of this disease in Sri Lanka. A prevalence study of hantavirus among rodents (e.g., *Bandicota* sp.) would also be very useful in order to improve prevention and control measures.

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Review
Future Virology

Recent progress in the treatment of Crimean–Congo hemorrhagic fever and future perspectives

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Crimean–Congo hemorrhagic fever (CCHF) caused by the CCHF virus, a member of the family *Bunyaviridae*, genus *Nairovirus*, is a tick-borne acute viral hemorrhagic fever with a high case-fatality rate. Ribavirin has been used as a treatment for patients with CCHF. Although the efficacy of ribavirin in the treatment of CCHF has not yet been proven conclusively, its use in the early stage of the disease is recommended. A number of clinical and virological insights into CCHF have been revealed. Virus-associated hemophagocytic syndrome has been found to contribute to the exacerbation of CCHF in some patients, and the administration of methylprednisolone at high doses was observationally undertaken in patients with CCHF and virus-associated hemophagocytic syndrome, with promising results. It is expected that effective therapeutics and preventive measures will be developed in the future.

Crimean–Congo hemorrhagic fever (CCHF) virus (CCHFV) is a member of the family *Bunyaviridae*, genus *Nairovirus* [1]. CCHFV was first definitively recognized in Crimea at the end of World War II, when Russian troops returned to assist in the cultivation of tick-infested land, left fallow during the war. The agent was named Crimean hemorrhagic fever virus. It was subsequently found to be indistinguishable from a virus isolated in 1956 from a febrile child in Stanleyville (now Kisangani), Democratic Republic of Congo [2]. Therefore, this virus is named CCHFV. CCHF is an acute viral hemorrhagic fever with a high case–fatality rate (CFR) [1]. The CFR has varied from 10% to more than 50%, with most deaths occurring 5–14 days after onset [3]. This disease is also one of the most important tick-borne zoonotic viral diseases. Owing to the high CFR, and the increase in the number of patients with CCHF, an effective treatment for CCHF is urgently required. The development of the diagnostic systems for CCHF, such as quantitative real-time reverse-transcription (RT)-PCR is necessary for its proper treatment. In this article, recent progress in the field of epidemiology, diagnosis and treatment of CCHF, in both adults and in children, is reviewed.

Crimean–Congo hemorrhagic fever virus

The *Bunyaviridae* family of viruses comprises of more than 300 virus species, grouped into five genera, *Orthobunyavirus*, *Hantavirus*, *Phlebovirus*, *Nairovirus* and *Tospovirus*. CCHFV belongs to genus *Nairoirus* and is maintained in ixodid ticks. CCHFV virions are spherical, with a diameter of approximately 90–100 nm. CCHFV is an enveloped particle with a tripartite, ssRNA genome of negative polarity [1]. Each of the three genome segments contains one open reading frame, flanked by noncoding regions. Four structural proteins are encoded: the RNA-dependent RNA polymerase (L protein), expressed by the large (L)-genome segment; the mature glycoproteins G_N and G_C, encoded by the medium (M)-genome segment, and the nucleoprotein (N), encoded by the small (S)-genome segment. These three segments are encapsidated by nucleoprotein N, and associated with L protein, resulting in the formation of ribonucleoprotein particles [1]. The polyprotein encoded by the M-genome segment is cotranslationally cleaved into precursor molecules, $PreG_N$ and $PreG_C$, which are, subsequently post-translationally processed into the mature glycoproteins, G_N and G_C, respectively [4,5]. These mature glycoproteins play a role in virion attachment to currently unidentified receptors on the susceptible host cells [6].

Crimean–Congo hemorrhagic fever virus is maintained in ixodid ticks (genus *Hyalomma*, *Dermacenter* and *Phipicephalus*) through transovarial and -stadial transmission. Wild and domestic animals are usually infected with CCHFV through tick bites, forming a tick–vertebrate host cycle. Humans acquire infection through tick bites (genus *Hyalomma*)

Keywords
▪ Crimean–Congo hemorrhagic fever ▪ diagnosis ▪ ribavirin ▪ treatment ▪ viral hemorrhagic fever

primarily, or by close contact with fresh meat or blood from slaughtered viremic animals, including sheep, cattle, ostriches and goats. Human-to-human transmission is also documented, mostly through a form of nosocomial or in-house infection [7–17].

Epidemiology of CCHF

Crimean–Congo hemorrhagic fever has been reported in Africa, Eastern Europe, the Middle East and Central and Southern Asia [7,13,15,17,19–45]. CCHF has been confirmed in Abu Dhabi, Afghanistan, Albania, Bulgaria, China (Xinjiang Uygur Autonomous Region), Democratic Republic of Congo, Dubai, Greece, Iran, Iraq, Kazakhstan, Mauritania, Namibia, Oman, Pakistan, Russia, Saudi Arabia, Senegal, Serbia/Yugoslavia, South Africa, Tajikistan, Turkey, UAE and Uganda. The virus has also been isolated in Nigeria, Central African Republic, Kenya, Upper Volta, Madagascar and Ethiopia. The actual number of patients with CCHF is believed to be far higher than that reported, because the disease usually occurs in remote areas. CCHF usually occurs in the spring and early summer in endemic countries in the Northern hemisphere. The outbreak seasonality might be associated with tick density and activity, and with the increase in viral load in animals in the regions. It is noteworthy that, although CCHF had been recognized as a viral disease in the early 1940s in the Crimea Peninsula, Russia, the first case of CCHF in Turkey was identified in 2002, but the number of the patients exceeded 1820 by 2007 [43]. The geographic distribution of CCHF is closely associated with that of ixodid ticks, which are the natural reservoir of CCHFV. Possible causes for the emergence or re-emergence of CCHF are climate changes, which may have a significant impact on the reproduction rate of the vector ticks, and changes in human occupational and agricultural activities [45].

To date, two cases of CCHF imported from Africa to Europe have been reported: one was from Zimbabwe to the UK [101] and the other was from Senegal to France [102]. CCHF outbreaks have been reported commonly among agricultural and abattoir workers and shepherds, all of whom handle livestock, such as sheep, goats and ostriches [15,35,46–48]. The animals responsible for CCHFV transmission to humans in the Northern hemisphere were mainly sheep, while, in South Africa they were also ostriches, and in Namibia, pigs [103].

Clinical symptoms & pathophysiology

Patients with CCHF present with symptoms ranging in severity from fever only, or fever with flu-like symptoms, to hemorrhage with multiple organ failure, resulting in death. The time course of the typical infection consists of four phases: incubation (3–7 days), prehemorrhagic (1–7 days), hemorrhagic (2–3 days) and convalescent [49]. The incubation period is defined as the duration from exposure to CCHFV to the onset of symptoms. Incubation could differ, due to several factors, such as route of infection, dose of infected virus and age of the patients. For instance, it is reported that the average incubation time was 3.2 days when infected through a tick bite, while it was 5 and 5.6 days when infected through exposure to blood or tissue of livestock, and through exposure to blood from human cases, respectively. Patients usually have a prehemorrhagic period, which is defined as the duration from the onset of flu-like symptoms, such as fever, headache, myalgia and dizziness, but not hemorrhagic symptoms to the onset of hemorrhagic symptoms. Fever, joint pain, orbital pain, backache and headache are common symptoms. Elevation of liver enzymes has been demonstrated as well [49].

