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Review

Current knowledge on lower virulence of Reston Ebola virus (in French: Connaissances actuelles sur la moindre virulence du virus Ebola Reston)

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Abstract

Ebola viruses (EBOV) and Marburg virus belong to the family *Filoviridae*, order *Mononegavirales*. The genus *Ebolavirus* consists of four species: Zaire ebolavirus (ZEBOV), Sudan ebolavirus (SEBOV), Ivory Coast ebolavirus (ICEBOV) and Reston ebolavirus (REBOV). Three species of ebolaviruses, ZEBOV, SEBOV, ICEBOV, and Marburg virus are known to be extremely pathogenic in primates and humans and cause severe hemorrhagic fever leading up to case fatality rate of some 90%, while REBOV is thought to be pathogenic in Asian monkeys but not in African monkeys and humans. Recent studies indicated several factors involved in different virulence between African EBOV and REBOV. This article reviews the history, epidemiology, and virulence of REBOV.

Keywords: Reston ebolavirus; Virulence; Epidemiology

Résumé

Les virus Ebola (EBOV) et le virus Marburg appartiennent à la famille des *Filoviridae*, ordre des *Mononegavirales*. Le *virus Ebola* comporte quatre espèces: le virus Ebola-Zaïre (ZEBOV), le virus Ebola-Soudan (SEBOV), le virus Ebola-Côte d'Ivoire (ICEBOV) et le virus Ebola

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Reston (REBOV). Trois espèces de virus Ebola, ZEBOV, SEBOV, ICEBOV et le virus de Marbourg, sont extrêmement pathogènes chez les primates et les hommes, et se caractérisent par une fièvre hémorragique foudroyante entraînant la mort dans 90% des cas. Le virus REBOV, quant à lui, est pathogène chez les singes asiatiques, mais pas chez les singes africains et l'homme. De récentes études ont montré que la différence de virulence entre le virus africain EBOV et REBOV était liée à plusieurs facteurs. Le présent article analyse l'histoire, l'épidémiologie et la virulence du virus REBOV.

Mots clés: Le virus Ebola Reston; La virulence; Épidémiologie

Contents

1. Introduction

Epizootic caused by Reston Ebola virus (REBOV) was first recognized in 1989 at a quarantine facility in Reston, Virginia where a shipment of cynomolgus monkeys (*Macaca fascicularis*) from the Philippines was quarantined. Introduction of REBOV through infected cynomolgus monkeys from the Philippines was also identified in Philadelphia, Pennsylvania in 1989, Reston and Alice, Texas in early 1990, Siena, Italy in 1992, and Alice in 1996. Epidemiological study in the Philippines showed that REBOV-infected monkeys were originated from one monkey facility but not from other facilities in the Philippines. After the facility was closed in 1997, any epizootic or sporadic case of Ebola hemorrhagic fever-like disease caused by REBOV has not been documented. It is still unclear how the monkeys in the facility were infected with REBOV. Epidemiological study indicated that REBOV was avirulent in humans and experimental infection of nonhuman primates showed that REBOV was less virulent compared to ZEBOV and SEBOV.

Recently, many studies revealed the pathogenesis of ZEBOV especially the impairment of innate and adapted immune response, vascular disfunction and mechanism of virus infection to a variety of cell types. The recent review excellently summarized the current view on pathogenesis [1]. A majority of these studies did not answer the question why REBOV was less virulent compared to African EBOVs, however, several studies implicated the reason why REBOV was less virulent compared to African EBOV. These studies indicated that lower virulence of REBOV was, at least, due to several viral factors.

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2. Epizootics and epidemiology of Reston Ebola

In late 1989, an Ebola virus antigenically related to ZEBOV was identified in cynomolgus monkeys (*M. fascicularis*) at a quarantine facility in Reston, Virginia [2–5]. These wild-caught cynomolgus monkeys were imported from the Philippines, and a higher mortality rate was noted in the monkeys during quarantine. The clinical features were characterized by abrupt onset of complete anorexia. Splenomegaly, puffy eyelids, lacrimation, nasal exudates, and coughing were observed in some monkeys. Epistaxis, subcutaneous hemorrhage, dehydration, bloody diarrhea and fever were observed infrequently [6]. Marked increase in lactate dehydrogenase, asparatate aminotransferase and alanine aminotransferase, increase in alkaline phosphatase, blood urea nitrogen, creatine, and triglycerides, and decreased platelet counts were observed [6]. These clinical features were similar to simian hemorrhagic fever, which is caused by infection of simian hemorrhagic fever virus, a member of family *Arteriviridae.* Actually, simian hemorrhagic fever virus was isolated from some monkeys but many of them were also shown to be infected with an Ebola virus.

