in the upper respiratory tract of horses suggesting a recent avian origin of the strain[4]. Further evidence horse of susceptibility to avian like influenza viruses is the occurrence of two outbreaks in China in 1989 and 1990, caused by H3N8 virus, antigenically related to H3 viruses of avian origin [5]. Moreover, previous H3N8 influenza virus outbreaks in dogs [6], their isolation from a Bactrian camel in Mongolia [7], and some evidence for human infection [8], indicate that horses are not the only host for H3N8 viruses. Mongolia, with a current population of domestic horses exceeding 3 million, has suffered several EIV outbreaks [9]. The first two outbreaks, 1974-75 and 1983-84, were caused by H7N7 EIV and the last three, 1993-94, 2007-08 [10] and 2011 were caused by H3N8 EIV. After 1984 [11], H7N7 EIV was not isolated and is considered extinct in the region, while H3N8 seems to circulate in low prevalence [9].

In addition to domestic horses, Mongolia is home to the Przewalski's horse (*Equus ferus przewalskii)*, and hosts the biggest population of Asiatic wild ass (or khulan, *Equus hemionus hemionus*) in Central Asia [12]. The distribution of khulan overlaps with other free-living ungulate species, such as goitered gazelles (*Gazella subgutturosa*), Mongolian gazelles (*Procapra gutturosa*), and free ranging Bactrian camels (*Camelus ferus*). Most importantly their distribution overlaps with local livestock including domestic horses which outnumber wild ungulates by several orders of magnitude. Disease transmission between domestic and free-living populations is possible through sharing pasture and waterholes. EIV outbreak dynamics in wild equids from Central Asia are poorly understood. In 2007 an H3N8 influenza (A/equine/Xinjiang/4/2007) outbreak was reported in a Przewalski's horse population in the Chinese part of the Gobi with a 5% mortality rate [13]. Influenza exposure in khulans, however,

67 remains uncharacterized. Mongolia also has a high diversity of wild birds, including migratory
68 waterbirds, that use Mongolia as a stop-over during their annual migrations. The Central and the
69 East Asian flyways passing through Mongolia are critical to influenza ecology (Figure 1).
70 Therefore, we sought to investigate the exposure to influenza viruses in Mongolian khulan, as a
71 first step in understanding their role in the ecology of influenza viruses.

72

73 **Materials and Methods**

74 The study took place in the Southern Gobi Desert in Mongolia, and was approved by the
75 ethical committee of the University of Veterinary Science in Vienna (ETK-15/03/2016) and the
76 Mongolian Government (05/5656). Twenty-one adult khulan (8 stallions and 13 mares) were
77 anesthetized and nasal swabs, serum and blood samples collected from October 15-19, 2015. The
78 method of choice for efficient chemical capture in the Gobi desert is from a moving jeep. After
79 successful detection of khulan in the steppe, and initiation of the chase, there is a cut off time of
80 15 min for animal welfare reasons which determines when capturing will end. Subsequently, a
81 new khulan group needs to be found before continuing. The time to capture (from detection and
82 initiation of the chase to reversal of the anesthesia to being ready to resume the search for a new
83 animal) for individual animals ranged from approximately 1 hour to several days.

84 The animal sampling expedition was part of a radio collaring project, in which habitat
85 fragmentation, due to new mining-related infrastructures in Southern Gobi was investigated.
86 Khulans were captured in two different locations, one near the mining-infrastructure site and one
87 near the Ergeliin Zoo protected area (Figure 1). All animals were darted from a moving jeep,
88 using a Daninject JM CO2 darting gun [14]. None of the khulan demonstrated clinical symptoms
89 of EIV or other infectious diseases when handled. Samples were stored immediately at -20°C in

a portable freezer in Mongolia, transported on dry ice to Austria in full compliance with the Convention on International Trade in Endangered Species (CITES) and stored at −80 °C until laboratory analysis at the Research Institute of Wildlife Ecology, University of Veterinary Medicine, Vienna. Due to field conditions and the absence of a mobile laboratory, blood samples could not be processed on site, so that only 13 of 21 serum samples could be used and all were severely hemolysed.

A protein microarray (PA) technique as described previously [15,16,17,18],was used to identify the influenza virus strains in the khulan serum samples. Samples were inactivated in a water bath at 56° C for 4 hours due to regulations for testing of animal samples from foot and mouth disease endemic regions. Serum samples from 3 kulans were tested against different secondary antibodies in order to determinate the highest sensitivity; protein A, protein G and anti-horse. Anti-horse IgG showed highest overall response.