The hemorrhagic period in typical CCHF cases rapidly develops at between 3 and 5 days from disease onset. Oliguria is a common symptom, which is presumably associated with renal failure caused by the direct influence of CCHFV infections and/or by indirect influence through hypovolemic shock. Severe cases usually progress to disseminated intravascular coagulation (DIC), leading to the exacerbation of coagulopathy [49]. Virus-associated hemophagocytic syndrome (VAHPS), presumably an important factor, has recently been reported to contribute to the clinical manifestations and severity of CCHF [50,51]. VAHPS is a rare and severe disease syndrome, characterized by fever, hepatosplenomegaly, cytopenia, elevated ferritin, lactate dehydrogenase and triglyceride levels and, most importantly, hemophagocytosis in the bone marrow, liver and lymphnodes. These events, seen in patients with VAHPS, are associated with the excessive production of various cytotokines by highly activated T-helper lymphocytes and macrophages. It was reported that VAHPS is possibly one of the major factors responsible for the high CFR rate in patients with CCHF [50]. It was reported that, of all the cytokines tested, TNF-α, soluble TNF receptor, IL-6 and IL-10 were significantly elevated in a fatal case of CCHF, but not in a nonfatal

case [52]. Similar findings were also reported elsewhere [53]. Serum levels of TNF-α, IL-6 and IL-10 were measured in three fatal cases of CCHF, and were compared with those of 27 nonfatal cases. The levels of TNF-α and IL-6 were significantly higher in fatal cases than those of nonfatal cases. TNF-α production contributes to macrophage activation, resulting in hemophagocytosis. These findings support the evidence that VAHPS contributes to the pathophysiology on CCHF.

Although the number of children with CCHF is relatively small, the clinical features of CCHF in 21 children have been reported [28]. CCHF was more common in males, and approximately 70% of cases had a history of a tick bite. Fever, nausea, malaise and headache were common symptoms. Thrombocytopenia, anemia and elevated liver enzymes were also demonstrated in most cases. VAHPS was demonstrated in some children. Pulmonary hemorrhage developed in two children, and one child died.

The details of the clinical course of CCHF in children were assessed on 31 children with CCHF by researchers in Turkey [54]. Male:female ratio was 19:12. The majority of the patients (87%) had a history of a tick bite. Fever, malaise, nausea/vomiting, diarrhea, tonsillopharingitis, headache and myalgia were common symptoms. No patients died. It was documented that VAHPS was demonstrated in bone marrow aspirate in one child, indicating that VAHPS also contributes to the pathophysiology of CCHF in children.

On the contrary, the CFR of CCHF in children in Iran (26.5%; nine out of 34) [55] was reported to be higher than those in Turkey [28,54].

Although further studies are needed, the clinical features of CCHF in children are essentially the same as those observed in adults.

Diagnosis

The precise diagnosis of CCHF is necessary in order to reduce the mortality and morbidity associated with CCHF. The establishment of diagnostic systems for CCHFV infections in regional laboratories and reference hospitals near the sites of CCHF outbreaks is, therefore, an issue that needs to be resolved. CCHFV is regarded as a biosafety level-4 (BSL-4) pathogen in some countries, indicating the manipulation of CCHFV is restricted to high-containment laboratories. The materials for virological tests should be handled very carefully to reduce the risk of nosocomial infections. The detection of both IgG and IgM CCHFV-antibodies is required for diagnosis. CCHFV antibody detection tests such as indirect immunofluorescent assay (IFA) and ELISA, are available only in a limited number of laboratories. CCHFV antigen-detection assay is useful for the proper diagnosis of CCHF and assessment of the clinical course of CCHF in patients [56,57]. CCHFV genome amplification tests are also important and practically useful virological tests for the diagnosis of CCHF [12,47,58,59]. Nested RT-PCR is commonly used for diagnostic purposes, while, recently, quantitative real-time RT-PCR assays for the amplification of the CCHFV genome were also reported [60–62]. Although the sensitivity and specificity of the real-time RT-PCR require further evaluation, this assay might prove to be an efficacious tool for the assessment of the clinical course of CCHF and its outcome in patients.

As mentioned previously, CCHFV must be handled in a high-containment laboratory. This restriction makes it difficult to prepare diagnostic materials and to perform virological tests for CCHFV infections. To overcome this difficulty, recombinant viral antigen-based antibody- and antigen-detection assay systems have been developed. A recombinant CCHFV nucleoprotein N-based ELISA for the detection of IgG and IgM antibodies was developed, and was shown to have high sensitivity and specificity [44,47,63,64]. A recombinant CCHFV nucleoprotein N-based indirect IFA has also been developed [65]. Garcia *et al.* also developed a recombinant N-based antibody detection assays (IFA and ELISA) using the recombinant nucleoprotein N expressed in mammalian cells via the recombinant Semliki Forest α-virus replicon [66]. Furthermore, a CCHFV antigen-detection sandwich ELISA was developed using a novel monoclonal antibody to recombinant CCHFV nucleoprotein N [56]. The advantage of these recombinant protein-based diagnostic systems is that these diagnostics can be employed in regional and reference institutes without a BSL-4 laboratory.

Therapy

General considerations

No specific therapies have been confirmed to be efficacious in the treatment of patients with CCHF. Differential diagnosis is extremely important, and it is necessary to consider other possible causes of the symptoms. After performing a septic work-up, broad-spectrum antibiotics should be considered. Supportive therapies to improve hemostasis are required, and red blood cells, fresh–frozen plasma and/or thrombocyte

solutions should be administered, if necessary [67]. Preventive measures should be installed to minimize the risk of nosocomial infections.

Supportive therapies

Essentially, maintenance therapies, such as hydration, blood transfusion and other specific supportive therapies (i.e., the administration of diuretics and/or antibiotics – if necessary), should be initiated as soon as possible. If findings consistent with DIC are demonstrated, the treatment strategy becomes more complicated because the treatment for DIC itself carries the risk of exacerbating the tendency toward bleeding. Recently, it was reported that the administration of high doses of methylprednisolone showed promise for CCHF patients, in whom findings consistent with VAHPS were demonstrated [51]. Methylprednisolone was administered to five patients at a dose of 20–30 mg/kg/day intravenously for approximately 5 days. Although one of the five patients died due to septicemia, the prognosis was good in the other patients. Although methylprednisolone administration is still purely observational, this therapy should be one of the choices for CCHF patients with VAHPS.

Specific therapies

Antiviral drug: ribavirin

Ribavirin is the only antiviral drug that has been used to treat CCHF. Ribavirin inhibits the replication of CCHFV *in vivo* and *in vitro* [68,69]. Ribavirin has been administered to patients orally and intravenously [10,70–78] and, although there are a relatively large number of case reports in which ribavirin was used for the treatment of CCHF, and clinical studies in which the efficacy of ribavirin was assessed, we have not yet obtained conclusive results on its efficacy. The papers describing the efficacy of ribavirin in patients with CCHF are summarized in Table 1. Some studies demonstrated its efficacy, especially when administered in the early stage of disease onset [71,72,77,78], although other studies did not [70,73,76]. Since there are some reports on the efficacy of ribavirin, especially when prescribed at an early phase of illness, ribavirin is considered to be one of the choices for patients with CCHF under the present circumstances. A current recommended regimen, adjusted for bodyweight, is 30 mg/kg as an initial loading dose, then 15 mg/kg every 6 h (4 × 1 g) for 4 days, and 7.5 mg/kg every 8 h (4 × 0.5 g) for 6 days [67]. Ribavirin should be one of the choices for treatment of CCHF, and should be administered as early as possible.

Hematological and neurological abnormalities are common side effects induced by ribavirin treatment. In the treatment of patients with CCHF by ribavirin, adverse events due to ribavirin treatment were described, and severe adverse events were not reported in the randomized study on the efficacy of ribavirin in the treatment of CCHF patients [73,74,79].