The virus, named Reston Ebola virus (REBOV), was shown to be closely related but clearly distinct from previously known ZEBOV and SEBOV. Transmission of REBOV among monkeys were mainly caused by direct contact with blood or secretion, or by injection with virus-contaminated syringes, however, droplet or aerosol transmission was also suspected since the disease among monkeys spread within rooms despite discontinuation of all direct contact with animals [6]. Similar outbreak was also reported in Philadelphia, Pennsylvania in 1989, and REBOV was also isolated from monkey specimens. Antigen capture ELISA showed that 52.8% of 161 monkeys that died over a 2.5-month period at export facilities in the Philippines [7]. A case fatality rate of 82.4% was documented for the monkeys infected with REBOV and simian hemorrhagic fever virus at an export facility. Any illness associated with the epizootic in monkeys was not observed in any human, however, four animal handlers at the quarantine facility in Reston had serologic evidence of recent infection with the virus [8,9]. Seroepidemiological survey in the Philippines showed that three out of five workers in the animal hospital at the export facility at Laguna, with which the epizootics in the USA were associated, were positive for REBOV antibodies. In the facility, there was no illness associated with REBOV [10]. These indicated the REBOV can be transmitted to humans when heavily exposed to the infected monkey or its specimens but may not induce illness in humans. In 1990, REBOV was again introduced to primate facilities in Reston, Virginia and Alice, Texas by infected monkeys imported from the same export facility in the Philippines.

In 1992, REBOV was introduced in Siena, Italy and the virus was isolated from monkeys imported from the same export facility the Philippines [11]. It has been reported that all monkeys in the export facility in the Philippines has been destroyed following the epizootics in 1989–1990 [7]. It has been suggested that depopulation of the monkeys was not completely carried out [12].

In 1996, 4 cynomolgus monkeys among 100 monkeys imported from the Philippines were shown to be infected with REBOV in a quarantine facility in Alice, TX, USA [13]. Epidemiological study in the Philippines showed that 113 out of 353 dead or

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moribund monkeys collected during May–September in 1996 were REBOV antigen positive, and 18 out of 465 healthy monkey were shown to be antigen positive [12,14]. All the monkeys from the other four facilities were negative for virus antigen. After the complete depopulation of the facility, in which the REBOV-infected monkeys found, was executed by the Philippine government and eventually the facility was closed in 1997. Since then, any epizootic or sporadic case of Ebola hemorrhagic fever-like disease caused by REBOV has not been documented. It is still unclear how the monkeys in the facility were infected. However, a recent study in Gabon and the Republic of the Congo during human and ape Ebola outbreaks between 2001 and 2005 [15,16] has demonstrated ZEBOV RNAs in liver and spleen specimens of three different species of fruit bats, *Hypsignathus monstrosus*, *Epomops franqueti* and *Myonycteris torquata* [17,18]. These bats were asymptomatically infected and their distribution in Africa included regions where human Ebola outbreaks were documented. These findings support earlier studies that indicated bats as potential reservoirs of filoviruses [19,20]. An in-depth survey especially focused on bat species in south Asia is necessary to elucidate reservoir wildlife of REBOV as to whether the virus originated in south Asia or introduced from Africa.

3. Molecular structure of REBOV

Filoviruses contain a single stranded, negative sense RNA genome with approximately 19 kb. The genome of REBOV is composed of seven genes as like other filoviruses; major nucleoprotein (NP), P protein (VP35), matrix protein (VP40), glycoprotein (GP), minor nucleoprotein (VP30), minor matrix protein (VP24) and RNA-dependent RNA polymerase (L) [21,22]. Phylogenetically REBOV is clearly distinct from other EBOVs, even though REBOV is relatively related to SEBOV. Nucleotide and amino acid sequences among REBOV isolates were highly conserved. However, significant differences in GP sequence were observed among REBOVs isolated in different year, while no amino acid substitutions were observed within the different isolates in the same year. This strongly indicated independent introduction of REBOV into the monkey facility from yet-identified reservoir.

4. Virulence of REBOV *in vivo*

As described above, epidemiological study indicated that REBOV is pathogenic in cynomolgus monkeys but not pathogenic in humans. Similar differences in pathogenicity between cynomolgus monkeys and humans were also observed in experimentally infected nonhuman primates. Intraperitoneal infection of 1000 $TCID_{50}$ of ZEBOV, SEBOV, or REBOV in cynomolgus monkeys and African green monkeys (*Cerocopithecus aethiops*) demonstrated that ZEBOV caused lethal infection in both monkeys while SEBOV and REBOV caused lethal infection only in cynomolgus monkeys. Clinical disease in monkeys caused by REBOV infection was similar to that caused by ZEBOV and SEBOV, but the former was characterized by slower onset of disease and viremia [23]. Higher doses of subcutaneous infection

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of REBOV in cynomolgus monkeys resulted in higher mortality. REBOV-infected monkeys died within 8–14 days after infection, while ZEBOV and SEBOV-infected monkeys died more rapidly, within 8 days after infection [24]. These results indicated that REBOV was less pathogenic compared to African EBOVs.