Briefly, thirty-two recombinant proteins of different influenza A virus antigens were printed on 16-pad nitrocellulose Film-slides (Oncyte avid, Grace Bio-labs, Bend, OR, USA). All presently known influenza A virus HA-types are represented on the array (Table 1), except for bat hemagglutinin type 17 and 18. Slides were treated with Blotto-blocking buffer to avoid non-specific binding (Thermo Fischer Scientific Inc., Rockford, MA, USA) for 1 hour at 37°C in a moist chamber. After washing the slides were incubated with a fourfold dilution series of the khulan serum starting from 1:20 to 1:1280. After 1 hour incubation at 37 °C, slides were washed and incubated with a 1:500 dilution of the anti-horse IgG conjugated to Alexafluor 647 (Jackson immunoresearch). A last washing step was done to remove unbound conjugate, after which the slides were dried and scanned using a Powerscanner (Tecan). Spot intensities were determined,

and titer heights were calculated by curve fitting using R (R Statistical Computing, version 3.1.0, Vienna, Austria). Since the serum dilutions start from 1/20, titers less than 20 were set to 20.

Investigation of viral shedding was attempted from nasal swabs (see Supplementary material); however, as no viral material was detected, genome sequences were unavailable introducing uncertainty as to which strains to use in confirmatory serological assays. Protein microarray results were confirmed depending on the availability of strains with a probability of being related to the field strains, e.g. horse or avian derived. We used three available strains: one H3 strain (A/equine/Richmond/1/07) that is the equine influenza strain known to circulate worldwide and was included in the protein microarray and two H7 strains, A/Equine Prague/1/56 and A/Mallard/Netherlands/12/00). A/Mallard/Netherlands/12/00 is closely related to the H7N7 strain A/Chicken/Netherlands/1/03 that was found on the PA. No closely related strain to the H7N7 on the PA was available and A/Equine Prague/1/56 was chosen because of its equine origin.

Hemagglutination inhibition assays (HI) (see Supplementary material), Virus Neutralization Test (VNT) (Supplementary material) and Single Radial Hemolysis Assay (SRH) were used to confirm the results of PA on a subset of samples.

Seven khulan serum samples (both microarray positive and negative) were tested against strains H3N8 (A/equine/Richmond/1/07), H7N7 (A/Equine Praque/1/56), and H7N3 (A/Mallard/Netherlands/12/00) using SRH according to the OIE recommendations [19]. Positive and negative reference anti-equine influenza serum were used in each plate as controls. Fresh sheep erythrocytes (obtained from Berlin zoo) and/or chicken erythrocytes (Labor Dr. Merk, Germany) were washed and prepared to a final concentration of 8% in saline/HEPES buffer. Erythrocytes were sensitized with each virus independently, mixed with guinea pig complement

(Sigma) and 1% agarose gel, and finally spread on a plate. Holes were punched in the gel and 10 µl of heat inactivated (56°C for 30 min) serum, including positive and negative controls, were added to each well and plates were incubated at 34°C for 20 hours in a humid box. The area of hemolysis was measured and expressed in mm^2. The assay was repeated two times independently.

Results

Viral detection was attempted from nasal swabs using qPCR but no virus could be detected. The result is not surprising as 460 domestic free-ranging Bactrian camels were similarly screened yielding a single influenza A virus positive individual [7]. Considering none of the animals displayed clinical symptoms of infection, the lack of actively shedding individuals is consistent with expectations.

To detect exposure to influenza in non-shedding individuals, a protein microarray (PA) technique testing 32 hemagglutinin recombinant proteins (HA1-part) from type H1 to H16, as described previously [15,16,17,18], was used to profile the antibodies to influenza viruses in the khulan serum (Table 1). Six animals were negative, whereas 7 animals had reactivity detectable by microarray to one or more antigens. These were low levels of reactivity to H5 (2 animals), H8 and H10 antigen (1 animal each), and low to moderate titers against H1 (1 animal), and H7 (2 animals). Five khulans showed reactivity to H3-08, which is the horse influenza strain known to circulate in Mongolia. This reactivity was specific for the EIV H3 antigen, other antigens (representing strains isolated from humans) were negative (Figure 2). The two khulan serum samples which reacted with H7 antigen, reacted specifically to the Dutch H7N7 strain (H7-03, A/Chicken/Netherlands/1/03), but not to the Chinese H7N9