Passive antibody transfer

Since viremia is prominent in the early stage of CCHF, the transfer of antibodies to CCHFV is expected to be effective as a treatment [67]. Vassilenko *et al.* reported the prompt recovery of seven severely ill CCHF patients treated with the passive transfer of two different immunoglobulin preparations, CCHF bulin (for intramuscular use) and CCHF venin (for intravenous use), which were prepared from the plasma of CCHF survivor donors, boosted with one dose of CCHF vaccine [80]. It appears that this therapy, based on the intramuscular and intravenous transfer of human immunoglobulin active against CCHFV, was effective as a treatment. As the number of patients treated with this drug was too small to draw definite conclusions, the treatment of CCHF with the passive transfer of immunoglobulin remains controversial. In addition, as there are no standard therapeutics with this kind of drug to date, further study, such as a placebo-controlled trial of this therapy, is required to assess its efficacy. The passive transfer of immunoglobulin against CCHFV should be administered as early as possible, once it becomes available.

Interferon

It was reported that IFN-α inhibited the growth of CCHFV in human endothelial and hepatoma cells [81,82]. It was demonstrated that the IFN-induced MxA, which is induced exclusively by α and β IFNs and belongs to the dynamin superfamily of large GTPases, is a major factor mediating the antiviral effect against CCHFV. CCHFV replication was inhibited in the cells, in which recombinant MxA was inhibited, and the inhibition was due to the interaction of MxA with the viral nucleocapsid protein. However, IFN therapy in CCHF patients have not been reported, except for in one paper [17]. In the literature, IFN therapy was terminated owing to severe side effects. IFN therapy is experimental for patients with CCHF.

Conclusion & future perspective

Crimean–Congo hemorrhagic fever often appears to be a neglected disease, as it usually occurs in remote areas in developing countries.

Table 1. Summary of the reports on the efficacy of ribavirin in treatment of CCHF, route of administration, dose and number of patients.

Study (year)	Route of administration	Dose	Subjects (n)	Comments	Ref.
Fisher-Hoch *et al.* (1995)	p.o.	4 g/day in four divided doses for 4 days followed by 2.4 g/day for 6 days	3	Subjects were two surgeons and a hospital worker. All patients became afebrile, and their laboratory findings returned to normal within 48 h of ribavirin treatment	[10]
Mardani *et al.* (2003)	p.o.	30 mg/kg as an initial dose, 15 mg/kg every 6 h for 4 days, followed by 7.5 mg/kg every 8 h for 6 days	187 (81 confirmed and 106 suspected subjects)	The efficacy was evaluated with the historical data. The CFR among ribavirin treatment subjects was lower than that calculated from the history control, suggesting efficacy of ribavirin	[71]
Ozkurt *et al.* (2005)	p.o.	2 g as an initial dose, 4 g/day in 4 divided doses for 4 days followed by 2.0 g/day for 6 days	60 (22: ribavirin group; 38 control group)	It was reported that the efficacy of ribavirin was not statistically demonstrated	[73]
Cevik *et al.* (2008)	iv.	17 mg/kg as an initial dose followed by 17 mg/kg every 6 h for 4 days and the 8 mg/kg every 8 h for 6 days	25 (9: ribavirin group; 16: control group)	It was reported that the efficacy of ribavirin was not statistically demonstrated	[74]
Elaldi *et al.* (2009)	p.o.	30 mg/kg as an initial dose, 15 mg/kg every 6 h for 4 days, followed by 7.5 mg/kg every 8 h for 6 days	218 (126: ribavirin group; 92: control group)	Ribavirin treatment did not improve the survival rate, suggesting no efficacy of ribavirin	[76]
Izadi *et al.* (2009)	p.o.	2 g as an initial dose, 4 g/day in 4 divided doses for 4 days followed by 2.0 g/day for 6 days	63 (all were treated with ribavirin)	Survival subjects were prescribed with ribavirin 24 h in average earlier than fatal cases. It was suggested that early prescription of ribavirn improved prognosis	[77]
Tasdelen Fisgin *et al.* (2009)	Not mentioned	Not mentioned	52 (21: subjects prescribed with ribavirin within 4 days from onset; 20: subjects prescribed with ribavirin after 5 days from onset; 11: control group)	Subjects who were prescribed with ribavirin within 4 days of onset had better prognosis, suggesting that early prescription of ribavirln showed efficacy	[78]
Koksal *et al.* (2010)	p.o.	30 mg/kg as an initial dose, 15 mg/kg every 6 h for 4 days, followed by 7.5 mg/kg every 8 h for 6 days	136 (164: ribavirin group; 72: control group)	This study was conducted prospectively and in a randomized fashion. The efficacy was not demonstrated. Serious anemia was observed in two patients	[70]

CFR: Case fatality rate; iv.: Intravenously; p.o.: Oral route.

CCHF is one of the viral hemorrhagic fevers with a high CFR and is tick-borne; therefore, its eradication is impossible. The number of patients with CCHF in Turkey has dramatically increased since 2002, in which the first patient with CCHF in Turkey was identified. These facts indicate the urgent need of therapeutics for CCHF.

Ribavirin has been used as a treatment for patients with CCHF. Unfortunately, the efficacy of ribavirin in the treatment of CCHF has not yet been established conclusively. Owing to the high CFR and relatively small number of patients with CCHF, it is quite difficult to conduct a sophisticated randomized trial to assess the efficacy of ribavirin. Therefore, ribavirin should be used as a treatment of CCHF, if available, and the treatment should be initiated in the early stage of the disease.

The intravenous passive transfer of immunoglobulin active against CCHFV is expected to be an effective treatment. Immunoglobulin products, which are prepared from sera collected from survivors and are qualified for use, are expected to become available in the near future. Further studies, however, are still necessary.

Owing to the recent increase in the number of patients with CCHF in Turkey, many clinical and virological insights into CCHF have been revealed. For instance, VAHPS has been found to contribute to the exacerbation of CCHF in some patients, and the administration of high-dose methylprednisolone has been undertaken. CCHFV seems to be a neglected infectious disease. It is expected that clarification of the pathophysiology of CCHF will facilitate the further development of specific therapeutics against CCHF. The effort for the development of vaccines for CCHF is still limited. One reason might be the lack of a suitable animal model for CCHFV infection. Development of an efficacious vaccine against CCHF is expected.

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Executive summary

Epidemiology

- Crimean–Congo hemorrhagic fever (CCHF) is an acute viral hemorrhagic fever caused by CCHF virus.
- There have been many outbreaks of CCHF in countries ranging from South Africa to the Western part of China, including Eastern European and Middle Eastern countries.
- The number of the patients with CCHF is on the rise. Furthermore, newly recognized foci of CCHF have emerged recently.
- Surveillance system for CCHF should be established in CCHF-endemic countries.

Necessity for proper diagnosis

- Improved surveillance with better diagnostics enables more efficient determination of CCHF patients.
- Accurate and rapid diagnosis is required for proper treatment. However, CCHF virus must be handled in a high-containment laboratory.
- Therefore, recombinant viral antigen-based antibody- and antigen-detection assay systems were developed. The advantage of these recombinant protein-based diagnostic systems is that such diagnostic methods can be employed in regional and reference institutes without a BSL-4 laboratory.

Limitations in drug-based treatment for CCHF under present conditions

- An effective treatment for CCHF is urgently required.
- However, no efficacy-proved specific therapeutic strategies have yet been developed.
- Ribivirin has been used for the treatment of CCHF, but efficacy has not yet been established conclusively.
- Nonetheless, ribavirin should be used for CCHF, and this treatment should be initiated in the early stage of the disease.

Future perspective

- Alternative therapies effective in the treatment of CCHF, including immunoglobulin products, are expected to become available in the future.
- It is expected that further clarification of the pathophysiology of CCHF will facilitate the development of specific therapeutics against CCHF.
- Although the development of a CCHF vaccine is difficult and far from clinical use, a CCHF vaccine should be developed in the future.
- We believe that development of a CCHF vaccine is the best way to reduce the mortality and morbidity due to CCHF in the endemic regions, and its development is anticipated.