5. Pathogenesis of EBOV and factors contributed to lower virulence of REBOV

Many studies on pathogenesis of ZEBOV illustrated EBOV pathogenesis which were precisely reviewed by Hoene et al. [1]. Briefly, monocytes and tissue macrophages, one of the primary target cells for EBOV infection, was activated and produced a variety of proinflammatory cytokines, nitric oxide and tissue factors, while the other primary target cells, dendric cells, were impaired in their function. Lymphocytes were depleted by apoptosis even they were not susceptible for EBOV infection, and the apoptosis is likely to be due to impairment of dendric cells, proapoptic factors from activated monocytes/macrophages and direct effect of viral glycoproteins. Impairment of dendric cell function and depletion of lymphocytes were thought to result in impairment of adaptive immune response. Endothelial cells were infected with EBOV and increased endothelial permeability was noted. The virus infection to endothelial cells may have some effect on their function, but the cells were thought to be mainly affected by proinflammatory cytokines, such as TNFα released by macrophages/monocytes. Other factors might contribute to disseminated intravascular coagulation (DIC) which was often observed in human patients and infected primates, however, tissue factor released from virus-infected macrophages/monocytes was thought to play a crucial role in induction of DIC. Infection and apoptosis of hepatocytes and adrenal cortical cells may also contribute to EBOV pathogenesis.

A majority of these studies were focused on pathogenesis of ZEBOV, however, several studies implicated the reason why REBOV was less virulent compared to African EBOV. Significant differences in virus growth in cultured African green monkey cells, Vero cells, between REBOV and ZEBOV may reflect the difference in pathogenesis [25]. REBOV replication and transcription was also shown to be less efficient compared to ZEBOV using reconstituted minigenome replication/transcription systems [25].

Earlier study showed that a cleavage of viral GP to mature G_1 and G_2, which are disulfide linked to form a mature spike protein, $G_{1,2}$. The GP was shown to be cleaved by furin or furin-like endoprotease at C-terminal of consensus furin recognition motif of R-X-K/R-R to generate G_1 and G_2. REBOV GP, however, has a suboptimal sequence of K-Q-K-R and has been shown to be cleaved at reduced level, thus the difference in GP processing between REBOV and other three EBOV was thought to be responsible for different virulence among EBOVs [26]. However, this hypothesis has been contradicted to the observation that furin-cleavage was not essential for EBOV infectivity [27,28] and not critical for virus infectivity and virulence in nonhuman primates [29].

Many studies indicated that EBOV glycoprotein ($GP_{1,2}$) plays a crucial role in pathogenesis [1]. Earlier study showed that ZEBOV GP expression using an

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adenovirus vetor caused endothelial cell damage in explanted human saphenous vein and cynomolgus monkey carotid arteries leading to enhanced permeability of the vessels, while REBOV GP expression exerted a less effect on cynomolgus monkey vessels but no effect on human vessels [30]. A mucin-like domain within ZEBOV GP was shown to be responsible for vascular injury [30]. This may be caused by down regulation of adhesion molecules such as integrin $\beta 1$ but not due to endothelial cell death [31,32]. The down regulation of such molecules was significantly weaker in REBOV GP expressed cells compared to ZEBOV GP expressed cells.

Several cellular proteins [1], such as $\beta 1$-integrin receptor, variety of C-type lectins (DC-SIGN, L-SIGN, MGL), DC-SIGN-related factors, and Tyro3 receptor tyrosine kinase family [33], are thought to be involved in EBOV infection. Among these proteins, human MGL which is expressed on macrophages and monocyte-derived immature dendric cells, has shown to promote EBOV infection through of mucin-like domain within GP, however, infectivity of REBOV GP pseudotyped virus was significantly lower than that of ZEBOV, SEBOV, and ICEBOV GP pseudotyped viruses [34]. Since EBOV infection in monocytes/macrophages plays a crucial role in hemorrhage and DIC through high level of expressions of proinflammatory cytokines and tissue factor [1], lower level of REBOV infection utilizing hMGL may partly contribute to lower virulence of REBOV.

Antibody-dependent enhancement of ZEBOV infection in macrophages though Fc-receptor and in endothelial/epithelial cells through C1q/C1q-receptor was also demonstrated, but significantly weak antibody-dependent enhancement was demonstrated for REBOV [35–37]. These may also contribute different pathogenesis between REBOV and African EBOVs.

Lymphocytes were shown to refractory to EBOV infection, however depletion of both CD4 and CD8 cells were observed in human patients and nonhuman primates exposed to EBOV. Recent study showed that a 17 amino acid domain within ZEBOV GP, which is similar to an immunosuppressive motif of retrovirus glycoprotein, is responsible for apoptosis of these cells, inhibition of progression of CD4 and CD8 cell cycles, decreased expression of interleukin-2, interferon-γ and interleukin-12-p40, and increased expression of interleukin-10. Corresponding peptide of REBOV GP, however, caused similar effect on rhesus T cells but not on human cells [38].

Recent study using microarray technique showed that ZEBOV infection to human hepatoblastoma cells, Huh7 cells resulted in down regulation of interferon-stimulated gene expression and also down regulation of type I interferon stimulated gene expression, while REBOV-infected cells showed reduced ability to down regulate these gene expression [39]. This may be related to no or lower virulence of REBOV in human.