158 Confirmation of the PA results using hemagglutination inhibition assays was not possible
159 because the serum was severely hemolysed and agglutination was detected in the control well
160 without virus. VNT was tested on a subset of serum samples (Table 2 and 3) using one H3N8
161 equine, and two H7 strains (see supplemental information). Although minimal replicates were
162 used, VNT did confirm the H3N8 result of the PA, but failed to confirm the H7 result (Table 2
163 and 3).
164 Single radial hemolysis (SRH) was performed using A/equine/Richmond/1/07, A/Equine
165 Praque/1/56 and A/Mallard/Netherlands/12/00. Two of four H3 positive khulan in the protein
166 microarray reacted with A/equine/Richmond/1/07 with a hemolysis area of 35 and 60 mm^2. The
167 two samples that could not be confirmed had lower antibody titer as determined by the protein
168 microarray and were likely below the detection limit of the SRH assay (Table 2 and 3). The H7
169 positive khulan in the protein microarray, reacted with both A/Equine Praque/1/56 and
170 A/Mallard/Netherlands/12/00; the hemolysis area was 35 mm^2. The hemolysis areas of positive
171 and negative control serum were 148 and 12 mm^2, respectively.
172
173 **Discussion**

174 Although we could not detect viral genomes to further define the strains circulating
175 among wild equids, our serological results suggest that equids may be exposed to more influenza
176 viruses than previously considered. Virus detection is often limited by the short window in which
177 the virus is present, and therefore screening for antibodies, which often persist longer than the
178 virus itself, provides information about past infections and virus diversity in animal populations
179 [17]. While sampling of twenty-one individual animals may seem low, one has to keep in mind
180 that khulans are extremely skittish animals, and normally flee human presence even when

separated by several kilometers distance. Anesthesia and sampling of non-domestic equids, particularly under the physically challenging and remote environment of the Gobi Desert, can be difficult, for both animals and humans, and not always successful [14]. Furthermore, khulans are a red list species globally and nationally and capture permits are granted only after careful evaluation of the risks and benefits. On these grounds, capture permits for the mere sampling of an endangered species without an imminent need have little chance of approval.

The difficult terrain, with dry river beds, low mountains, bushes, shrubs and desert basins, severely restricts successful outcomes. Capture (from detection and initiation of the chase to reversal of the anesthesia to being ready to resume the search for a new animal) for one individual takes approximately 1 hour under the best conditions but ranges to several days, if khulans are not found in the vast Gobi ecosystem [19]. In our study, the number of animals captured exceeded the expectations for our short 2-week window. These challenges need to be taken into account when evaluating this study.

Our findings suggest that khulans might be susceptible to more influenza A viruses than previous thought, although they may not show any signs of disease. Zhu et al reached a similar conlusion, where he showed in his study, that Mongolian horses are being infected with different AIV, without that resulting to new outbreaks or clinical signs [20]. Despite the low titer values observed, the PA and the other confirmatory assays were largely congruent. The animals, which had higher titers as determined by the protein microarray, could generally be confirmed by additional serological methods. Those with very low titers as determined by the PA were either unconfirmed or only confirmed by one of the additional assays employed. Several other factors also played a role in the outcome of our confirmatory experiments. Besides low titer values, bad field storage conditions and the long inactivation time of serum for several hours at 56°C likely

204 degraded the samples and affected the readout of the assays. Moreover, without genomic
205 information, it is also unclear which exact H3 and H7 strains infected the khulans which likely
206 reduced the detection limit of the confirmatory assays as it is unlikely we used the same antigens
207 that confronted the khulans. Titers were also likely low because all animals were clinically
208 healthy when captured and sampled [21,22].
209
210 The most commonly detected antibodies were against H3 EIV HA1 antigens, consistent
211 with data on low vaccination rates and therefore an ongoing circulation of these viruses among
212 Mongolian horses [9,10]. We found evidence for exposure to influenza viruses with a
213 hemagglutinin of subtype H7. H7N7 equine influenza is considered extinct in the region,
214 although some studies still report serological evidence of the strain [23]. The protein microarray
215 and confirmatory SRH reacted both with H7N7 A/Equine Praque/1/56 and H7N3
216 A/Mallard/Netherlands/12/00. SRH, used in our study as a confirmatory test, has been
217 demonstrated to be the most sensitive serological assay for equine influenza viruses [24]. SRH is
218 able to distinguish closely related strains, and detect small quantities of viral antigen [25].
219 Considering our results, co-circulation of both subtypes cannot be excluded. Other H7 subtypes
220 circulate in wild birds in Southeast Asia and viral isolation would further clarify to which
221 specific H7 influenza virus khulan might be exposed in Mongolia. From our results, we could
222 not determine whether the khulans were infected with equine derived H7 strains or were directly
223 infected by waterfowl. Positive khulans against H1 strains may have been infected during an
224 H1N1 pandemic in 2009 [26]. Sajid et al. [27] reported similar results in horses in Pakistan
225 during an EIV outbreak. Two khulans had antibodies against H5 influenza viruses. Similar