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Short Communication

Molecular Epidemiology of Rabies Virus in Mongolia, 2005–2008

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SUMMARY: The objective of this study was to determine the genetic diversity of rabies virus (RABV) in Mongolia based on the nucleotide sequences of viral N gene. A total of 24 rabies-positive samples from seven different domestic and wild animal species collected in western and central Mongolia between 2005 and 2008 were examined for their N gene sequences. The results showed that the endemic Mongolian RABVs could be divided into two different groups closely related to the Steppe-type and Arctic-like viruses isolated in Russia.

Rabies is a viral disease, characterized by fatal encephalitis in virtually all mammals, including humans, caused by rabies virus (RABV), which belongs to the *Lyssavirus* genus of the *Rhabdoviridae* family. Rabies is endemic in almost all parts of the world, except for a few countries or territories such as New Zealand, Australia, Hawaii, the United Kingdom, and Japan. Annually, at least 50,000 human deaths are estimated to occur, mainly in Asia and Africa.

Rabies is still a public health problem in Mongolia, with 34 human deaths reported from 1972 to 2004. Fifteen of these cases were a result of exposure to rabid dogs, whereas seven were a result of exposure to rabid wolves. A further 2,000 people receive post-exposure treatment every year. Nearly 6,000 animal cases were reported from 1972 to 2006 (1).

Since Mongolia borders the Russian Federation (Russia) and the People's Republic of China (China), both of which are rabies-endemic countries, it seems important to understand the possible route of introduction of RABV from these countries in order to establish effective strategic plans to mitigate the burdens imposed by rabies. To this end, the molecular epidemiology of RABV would appear to be a prerequisite, therefore we conducted nucleotide sequence analyses of RABVs isolated in Mongolia to better understand the molecular epidemiology of RABV circulating in that country.

Total RNA was extracted from 24 brain samples collected from animals (Fig. 1) which tested positive for RABV by FITC-labeled anti-rabies monoclonal antibody (mAb) (2) (Fujirebio Diagnostics, Malvern, Pa., USA) captured in Zavkhan, Khuvsgul, Govi-Altai, Bayan-Ulgii, and Tuv provinces between 2005 and 2008 (Fig. 1) using the QIAmp Viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. These total RNA samples were then converted into cDNA using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, Wis., USA) in the presence of the P1 primer (3). The resultant cDNA was amplified by conventional PCR using the TaKaRa ExTaq PCR kit (Takara, Shiga, Japan) and P1 and 304 (4) primers under the following conditions: 95°C for 5 min; then 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s; followed by further extension at 72°C for 10 min. The PCR product was separated by agarose gel electrophoresis and detected under a UV transilluminator after ethidium bromide staining.

All RT-PCR products were purified with on a QIAquick column (Qiagen) and used for sequencing of the entire N genes with the ABI Prism Big Dye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, Calif., USA) and a 3130 Genetic Analyzer (Ap-

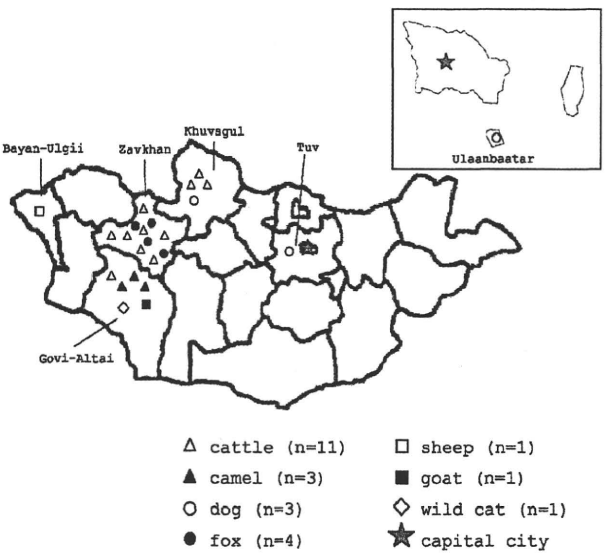

Fig. 1. Geographical distribution of the rabies viruses in Mongolia.

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Table 1. Primers used in this study

Primer	Nucleotide sequence	Position	Sense[6]	Use
P1[1]	ACAGACAGCGTCAATTGCAAAGC	28-50	G	RT-PCR and sequencing
JW12[2]	ATGTAACACC(C/T)CTACAATG	55-73	G	sequencing
B1c[3]	CTTTTGTTAAAATCGTGGAGCACC	530-553	G	sequencing
113[4]	GTAGGATGCTATATGGG	1012-1029	G	sequencing
B3[3]	TAGCTGGTCCAGTCTTCC	281-264	M	sequencing
304[5]	TTGACGAAGATCTTGCTCAT	1533-1514	M	RT-PCR and sequencing

[1]: Goto et al., 1994 (3).
[2]: Heaton et al., 1997 (18).
[3]: designed by nucleotide sequence of EF614254 (Table 2).
[4]: Smith, 1995 (4).
[5]: Smith, 1995 (4).
[6]: G, genomic; M, messenger.

plied Biosystems) using the sequencing primers shown in Table 1. ClustalX version 2 was used to generate the alignments, and the phylogenetic trees were constructed by the neighbor-joining method (5). These trees were drawn using the TreeView software based on a genetic distance matrix constructed by ClustalX version 2 (6). Forty-three N gene sequences of lyssaviruses were obtained from GenBank (Table 2) and the N gene of Australian bat lyssavirus (ABL) (GenBank accession no. NC003243) was used as an outgroup.

The nucleotide sequences of the full-length N genes (1,353 nucleotides) of 24 Mongolian RABVs were determined after PCR amplification and compared with those of 43 RABVs deposited in GenBank, including the RABV strains reported by Botvinkin et al. (10) recently. The results of the present study showed that the Mongolian RABVs could be divided into two different groups (Fig. 2). Thus, Group A consisted of viruses mainly isolated in the western part of Mongolia, which were aligned with the steppe-type viruses isolated in Rùssia and Kazakhstan, whereas only one virus (MGL 22), derived from central Mongolia, was found to belong to Group B. MGL 22 was closely related to the viruses isolated in Russia, Greenland, and South Korea, which were classified as Arctic-like viruses. The nucleotide sequence homology was greater than 98.3% within Mongolian isolates. When the deduced amino acid sequences of the N proteins of Mongolian RABV strains were aligned with those of other strains found in GenBank, it was noted that Ser 389 of the N gene, which is responsible for phosphorylation (7), was conserved not only among all Mongolian isolates but also among other RABV strains (Table 3). Antigenic sites I (amino acids 359-366) and IV (amino acids 375-383) (7) were also conserved among all Mongolian isolates. Examination of the amino acid sequence of the entire N protein revealed that there were 11 amino acid differences between MGL22 and the other Mongolian RABVs analyzed. The amino acid sequence homology was greater than 97.6% within the Mongolian isolates, all of which could be diagnosed using the direct immunofluorescent antibody (DFA) test.

Mongolia is located in north-central Asia and is bordered by Russia to the north and China to the south. It contains four different ecosystems, depending on the latitude, namely forest-steppe, steppe, semi-desert, and desert. The steppes predominate, covering more than three-quarters of Mongolian territory. The eastern and western Mongolian steppes extend to the Chita and Altai oblasts and Russia, respectively. The ecological landscape of the northeastern part of China is similar to Mongolian-type steppes. The traditional nomadic Mongolian lifestyle still continues in countryside regions, and people tend to come into close contact with both domestic and wild animals.

The first official report of rabies appeared in 1968 (8), and since then both human and animal rabies cases have occurred every year in Mongolia, although there are very few official reports related to rabies cases in Mongolia (9,10). Botvinkin et al. recently reported the isolation of Mongolian RABVs closely related to the Russian steppe-type viruses (10). Furthermore, several papers have described rabies cases in both southern (11,12) and northern parts of China (13).