Even though, many other factors may also be involved in difference in virulence between African EBOVs and REBOV, these studies strongly indicated that several factors at least contributed to the lower virulence of REBOV in human. In this regard, REBOV may not be a threat to humans even though we cannot rule out the possibility to cause severe disease in humans when accidental laboratory infection of high doses of REBOV occurs. Recent advances in reverse genetic system of

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filoviruses allowed to study biology and pathogenesis of filoviruses. More precise mechanisms of lower virulence of REBOV will be clarified when the reverse genetic system is applied to REBOV in near future.

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Recent progress in molecular biology of Crimean–Congo hemorrhagic fever

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Abstract

Crimean–Congo hemorrhagic fever (CCHF) is a severe hemorrhagic fever in humans with a case fatality rate of up to 50%. A causative agent of CCHF is CCHF virus, which is a tick-borne virus in the family *Bunyaviridae*, genus *Nairovirus*. The virus is transmitted to humans through infected tick bites, squashed ticks or from direct contact with viremic animals or humans. Outbreaks of CCHF have been documented in Africa, the Middle East, Eastern Europe and Western Asia where the vector and/or reservoir ticks of *Hyalomma* spp. are distributed. Recent advances in molecular and biochemical analyses of CCHF virus revealed that the virus encodes larger proteins compared to other genus of Bunyavirus and the processing of viral proteins are complicated. Recent studies also showed that the CCHF viruses are relatively divergent in its genome sequence and the viruses are grouped in seven different clades. In general, these phylogenetic analyses based on sequences of S-RNA and L-RNA segment of CCHF viruses indicate that the seven clades correlate with their geographical location. The phylogenetic topology based on M-RNA segment sequences of CCHF viruses is different from those based on S-RNA and L-RNA segments. These analyses indicate that M-RNA segment reassortment events occur more frequently than those in S- and L-RNA segments.

Keywords: Crimean–Congo hemorrhagic fever (CCHF); CCHF virus; Tick; Epidemiology; Phylogenetic analysis; Reassortment

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Résumé

La fièvre hémorragique de Crimée–Congo (FHCC) est une fièvre hémorragique foudroyante qui s'attaque à l'homme et entraîne la mort dans 50% des cas. L'agent responsable de la FHCC est un virus de type *Nairovirus de la famille des Bunyaviridae* qui se transmet par les tiques. Ce virus est transmis aux hommes lors de la morsure d'une tique infectée, par une tique écrasée ou par contact direct avec des animaux ou des personnes infecté(e)s. Les tiques *Hyalomma spp.* ont joué le rôle de vecteur et/ou de réservoir en répandant la FHCC en Afrique, au Moyen Orient, en Europe de l'Est et en Asie occidentale. Les récentes découvertes en matière d'analyses moléculaires et biochimiques sur le virus de la FHCC ont révélé qu'il encodait des protéines plus grosses que celles des autres virus de la famille des Bunyavirus, et que la fabrication des protéines virales était compliqué. De récentes études ont également montré que la séquence génomique des virus de la FHCC était assez différente, et que les virus étaient répartis en sept clades. En général, les analyses phylogénétiques, basées sur des séquences de segment d'ARN court et long des virus de la FHCC, indiquent que les sept clades sont liées à leur situation géographique. La topologie phylogénétique, basée sur les séquences du segment d'ARN moyen des virus de la FHCC, diffère de celle basée sur les segments d'ARN court et long. Ces analyses montrent que les évènements de réassortiment du segment d'ARN moyen se produisent plus fréquemment que ceux des segments d'ARN court et long.

Mots clés: La fièvre hémorragique de Crimée–Congo (FHCC); le virus de la FHCC; tictaquer; épidémiologie; phylogenetic analyse; reassortment

1. Introduction

Crimean–Congo hemorrhagic fever (CCHF) is a severe hemorrhagic fever in humans with a case fatality rate of up to 50%. A causative agent of CCHF is CCHF virus, which is a tick-borne virus in the family *Bunyaviridae*, genus *Nairovirus*. The virus is transmitted to humans through infected tick-bites or direct contact with the viremic animals or humans. Outbreaks of CCHF have been documented in Africa, the Middle East, Eastern Europe and western and central Asia, where the vector and/or reservoir ticks, *Hyalomma* spp., *Rhipicehpalus*, *Ornithodoros*, *Boophilus*, *Dermatocentor*, and *Ixodes* spp. are prevalent. Among these ticks, *Hyalomma* spp. is the most important vector for the virus. Clinical features of CCHF patients are characterized by a sudden onset of fever, myalgia, headache, dizziness, sore eyes, photophobia and hyperanemia. In severe cases, hemorrhagic manifestations develop several days after onset of disease. Course of CCHF virus infection and clinical features are recently reviewed more in detail [1,2]. In this review, epidemiology and ecology of CCHF, recent advances in biology of CCHF virus, molecular epidemiology of the virus, and diagnosis are reviewed.