226 results have been reported for donkeys, a domestic equid, suggesting equids are susceptible to
227 highly pathogenic H5N1 influenza strains [28].
228 Individual animals were positive for HAs of influenza A H8 and H10, suggesting that
229 sporadic infections with viruses belonging to these subtypes have occurred. However, we cannot
230 exclude that multiple known or unknown strains of H1, H5, H8 and H10 cross reacted in the
231 microarray assay due to the haemolytic nature of the khulan serum or that the viral strains
232 eliciting the immune response are divergent from known strains. The lack of knowledge of
233 strains circulating in wild animal populations constrains assay confirmation and represents a
234 potential area where further research would be beneficial.
235 A possibility is that these viruses co-circulate with H3N8 among equids in Central Asia,
236 but occasional introduction from exposure to wild birds, other mammals or their droppings is a
237 possible alternative [29]. In a harsh steppe-desert ecosystem such as the Gobi Desert, water can
238 be scarce resulting in diverse species congregation at waterholes which may increase disease
239 transmission. The upper respiratory tract of the horse expresses both sialic acid 2,3-Gal and 2,6-
240 Gal receptors, which are similar to those in wild aquatic birds. Because of this similarity in avian
241 and equine respiratory biology [30], it is possible that equids are susceptible to a broader
242 spectrum of influenza viruses than other mammals. Furthermore, the presence and free
243 movement of the domestic and wild species may be risk factors associated with the influenza
244 exposure and transmission.
245
246 The Results reported here should be considered in the light of some of the field and laboratory
247 limitations. The lack of research on the topic , the low animal numbers that could be collected
248 and the nature and preservation of the serum samples exclude statistical analysis, modeling of the

data and limit the conclusions that can be drawn. Although susceptibility of wild equids to new influenza strains may not pose a threat to their conservation status, it might represent an overlooked ecological niche for influenza virus and an alternative route of infection for other wild and domestic animals. Further epidemiological investigation of wild equids from Central Asia should clarify the diversity of influenza virus strains that infect wild equids and help to establish the monitoring of influenza virus transmission between wild and domestic equids in the area.

Authors and contributors

AG, CW conceptualized the study and supervised the study. SES, BB, PK, CW conducted the investigation on capturing and sampling the animals. SES, EdB, KE, WA, NO, GC analyzed the data. SES, GÁC and ADG wrote the original draft. All co-authors contributed to the writing, review and edited of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest

Funding information

ESS, NO, GÁC, CW and ADG were funded by a grant from the Leibniz Gemeinschaft (SAW-2015-IZW-1 440).

Acknowledgments

We acknowledge the FUW-Advanced Design Studio for their insightful suggestions for the map illustration. We thank the Ministry of Nature, Environment and Tourism of Mongolia, Dashzeveg Tserendeleg, Otgonsuren Avirmed, Enkhtuvshin Shiilegdamba (WCS), Nyamdorj Barnuud (SEA), Dennis Hosack, and Purevsuren Tsolmonjav (OT) for the logistical and practical support during khulan capture.

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383 **Figure legends**

384 Figure 1: Relation between khulan distribution, protected areas, sampling sites and the major
385 migratory flyways in Mongolia. The arrows represent the outside border of each migratory
386 flyway.