The Mongolian RABV isolates described in a previous report were shown to be genetically close to RABVs found in East Siberia, Tuva, Russia, and Kazakhstan (14). We were able to confirm the above findings of Kuzmin et al., namely that RABVs belonging to Group A exist in western Mongolia. Since this is a mountainous region, it seems likely that the Altai Mountains form a natural barrier which prevents the spread of RABV from neighboring areas across the mountains. The major wildlife reservoirs of RABV in both Mongolia and Russia are considered to be Mongolian-type steppe animals such as red foxes and steppe foxes (14). However, the RABVs isolated from Mongolian-type steppe animals in Russia (Chita region) were phylogenetically close to Arctic-like viruses (10). Interestingly, the MGL 22 isolate (Group B), which was obtained from a dog in Tuv province (central Mongolia), showed genetic similarity to Arctic-like viruses (Fig. 2) such as 248c and 304c (GenBank accession nos. AY352460 and AY352459) obtained from steppe foxes in the Chita region of Russia (14). Geographically, the Mongolian steppes extend to the Chita region in the east of Russia. During the large-scale rabies outbreaks involving wolves and foxes that occurred from 1972 to 1981 in central and eastern Mongolia (1), rabies cases in red foxes and steppe foxes were also observed in eastern Siberia near the border between Mongolia and Russia (15). This would suggest that Arctic-like RABVs may have been introduced from Russia to Mongolia by the movement or migration of wildlife such as foxes and wolves.

There have been no reported rabies cases caused by bats in Mongolia, although Russian scientists detected

Table 2. Lyssavirus isolates used for genetic analysis

Virus name	Host	Country	Year	Accession no.
MGL 2	cattle	Mongolia (Govi-Altai)[3]	2005	AB570995
MGL 5	red fox	Mongolia (Zavkhan)	2006	AB570996
MGL 10	cattle	Mongolia (Khuvsgul)	2006	AB570997
MGL 11	cattle	Mongolia (Zavkhan)	2006	AB570998
MGL 12	camel	Mongolia (Govi-Altai)	2006	AB570999
MGL 13	cattle	Mongolia (Zavkhan)	2008	AB571000
MGL 17	cattle	Mongolia (Zavkhan)	2006	AB571001
MGL 20	cattle	Mongolia (Zavkhan)	2006	AB571002
MGL 21	red fox	Mongolia (Zavkhan)	2008	AB571003
MGL 22	dog	Mongolia (Tuv)	2007	AB571004
MGL 23	camel	Mongolia (Govi-Altai)	2008	AB571005
MGL 24	cattle	Mongolia (Khuvsgul)	2008	AB571006
MGL 25	dog	Mongolia (Khuvsgul)	2008	AB571007
MGL 26	cattle	Mongolia (Zavkhan)	2006	AB571008
MGL 27	red fox	Mongolia (Zavkhan)	2006	AB571009
MGL 28	cattle	Mongolia (Zavkhan)	2006	AB571010
MGL 29	goat	Mongolia (Govi-Altai)	2008	AB571011
MGL 30	cattle	Mongolia (Khuvsgul)	2007	AB571012
MGL 31	cattle	Mongolia (Zavkhan)	2005	AB571013
MGL 32	red fox	Mongolia (Zavkhan)	2005	AB571014
MGL 33	camel	Mongolia (Govi-Altai)	2005	AB571015
MGL 34	wild cat	Mongolia (Govi-Altai)	2005	AB571016
MGL 35	sheep	Mongolia (Bayan-Ulgii)	2005	AB571017
MGL 36	dog	Mongolia (Ulaanbaatar)	2005	AB571018
Mongolia 3[1]	cattle	Mongolia (Zavkhan)	2006	EF614257
Mongolia 4[1]	wolf	Mongolia (Zavkhan)	2006	EF614256
Mongolia 6[1]	red fox	Mongolia (Govi-Altai)	2006	EF614254
Mongolia 7[1]	camel	Mongolia (Govi-Altai)	2006	EF614255
857r	raccoon dog	Russia	2004	AY352458
304c	steppe fox	Russia	2004	AY352459
248c	steppe fox	Russia	2004	AY352460
3561d	dog	Russia	2004	AY352481
686cattle	cattle	Russia	2004	AY352482
765w	wolf	Russia	2004	AY352483
409f	red fox	Kazakhstan	2004	AY352489
RV259	red fox	Kazakhstan	2004	AY352491
8684GRO	arctic fox	Greenland	1981	U22654
8618POL	raccoon dog	Poland	1985	U22840
9342EST	raccoon dog	Estonia	1999	U43432
9215HON	human	Hungary	1999	U43025
9202ALL	red fox	Germany	2005	U42701
9223FRA	red fox	France	1999	U43433
86107YOU	red fox	Yugoslavia	1999	U42703
8653YOU	wolf	Yugoslavia	1999	U42704
Guangxi_Yl66	dog	China	2006	DQ666287
Guizhou_A148	dog	China	2006	DQ666291
Henan_Sq10	dog	China	2006	DQ666300
Hunan_Xx33	dog	China	2006	DQ666317
SKRRD0406CC	raccoon dog	South Korea	2004	DQ076126
Yokohama	human	Japan[4]	2006	AB573763
Kyoto	human	Japan[4]	2006	AB573762
NNV-RAB-H	human	India	2007	EF437215
SRL1145	buffalo	Sri-Lanka	1996	AB041969
8681IRA	dog	Iran	1995	U22482
9001FRA	dog	Guyana	1990	EU293113
Eth2003	wolf	Ethiopia	2003	AY500827
8721AFS	human	South Africa	1995	U22633
8480FX	red fox	Canada	1993	U03768
3789	skunk	USA	1998	AF461045
Chile	bat	Chile	1999	AF070450
pehm3230	human	Peru	1999	AF045166
Argentina	bat	Argentina	1997	EU293116
PV		Lab strain		M13215
CVS-11		Lab strain		AB069973
ERA		Lab strain		EF206707
ABL[2]	bat	Australia	2002	NC003243

[1]: described previously by Botvinkin et al., 2008 (10).
[2]: Australian bat lyssavirus.
[3]: Province of Mongolia.
[4]: Japanese bitten by the dog in the Philippines.