2. Epidemiology and ecology

CCHF was first reported in the Crimean peninsula in 1940s, when a large outbreak of severe hemorrhagic fever with a case fatality rate of 10% was identified

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Table 1
A major tick species associated with CCHF virus

Country or region	Tick species
West and southern Africa	*Hyalomma marginatum rufipes* and *H. turanicum*
Madagascar	*Boophilus microplus*
China	*H. asiaticum asiaticum*
Uzbekistan	*H. asiaticum asiaticum*
Tajikistan	*Dermacentor niveus*
Pakistan	*H. anatolicum*
Russia, Balkan	*H. marginatum marginatum*
Turkey	*H. marginatum marginatum, Rhipicephalus bursa*
Greece	*Rhipicephalus bursa*

[1,2]. Disease was designated as Crimean hemorrhagic fever and later the disease was reported throughout the European and central Asian republics of the former Soviet Union, and other countries. The Crimean hemorrhagic fever virus was isolated by inoculating patient specimens in newborn mice [3]. The virus was later shown to be antigenically identical to Congo virus which was isolated from a febrile patient in Democratic Republic of Congo in 1956 [4] and subsequently became named CCHF virus.

The epidemiology of CCHF reflects the geographic distribution of the ixodid ticks, particularly those of the genus *Hyalomma* [5,6] (Table 1). The virus and/or CCHF is reported in over 30 countries in Africa (Democratic Republic of Congo, Uganda, Mauritania, South Africa, Tanzania, Nigeria, Senegal, etc.), southeast Europe (Russia, Bulgaria, Kosovo, Turkey, Greece, etc.), the Middle East (UAE, Iraq, Iran, Saudi Arabia, Oman) and Asia (China, Kazakhstan, Tajikistan, Uzbekistan, Pakistan) [1,5] (Fig. 1).

CCHF virus persists in the host ticks through its life stages from larvae to nymph to adult (transstadial transmission) [7–11]. The virus can also be transmitted transovarially [1,12,13]. However, the virus generally circulates in an enzootic tick–vertebrate–tick cycle and vertebrates such as sheep, goats, cattle are thought to amplify the virus in the cycle. Smaller mammals, such as hares, hedgehogs, rodents, are known to be infested by immature stages of the ticks and may play a role in the lifecycle of the ticks [5]. Even though a variety of animals was demonstrated to be infected, the virus only causes disease in human and newborn mice [13]. In contrast, birds are generally refractory to CCHF virus infection, even though some species of birds are demonstrated to support large number of the virus infected ticks [5]. This has also been demonstrated in some birds experimentally infected with CCHF viruses [5]. However, many migrating bird species are known to be infested by immature ticks such as *H. marginatum marginatum* in Eurasia, and *H. m. rufipes*, *H. truncum* and some other *Hyalomma* spp. in Africa [5], thus the birds may play an important role in virus dissemination. Among birds, ostrich is only known to be susceptible to virus infection, and two CCHF outbreaks associated with slaughtering of Ostriches in South Africa have been reported [14,15]. Trade in livestock is shown to be associated with several outbreaks of CCHF [16–19].

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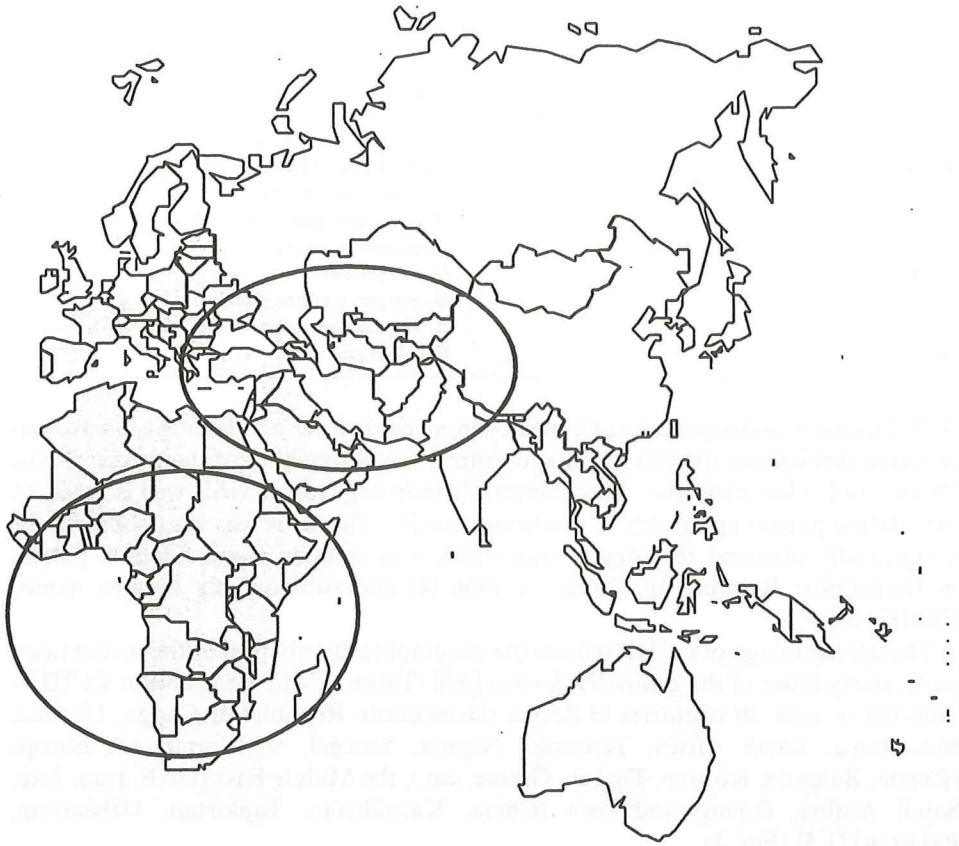