387

388

389

390 Figure 2: Antibody profiles in sera from khulans, expressed as titers (Y axis) of IgG reactivity to
391 a range of influenza A HA1 antigens (X axis). Animal number corresponding to ID's: 1: 19742;
392 2:19850; 3:19845; 4:19842; 5:19852; 6:19555; 7:19848

393

394

395

396 Table 1. Recombinant HA1-proteins included in the protein microarray.

CODE	SUBTYPE	STRAIN
H1-1918	H1N1	A/South Carolina/1/18
H1-1933	H1N1	A/WS/33
H1-1977	H1N1	A/USSR/92/1977
H1-2007	H1N1	A/Brisbane/59/2007
H1-2009	H1N1	A/California/6/2009
H2-2005	H2N2	A/Canada/720/05
H3-1968	H3N2	A/Aichi/2/1968(H3N2)
H3-2009	H3N9	A/VICTORIA/210/2009
H3-2013	H3N2	A/Switzerland/9715293/2013
H3-2008	H3N8	A/equine/Gansu/7/2008
H4-2002	H4N6	A/mallard/Ohio/657/2002
H5-2997	H5N1	A/Hong Kong/156/97
H5-2002	H5N8	A/duck/NY/191255-59/2002(H5N8) LP
H5-2007	H5N3	A/duck/Hokkaido/167/2007
H5-2008	H5N1	A/chicken/Egypt/0879-NLQP/2008
H5-2010	H5N1	A/Hubei/1/2010
H5-2006	H5N1	A/Turkey/15/2006 (clade 2.2)
H6-1999	H6N1	A/quail/HK/1721-30/99
H7-2003	H7N7	A/Chicken/Netherlands/1/03
H7-2013	H7N9	A/chicken/Anhui/1/2013
H7-2012	H7N3	A/chicken/Jalisco/CPA1/2012

H8-1979	H8N4	A/pintail duck/Alberta/114/1979
H9-1999	H9N2	A/Guinea fowl/Hong Kong/WF10/99
H9-1997	H9N2	A/chicken/Hong Kong/G9/97 (G9 lineage)
H9-2011	H9N2	HA1 (H9N2) A/Chicken/India/IVRI-0011/2011
H10-2007	H10N7	A/blue-winged teal/Louisiana/Sg00073/07
H11-2002	H11N2	A/duck/Yangzhou/906/2002
H12-1991	H12N5	A/green-winged teal/ALB/199/1991
H13-2000	H13N8	A/black-headed gull/Netherlands/1/00
H14-1982	H14N5	A/mallard/Astrakhan/263/1982new
H15-1983	H15N8	A/duck/AUS/341/1983
H16-1999	H16N3	A/black-headed gull/Sweden/5/99

Table 2. Results of the protein microarray (PA), virus neutralization test (VNT) and single radial hemolysis assay (SRH) against A/equine/Richmond/1/07 (H3N8) influenza virus strains.

Animal ID	**PA H3N8**	**VNT H3N8**	**SRH H3N8**
19850	**Positive**	**Positive**	**Positive**
19845	**Positive**	**Positive**	**Positive**
19555	**Positive**	**Positive**	**Negative**
19742	**Positive**	NT	**Negative**
19848	**Negative**	**Positive**	**Negative**
19836	**Negative**	NT	**Negative**
19841	**Negative**	NT	**Negative**

401 Table 3. Results of the protein microarray (PA). virus neutralization test (VNT) amd single radial
402 hemolysis assay (SRH) against A/Equine Praque/1/56 (H7N7) and A/Mallard/Netherlands/12/00
403 (H7N3) influenza viruses.

Animal ID	PA H7	VNT H7N7	VNT H7N3	SRH H7N7	SRH H7N3
19850	**Positive**	**Negative**	**Negative**	**Positive**	**Positive**
19845	**Negative**	NT	NT	**Negative**	**Negative**
19852	**Negative**	**Negative**	**Negative**	NT	NT
19555	**Negative**	NT	NT	**Negative**	**Negative**
19742	**Negative**	NT	NT	**Negative**	**Negative**
19848	**Negative**	NT	NT	**Negative**	**Negative**
19842	**Positive**	**Negative**	**Negative**	NT	NT
19840	**Negative**	**Negative**	**Negative**	NT	NT
19836	**Negative**	NT	NT	**Negative**	**Negative**
19841	**Negative**	NT	NT	**Negative**	**Negative**