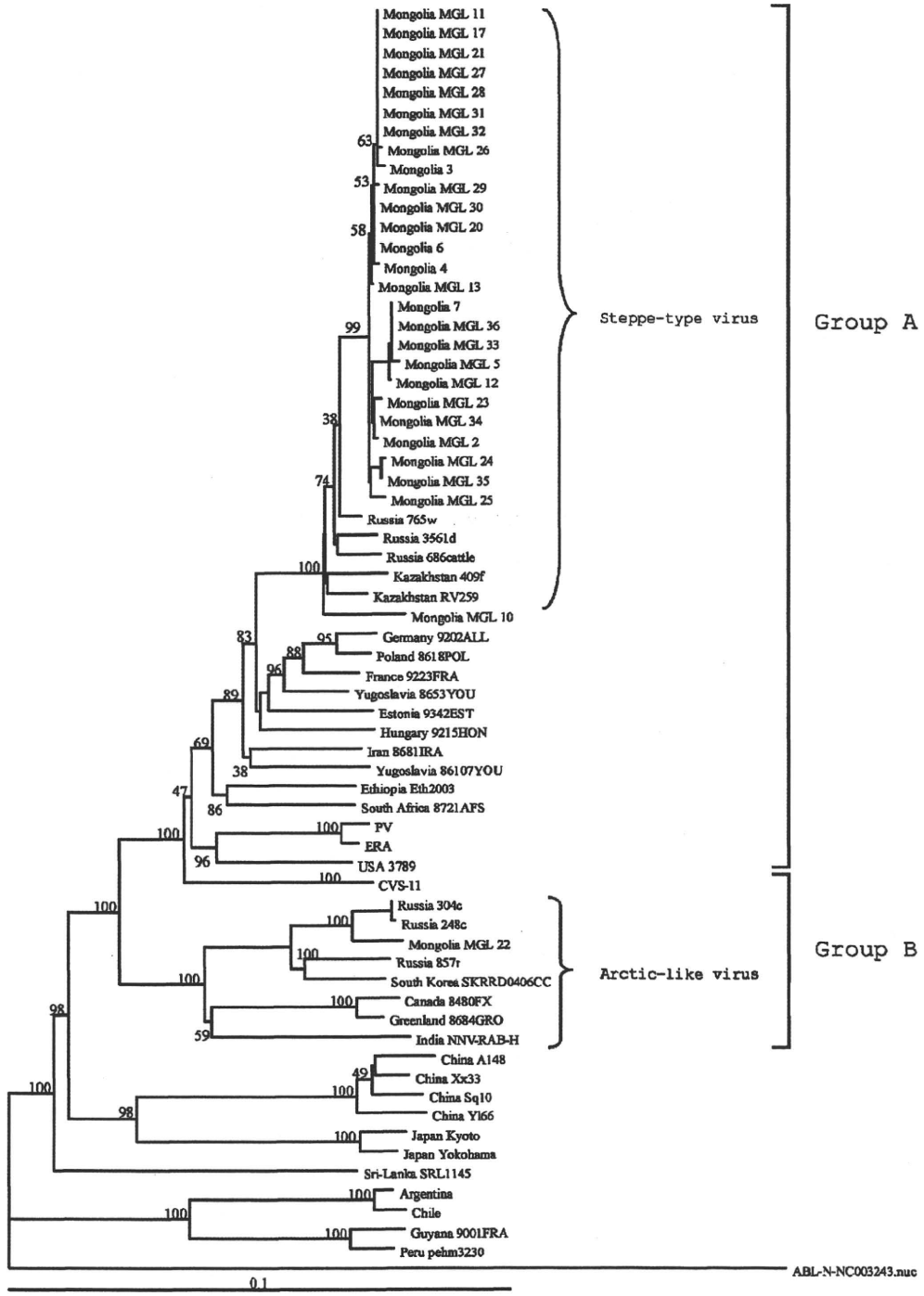

Fig. 2. Phylogenetic tree with complete sequences of N gene of rabies virus from Mongolia and other countries. ClustalX was used for sequence alignment and TreeView was used for drawing phylogenetic tree.

a rabies-related virus (Irkut virus) in bats (*Murina leucogaster*) thriving in Irkutsk adjacent to the Mongolian border (16,17). This bat species has also been found in the southern part of Siberia as well as the western part of Mongolia and Manchuria, which is located in the northern part of China. It therefore seems likely that bats in Mongolia are a possible reservoir of RABVs or related viruses, although further studies are required to confirm this hypothesis.

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Conflict of interest None to declare.

Table 3. Amino acid alignment of N protein

Virus name	358										367							375									383					389	
MGL 11	R	R	F	F	R	D	E	K	E	L	Q	E	H	E	A	A	E	L	T	K	T	D	V	A	L	A	D	D	G	T	V	H	S	
MGL 17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 31	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 32	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 26	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Mongolia 3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 29	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 30	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 20	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Mongolia 6	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Mongolia 4	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 13	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Mongolia 7	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 36	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 33	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 5	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 12	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 23	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 34	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 2	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 24	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 35	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 25	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
765w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
3561d	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
686cattle	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
409f	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
RV259	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
MGL 10	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
9202ALL	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
8618POL	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
9223FRA	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
8653YOU	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	M	—	—	—	—	—	—	—	—	N	—	
9342EST	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
9215HON	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
8681IRA	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
86107YOU	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
Eth2003	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
8721AFS	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
PV	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
ERA	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
USA 3789	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
CVS-11	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
304c	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	*
248c	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	*
MGL 22	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	*
857r	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	*
SKRRD0406CC	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	*
8480FX	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	N	—	*
8684GRO	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	N	—	*
NNV-RAB-H	—	—	—	—	—	—	—	—	—	—	—	—	Y	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	*
A148	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	L	—	—	—	—	—	—	—	—	N	—	
Xx33	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	L	—	—	—	—	—	—	—	—	N	—	
Sq10	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	L	—	—	—	—	—	—	—	—	N	—	
Yl66	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	L	—	—	—	G	—	—	—	—	N	—	
Kyoto	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
Yokohama	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	
SRL1145	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	—	I	—	—	—	—	—	—	—	—	N	—	
Argentina	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	E	M	—	—	—	—	—	—	—	—	N	—	
Chile	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	—	E	T	—	—	—	—	—	—	—	—	N	—	
9001FRA	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	A	E	T	—	—	—	—	—	—	—	—	N	—	
pehm3230	—	—	—	—	—	—	—	—	—	—	—	—	Y	—	—	—	—	—	—	—	A	E	T	—	—	—	—	—	—	—	—	N	—	
ABL	—	—	—	—	—	—	—	—	—	—	—	D	Y	—	—	—	—	A	M	—	I	—	L	—	—	—	—	—	—	—	—	N	—	

The dashes indicate amino acid identity; the open box shows the antigenic site I.
The gray boxes show the antigenic site IV.
Asterisks show Arctic-like viruses.
389 in boldface type is phosphorylation site.
The virus name was aligned according to Fig 2.

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Rapid diversification by recombination in *Bartonella grahamii* from wild rodents in Asia contrasts with low levels of genomic divergence in Northern Europe and America

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Abstract

Bartonella is a genus of vector-borne bacteria that infect the red blood cells of mammals, and includes several human-specific and zoonotic pathogens. _Bartonella grahamii_ has a wide host range and is one of the most prevalent _Bartonella_ species in wild rodents. We studied the population structure, genome content and genome plasticity of a collection of 26 _B. grahamii_ isolates from 11 species of wild rodents in seven countries. We found strong geographic patterns, high recombination frequencies and large variations in genome size in _B. grahamii_ compared with previously analysed cat- and human-associated _Bartonella_ species. The extent of sequence divergence in _B. grahamii_ populations was markedly lower in Europe and North America than in Asia, and several recombination events were predicted between the Asian strains. We discuss environmental and demographic factors that may underlie the observed differences.

Keywords: *Bartonella grahamii*, population structure, phylogeography, recombination

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Introduction

Rodents represent a major reservoir for bacterial infectious diseases; however, we know remarkably little about the ecology and evolution of rodent-adapted bacterial populations. *Bartonella* is a genus of vector-borne bacteria that is particularly abundant in rodent populations all over the world, with an overall prevalence ranging from 6% in the Greater Jakarta area (Winoto *et al.* 2005) to 70% in the Russian far east (Mediannikov *et al.* 2005). Of the almost 30 identified species and subspecies of *Bartonella*, more than half have been isolated from rodents and several of these have been associated with human disease (Boulouis *et al.* 2005).

Bartonella grahamii is one of the most prevalent *Bartonella* species in wild rodents, and has been associated with two cases of human neuroretinitis (Kerkhoff *et al.* 1999; Serratrice *et al.* 2003). It is transmitted by the rodent flea *Ctenophthalmus nobilis* (Bown *et al.* 2004), and has been isolated from the rodent genera *Myodes*, *Apodemus*, *Microtus*, *Mus*, *Dryomus*, *Arvicola* and *Rattus* in many countries (Hsieh *et al.* 2009; Inoue *et al.* 2009), indicating a wide host range and a global distribution pattern. In rodents and other natural hosts, *Bartonella* cause asymptomatic infections of red blood cells. The genus also contains two pathogens adapted to humans: *Bartonella bacilliformis*, which causes Carrion's disease, and *Bartonella quintana*, the agent of trench fever. There

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are additionally several zoonotic pathogens, for example, the feline-adapted *Bartonella henselae*, which causes cat-scratch disease in incidentally infected humans.