Fig 1. The worldwide geographical distribution of CCHF virus and CCHF cases. Outbreaks of CCHF have been documented in Africa, the Middle East, Eastern Europe and Western and Central Asia, where the vector and/or reservoir ticks, especially *Hyalomma* spp., are prevalent.

3. Biology of CCHF virus

CCHF virus belongs to the family *Bunyaviridae*, genus *Nairovirus*. The genus *Nairovirus* is a tick-born virus and includes 34 viruses, which are grouped in seven serogroups. CCHF serogroup contains CCHF virus and Hazara virus. Recent study demonstrated the striking similarities between Nairovirus and tick phylogenies which indicate possible co-evolution of the viruses and their host ticks [20].

CCHF virus is an enveloped virus with a tripartite (small (S), medium (M), and large (L)), negative-sense single stranded RNA genome which encodes viral nucleocapsid (N), membrane glycoprotein precursor (GPC), and RNA-dependent RNA polymerase (L) proteins, respectively [21]. Basically, the structure and replication strategy of CCHF virus is indistinguishable from other Bunyaviruses [21].

N protein encoded in the S-RNA segment comprises 482 amino acids and a major component of nucleocapsid, however, mechanism of interaction of the N protein to

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viral RNAs is poorly understood. In the virus infected mammalian cells, the N protein is mainly localized in the perinuclear region, but not associated with Golgi apparatus. Recent analysis showed that the N protein is targeted to the perinuclear region without viral glycoproteins and native viral RNAs. It has been also demonstrated that the depolymerization of actin filaments by Cytochalasin D resulted in disruption of N protein localization in both CCHF virus infected cells and the N protein expressing cells, indicating that actin filaments are involved in the targeting of the N protein [22]. Human MxA protein has been shown to inhibit replication of CCHF virus by interacting with the N protein [23] and inhibition of the growth of CCHF virus in human cells by interferon-alpha is mediated by the interferon-induced MxA GTPase [24].

Recent research clarified the unique coding strategy of the GPC protein in the M-RNA segment and the complex processing of the GPC into mature viral glycoproteins, Gn and Gc. CCHF virus M-RNA segment encodes an unusually large polyprotein compared to that of other genera of the *Bunyaviridae* family, in which GPCs are co-translationally processed into Gn and Gc [21]. The processing of CCHF virus GPC is more complicated in that further post-translational cleavage of the glycoprotein precursor products is required for production of the mature glycoproteins, Gn and Gc. In CCHF virus infected cells, M-RNA derived mRNA translates a large precursor protein, which is thought to be co-translationally cleaved into 140 kDa PreGn and 85 kDa PreGc at the N-terminal and the fifth hydrophobic stretch of the precursor by signalase in the endoplasmic reticulum (ER). The PreGn and PreGc give rise to the two mature envelope proteins, 37 kDa Gn and 75 kDa Gc [25–27]. The PreGn is processed at the consensus motif, RRLL, by SKI-1 protease in the ER/*cis* Golgi network to give rise to the Gn and the N-terminal region consisting the hypervariable mucin-like domain and GP38 [26,27]. The latter is heavily O-glycosylated in its mucin-like domain and some of them are further processed by furin to generate GP38 and GP85/GP160 in the *trans* Golgi network [27]. Function of these three proteins is unknown but they are secreted in significant quantity [27]. The N-terminal regions of GP85/GP160 comprising 243–259 amino acids are mucin-like, highly variable (amino acid identity ranging 15.2–100%, similarity ranging 67.5–100%) [25,28] and extremely rich in serine/threonine/proline residues (43.1–51.8%) [25]. The C terminus of the PreGn is thought to be processed at the R(R/K)LL by unknown protease [26,27]. The PreGc is further processed into mature Gc at the motif of RKPL by unidentified SKI-1-like protease in ER/*cis* Golgi network [26,27]. The mature Gn and Gc are localized to the Golgi where assembly and release of the virion occur. The Golgi targeting/retention signal resides within the ectodomain of Gn and its N-glycosylation is important for its localization and transport [29,30].