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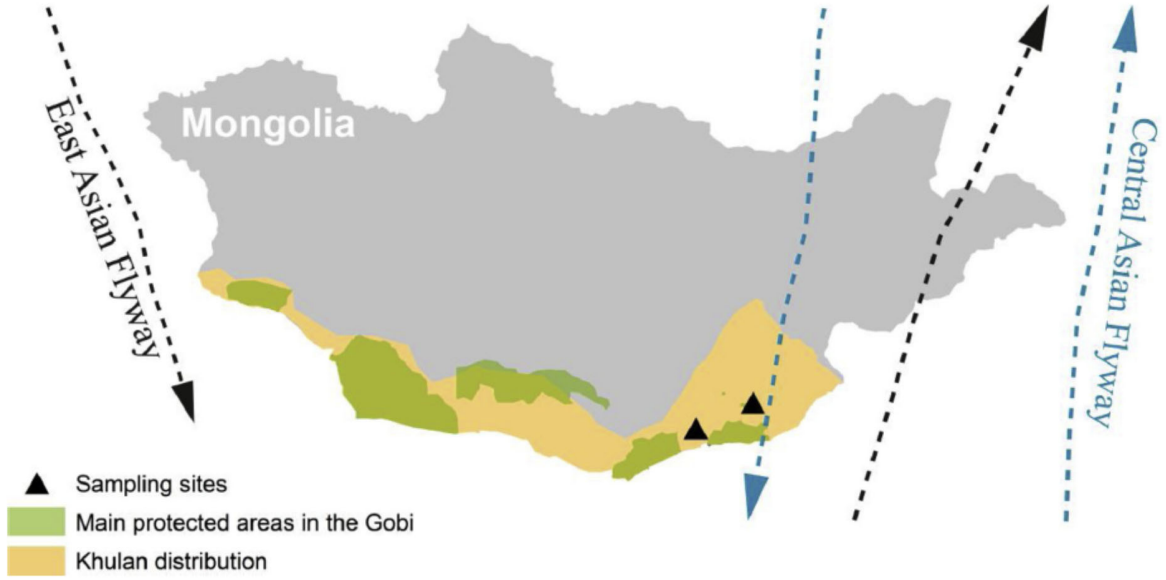

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407 Fig.1

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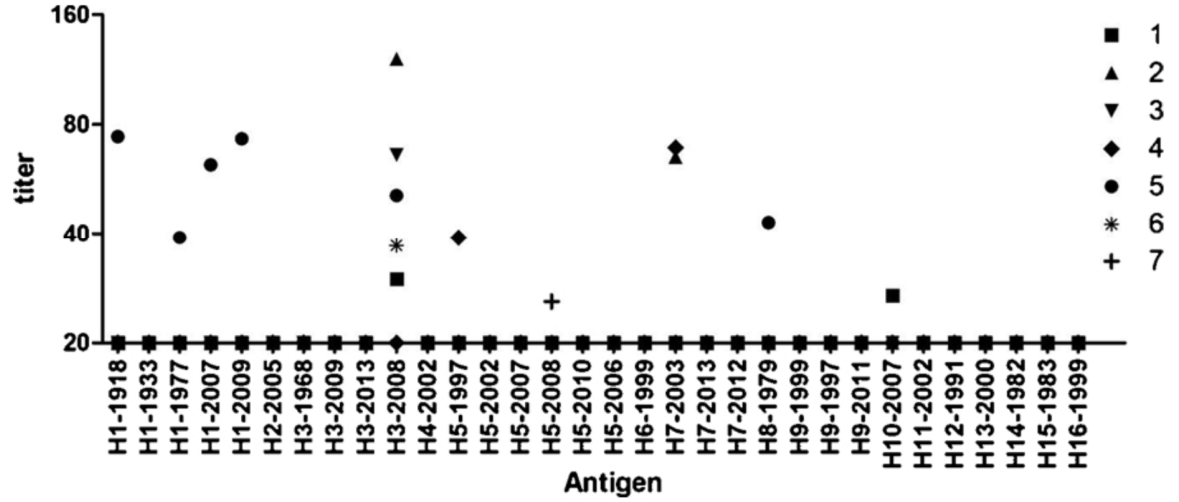
160
80
40
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titer
H1-1918
H1-1933
H1-1977
H1-2007
H1-2009
H2-2005
H3-1968
H3-2009
H3-2013
H3-2008
H4-2002
H5-1997
H5-2002
H5-2007
H5-2008
H5-2010
H5-2006
H6-1999
H7-2003
H7-2013
H7-2012
H8-1979
H9-1999
H9-1997
H9-2011
H10-2007
H11-2002
H12-1991
H13-2000
H14-1982
H15-1983
H16-1999
Antigen
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410 Fig. 2