The genome of *B. grahamii* strain as4aup, isolated from a wood mouse (*Apodemus sylvaticus*) in central Sweden (Holmberg *et al.* 2003), was recently sequenced, and found to consist of a 2.3 Mb chromosome and a 28 kb plasmid (Berglund *et al.* 2009). Sixteen genomic islands (BgGI 1–16), mainly containing horizontally transferred genes for surface proteins, secretion systems and phage genes, were identified by comparison to the previously published *Bartonella* genomes (Alsmark *et al.* 2004; Saenz *et al.* 2007; Berglund *et al.* 2009). Among the functionally most important secretion systems are the VirB and Trw type IV secretion systems (T4SSs) and the type V secretion system (T5SS) trimeric autotransporter BadA, which have been shown in other *Bartonella* species to be required for binding to and infect endothelial cells and erythrocytes (Schulein & Dehio 2002; Seubert *et al.* 2003b; Riess *et al.* 2004; Schmid *et al.* 2004; Zhang *et al.* 2004; Schulein *et al.* 2005; Schmid *et al.* 2006; Kaiser *et al.* 2008). Many of the genomic islands are clustered in a genomic region that is amplified by run-off replication and packaged into phage particles (Berglund *et al.* 2009). As this phage packages bacterial DNA randomly it was described as a gene transfer agent (GTA) (Berglund *et al.* 2009).

Previous multilocus sequence typing (MLST) based on six house-keeping genes resulted in 10–16 alleles per locus in 31 *B. grahamii* strains (Inoue *et al.* 2009). Phylogenetic analysis revealed a clustering according to geographic origin, with the Asian isolates clearly distinguished from the American and European (Inoue *et al.* 2009). The Japanese strains were split into two subgroups, one of which was suggested to originate from China and the other from Far Eastern Russia (Inoue *et al.* 2009). In comparison, MLST of more than 200 *B. henselae* strains identified only 14 STs (Sequence Types), with up to four alleles per locus, and no obvious correlation with geographic origin or host species (Iredell *et al.* 2003; Lindroos *et al.* 2006; Arvand *et al.* 2007), although variable number tandem repeat work has suggested that human-infecting strains are overrepresented among strains of genotype II (Bouchouicha *et al.* 2009). Sequencing of highly variable spacer regions (MST) yielded 39 variants in 126 cat isolates (Li *et al.* 2006) and 16 variants in 75 human isolates (Li *et al.* 2007). Microarray comparative genome hybridizations of a global collection of *B. henselae* strains revealed no differences in gene content between human and feline isolates, nor was a geographical pattern evident (Lindroos *et al.* 2006). In *B. quintana*, MST based on as many as 34 spacer regions identified only seven sequence variants in 74 strains (Foucault *et al.* 2005; Woolley *et al.* 2007). This low diversity was attributed to a bottleneck associated with a recent host switch (Foucault *et al.* 2005).

To learn more about the emergence of infectious diseases, it is important to examine the genomic diversity of bacterial species adapted to wild animals, and the evolutionary, ecological and demographic factors that shape this diversity. The aim of this study was to determine the diversity of the global population of *B. grahamii* infecting wild rodents on a whole-genome scale. We found a much higher genomic diversity in Asia than in Europe and North America, and a larger genome plasticity in *B. grahamii* compared with previous results from *B. henselae* and *B. quintana*. We discuss potential environmental and demographic factors that may underlie the observed differences.

Materials and methods

Bacterial strains and DNA isolation

The *B. grahamii* strains used in this study are listed in Table 1. Bacteria were grown for 4–5 days on chocolate agar plates and DNA was extracted as described previously (Lindroos *et al.* 2005).

PCR amplification and phylogenetic inference

Primers used for PCR amplification are listed in Table 2. In addition, a 379-bp fragment of the *gltA* gene was amplified with primers 5′-GGGGACCAGCTCATGGTGG-3′ and 5′-AATGCAAAAAGAACAGTAAACA-3′. PCRs were performed as described previously (Lindroos *et al.* 2006). Sequences were assembled and edited with Phred, Phrap and Consed (Ewing & Green 1998; Ewing *et al.* 1998; Gordon *et al.* 1998) and trimmed with Lucy (Chou & Holmes 2001). The homologous regions were also sequenced from *Bartonella vinsonii berkhoffii* strain Winnie (Kordick & Breitschwerdt 1998), and extracted from the previously sequenced genomes of *B. grahamii* as4aup and *Bartonella tribocorum* IBS 325 (GenBank entries NC_012846 and NC_010161, respectively).

For loci including a spacer region, the gene borders were identified manually and the sequence was cut at these positions. The different parts were aligned with Prank (Loytynoja & Goldman 2008), concatenated and overhanging ends were cut for each locus. Genic parts were aligned with the translate option. Phylogenetic trees were inferred for each locus, the concatenation of all loci, the concatenation of all gene sequences and the concatenation of all loci after removing all sites with gaps, with RAxML (Stamatakis 2006), substitution model GTRMIX, 1000 bootstrap replicates. A phylogenetic tree was also inferred using ClonalFrame (Didelot & Falush 2007), with the number of burn-in iterations

Table 1 Country of origin, host and date of isolation of the *Bartonella grahamii* strains analysed in this study

Strain	Origin*	Host animal†	Isolation	Reference
as4aup	Sweden	*Ap. sylvaticus*	Sep-1999	Holmberg *et al.* (2003)
AL1714yn	China	*Ap. latronum*	Sep-2000	Ying *et al.* (2002)
Ac1733yn	China	*Ap. chevrieri*	Sep-2000	Ying *et al.* (2002)
Cg4224alb	Canada	*My. gapperi*	Jul-2002	Inoue *et al.* (2009)
Cg4227alb	Canada	*My. gapperi*	Jul-2002	Inoue *et al.* (2009)
Cg4228alb	Canada	*My. gapperi*	Jul-2002	Inoue *et al.* (2009)
Cg4263alb	Canada	*My. gapperi*	Aug-2002	Inoue *et al.* (2009)
Cg4285alb	Canada	*My. gapperi*	Aug-2002	Inoue *et al.* (2009)
Mo12494sd	USA	*Mi. ochrogaster*	Aug-2005	Inoue *et al.* (2009)
Mo12658sd	USA	*Mi. ochrogaster*	Aug-2005	Inoue *et al.* (2009)
PTZA 30/3	Russia	*Ap. flavicollis*	Aug-2006	Markov *et al.* (2006)
PTZB 29/18	Russia	*Ap. uralensis*	Aug-2006	Markov *et al.* (2006)
Hokkaido 29-1	Japan	*Ap. speciosus*	Aug-2005	Inoue *et al.* (2008)
Nagano 14-1	Japan	*Ap. speciosus*	Sep-2003	Inoue *et al.* (2008)
Fuji 4-1	Japan	*Ap. speciosus*	Aug-2003	Inoue *et al.* (2008)
Ehime 5-1	Japan	*Ap. speciosus*	May-2006	Inoue *et al.* (2008)
Nakanoshima 39-1	Japan	*Ap. speciosus*	Oct-2004	Inoue *et al.* (2008)
MAC29	UK	*Ar. terrestris*	Jun-2002	This study
WM11	UK	*Ap. sylvaticus*	Sep-1999	This study
V2	UK	*My. glareolus*	Sep-1999	Birtles *et al.* (1995)
C162	UK	*Mi. agrestis*	Sep-2002	Telfer *et al.* (2007)
J142	UK	*Mi. agrestis*	Sep-2003	Telfer *et al.* (2007)
C066	UK	*Mi. agrestis*	Sep-2002	Telfer *et al.* (2007)
R170	UK	*Mi. agrestis*	Sep-2004	Telfer *et al.* (2007)
J019	UK	*Mi. agrestis*	Sep-2003	Telfer *et al.* (2007)
S116	UK	*Mi. agrestis*	Sep-2004	Telfer *et al.* (2007)

*The collection sites within each country were: as4aup, Hâtunaholm in central Sweden; Chinese strains, Yunnan; Canadian strains, Alberta; U.S. strains, South Dakota; Russian strains, Moscow region; Hokkaido 29-1, Hokkaido; Nagano 14-1, Nagano; Fuji 4-1, Shizuoka; Ehime 5-1, Ehime; Nakanoshima 39-1, Kagoshima; MAC29, Reisa Mhic Phaiden; WM11, Brimstage; V2, Shropshire; remaining UK strains, Kielder.
†Host species are abbreviated as follows: *Ap—Apodemus, Ar—Arvicola, Mi—Microtus, My—Myodes* (formerly known as *Clethrionomys*).

set to 50 000, and the number of MCMC iterations after the burn-in period set to 200 000. Two runs were performed and the convergence was tested with the Gelman-Rubin statistic. All parameters were found to be equal to or below 1.2, except delta, which was 2.5.