Early study has shown only minor antigenic differences among some CCHF virus strains originated in widely separated areas of the world by virus neutralization assay, but a slight difference has been observed between two strains, one isolated in Senegal and the other in Pakistan [31]. Recent analysis using a panel of monoclonal antibodies (mAbs) to Gn and Gc of CCHF virus IbAr10200 strain demonstrated broadly reactive and group-specific neutralizing and/or protective epitopes on Gn

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and Gc [32]. Interestingly, any of mAbs against Gn has no virus neutralizing in *in vitro* plaque reduction assays, many of them confer protection to CCHF virus challenge in suckling mice [29,32]. Passive immunization of the plasma of convalescent patients was shown to be effective for the treatment of CCHF in seven patients, indicating that an inactivated or subunit vaccine may be effective [33]. Actually, CCHF inactivated vaccine was produced from mice brain tissues and used in Russia in 1970s [5].

The complete nucleotide sequence of L-RNA segment has been recently determined [34,35]. The data showed that L-RNA is nearly twice the size (12164 nucleotides) of those of other genus of Bunyavirus. The L-RNA segment of the CCHF virus encodes a large protein with 3944 amino acids in which an ovarian tumor (OTU)-like protease motif is found at N terminus followed by zinc finger motif and helicase domain, and RNA polymerase catalytic domain locates in the central region, indicating that the large protein is autoproteolytically cleaved, even though such a processing is not yet proved in the virus infected cells or in recombinant L protein expressing cells.

The processing of viral proteins is more complicated compared with other Bunyaviruses as described above, thus an establishment of a reverse genetics system for CCHF virus may help to understand biology of CCHF virus more in detail. In the case of Bunyaviruses, Bunyamwera virus was the first generated from cloned cDNA using T7 RNA polymerase system [36]. Recently, a reverse genetics approach has been made for CCHF virus using RNA polymerase I system [37]. In this study, S-RNA segment based minigenomes were transcribed, replicated and encapsidated upon infection of helper CCHF virus infection. Further development of a reverse genetics system for CCHF virus to generate an infectious CCHF virus from entirely cloned cDNA will allow remarkable progress in understanding biology of CCHF virus and also to develop therapeutic and prophylactic measures against CCHF virus infections.

4. Phylogenetic relationship and geographic distribution of the CCHF viruses

Complete nucleotide sequence of S-RNA segment of Chinese isolate C68031 of CCHF virus was determined in 1992 [38]. Thereafter, nested reverse transcriptase polymerase chain reaction (RT-PCR) amplifying the partial S-RNA segment of CCHF virus was developed and used for analysis of CCHF viruses at 1994–1995 CCHF outbreak in the United Arab Emirates (UAE) [16,39]. Phylogenetic analyses based on the sequence data of the amplicons have revealed genetic diversity. The outbreak was indicated to be a multisource outbreak associated with importation of the virus infected livestock and ticks. The genetic diversity in S-RNA segment sequence was also demonstrated for many CCHF virus isolates from different regions of the world [40–55]. A partial sequence of S-RNA segment was often used for phylogenetic analyses. However, recent studies indicated the possibility of recombination of the S-RNA segments [55–57], although the recombination is relatively rare. Thus, it would be better to use the full sequence data of the S-RNA segment in a comprehensive phylogenetic analysis. The analysis showed that CCHF

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viruses were grouped in seven different clades; African clade 1 comprising isolates in Senegal, African clade 2 comprising those in Uganda and some in South Africa, African clade 3 comprising those in South and Western Africa, European clade 1 comprising those in Russia, Turkey, and Balkan region (Bulgaria, Kosovo), European clade 2 composed of a single Greek isolate AP92, Asian clade 1 comprising those in Middle East, Pakistan, Iran and Asian clade 2 comprising those in China, Uzbekistan, Kazakhstan. Among these clades, European clade 2 is most distantly related to other clades including European clade 1. In general, these phylogenetic analyses based on S-RNA segment sequences indicate that the seven clades correlate with their geographical location. It is worth mentioning that AP92 isolate was isolated from *Rhipicephalus bursa* ticks and there were no CCHF cases associated with this clade of the CCHF virus in Greece even though seroepidemiological survey demonstrated asymptomatic infection in human [58]. The genetic difference between European clades 1 and 2 may be due to genetic isolation of Greek isolate by adjacent mountain ranges [59] but not to the different tick species since an extensive survey of ticks in Turkey demonstrated that the CCHF viruses of European clade 1 were detected by RT-PCR in both *H. marginatum marginatum* and *R. bursa* [44]. In several cases (UAE [16], Oman [17], Saudi Arabia [18] and Madagascar [5,48]), CCHF viruses are thought to be introduced through import of the virus infected and/or tick-infested livestock such as sheep and cattle as mentioned above. Recently, it has been shown that two genetic lineages of CCHF viruses, Asian clade 1 and African clade 1, exist in Iran [42] and the latter is thought to be introduced by livestock trade [48]. Within the Asian clade 2, the viruses are clustered in two subclades, the first subclade including the viruses in China and Uzbekistan where *H. asiaticum* tick is a major vector, and the second in Tajikistan and Kazakhstan where *Dermatocentor niveus* is a major vector, indicating that a long-term association with a particular tick species plays a crucial role in genetic diversity among the clade [43]. Recent phylogenetic analyses based on L-RNA segment sequences showed that the L tree topology was similar to the S tree topology [55,60], however, L-segment reassortment has been suggested for some isolates in Senegal [55].