Phylogenetic trees for *gltA* and for the concatenation of *rrs*, *gltA* and *groEL* sequences from (Inoue *et al.* 2009) were inferred both with RAxML as described above, and with the NJ method with Jukes-Cantor parameters.

Pairwise sequence identities were calculated based on the complete concatenated alignment, excluding sites with gaps in each comparison. Sequence identities between and within groups were defined as the average of all relevant pairwise comparisons.

Detection of recombination

The SuperNetwork method in SplitsTree4 (Huson 1998; Huson & Bryant 2006) with default settings was used to detect conflicts between phylogenetic trees for individual loci. Each locus was tested for recombination using the methods implemented in RDP3 (Martin *et al.* 2005) with default settings. As all predicted recombination events took place between the Asian strains, the analysis was repeated with only these strains, in order to get a better alignment.

The MLST data set from (Inoue *et al.* 2009) was used to estimate the relative contribution of recombination and mutation in *B. grahamii* using the software ClonalFrame (Didelot & Falush 2007). Based on recommendations in (Vos & Didelot 2009), *rrs* sequences were excluded and only one strain of each sequence type was included. The mutational rate θ was set to Watterson's θ and 200 000 iterations were performed, of which 100 000 were discarded. Two runs were performed, and the convergence was tested with the Gelman-Rubin statistic and all parameters were found to be below 1.1.

Microarray comparative genome hybridizations

Microarrays were designed and manufactured as reported previously (Berglund *et al.* 2009) and cross-linked at

Table 2 Characteristics of the sequenced regions and primers used for PCR

No	Locus*	Position† and sequence (5′–3′) of primers	Size (bp)‡	No. of alleles
1	Bgr_05150 (*cycK*)	622901 GCGCTGCTTACTTTTTCCC	580	14
		623480 TCTTTCCCCATAGATCCGC		
2	Bgr_00610 (*batR*)	82636 CAATGGTGCGATCATCTACG	535	11
		83190 CGTCTTTATCTTTTGCGCTTG		
3	Bgr_00430	61980 ATGCACAGCTTTCTGGTCG	590	13
		62569 TCCTGCAATAAAACCATTTGC		
4	Bgr_03900 (*aldA*)	473807 TGTTTTCCATTTTTGAAACGC	725 (469)	15
	Bgr_03910 (*ftsK1*)	474531 CTTCTCTTGATGCACCTTTCG		
5	Bgr_05600 (*cspA*)	679952 TGAATCCGAAACCTTTTGTTG	617 (245)	9
	Bgr_05610 (*carB*)	680568 TTGGCTTTTCTGTTGTCGC		
6	Bgr_06060 (*pssA*)	734202 TAGGCGCTCTTGGTTTGG	727 (70)	13
	Bgr_06070	734928 TGGACGAGCCATTCTGTTATC		
7	Bgr_06380 (*uvrC*)	776004 CAATCATCCGGTAAACCCC	768 (397)	16
	Bgr_06390	776771 TGAAATGCGTATCCGAAAAAG		
8	Bgr_11560 (*maeB2*)	1305872 TTTTCGTGATCGTGTTTTTCC	723 (189)	10
	Bgr_11570 (*acpP2*)	1306594 GCCTGTTTTAAGGCAACGAG		
9	Bgr_18510 (*pgk*)	2129539 ACCCCATCACTGCTTCCTC	605 (514)	13
	Bgr_18520 (*gap*)	2130143 CGCGTTTTGGTTTGGTATG		
10	Bgr_19460 (*dnaJ2*)	2239347 GCAAAGATTCGCTCTGGAAC	708 (201)	11
	Bgr_19470 (*cobS*)	2240054 ATAGCCAGAAACCATCACACG		
11	Bgr_19730	2273643 CAAGGATTTCGTGCCCC	811 (144)	16
	Bgr_19740	2274453 TTATGTTTCGCGGTTGTTCTC		

*The locus_tag and gene name, if any, for the sequenced regions in *B. grahamii* as4aup. For intergenic regions, both the upstream and the downstream gene are shown.
†Genomic position of primers refer to the *B. grahamii* as4aup genome.
‡Size of the total PCR product refer to the *B. grahamii* as4aup genome. Within parentheses is the spacer size, if any.

250 mJ/cm^2. Genomic DNA from *B. grahamii* as4aup was used as reference, and two or three hybridizations were performed for each strain. Prehybridization, DNA labelling, hybridization, scanning and image analysis were performed as described previously (Berglund *et al.* 2009). The channel used for the reference strain is referred to as Ch1 and the channel used for the test strain is referred to as Ch2, and spots were filtered as described previously (Berglund *et al.* 2010). *M*-values were computed as $\log_2$(Ch2/Ch1) and normalization was performed as described previously (Lindroos *et al.* 2006). Median *M*-values of all replicate spots and arrays were computed for each strain. Probes were ordered according to the position in *B. grahamii* as4aup, classified as absent ($M \leq -2$), uncertain ($-2 > M \leq -1$) or present ($-1 > M$) and sorted into regions of contiguous probes with the same status as described previously (Lindroos *et al.* 2006). A maximum parsimony tree was inferred from this data with the PHYLIP package (Felsenstein 1989), with 100 bootstrap replicates.

Pulsed-field gel electrophoresis

Bacteria grown on chocolate agar plates for 4–5 days were harvested, suspended and washed twice in TNE buffer (10 mM Tris pH 8.0, 150 mM NaCl and 1 mM EDTA). The bacterial suspension was added to an equal volume of 2% SeaPlaque GTG agarose (Cambrex BioScience, East Rutherford, NJ) in 0.5 × Tris–borate–EDTA (TBE) buffer, and plugs were made using reusable moulds. After solidification, the agarose plugs were transferred into lysozyme solution (10 mM Tris [pH 8], 1 mM EDTA, 100 mM NaCl, 0.5% *N*-lauroyl sarcosine, RNAse A [0.5 mg/mL], Ready-Lyse lysozyme [50 kU/mL, Epicentre Technologies, Madison, WI, USA]) and incubated at 37 °C overnight. The plugs were rinsed with sterile water and incubated in proteinase K solution (10 mM Tris [pH 8], 100 mM EDTA, 1% SDS, 1% *N*-lauroyl sarcosine, 0.25% Triton X-100, proteinase K [1 mg/mL]) at 50 °C overnight. Subsequent to three washes in TE buffer (10 mM Tris [pH 8], 0.5 mM EDTA) at 4°C for 30 min, any remaining proteinase K was inactivated by incubation in TE buffer supplemented with 0.4 mg/mL phenylmethylsulfonyl fluoride at 50°C for 1 h. The ready plugs were either stored in 0.2 M EDTA at 4°C or equilibrated in TE buffer. Enzymatic digestions with 10 U of *Not*I or *Asc*I (New England Biolabs, Ipswich, MA) were performed as previously described (Lindroos *et al.* 2005). The DNA fragments were separated by electrophoresis