On the other hand, the phylogenetic topology based on M-RNA segment sequences of CCHF viruses is different from that based on S-RNA segments [28,32,55,60–66]. These analyses show that CCHF viruses are likely to be grouped in six different phylogenetic clades based on M-RNA sequences; clade M1 comprising isolates in China, Pakistan (Matin isolate), Oman, and South Africa (SPU97/85 and SPU415/85 isolates); clade M2 comprising those in Uzbekistan, Tajikistan, China, Pakistan, Iran, Iraq, South Africa and Nigeria; clade M3 comprising those in Congo (UG3010 isolate), Senegal, China (7001 and 79 121 isolates) and maybe Uzbekistan (Uzbek/TI10145 isolate which sequence is only partially determined (GenBank Acc. AY093627)); clade M4 comprising those in Russia, Kosovo and Turkey; other two clades are composed of Greek isolate AP92 and Mauritanian isolate ArD39554, respectively (Fig. 2). Essentially identical M tree can be obtained in phylogenetic analyses using partial sequence data of the M-RNA segment even when the extremely variable mucin-like domain is used [32,60], indicating that recombination

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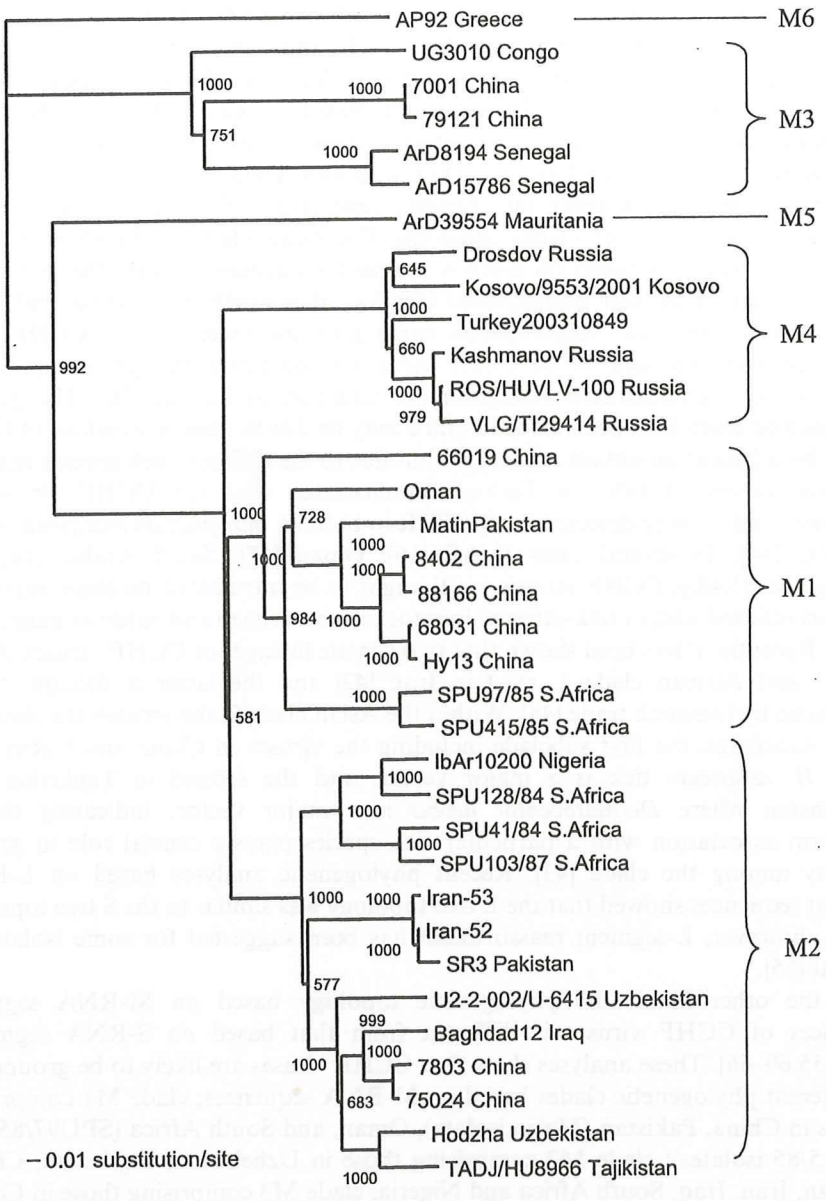

Fig 2. Phylogenetic relations of CCHF virus isolates based on the entire amino acid sequences of the M-RNA segment encoded protein. Phylogenetic analysis of CCHF virus isolates based on the entire amino acid sequences of the M-RNA segment encoded protein available on the GenBank using neighbor-joining method with Kimura's parameter was performed using the ClustalW program. The tree was drawn using the TreeView program. The numbers at the nodes are the bootstrap confidence level for 1000 replicates. The scale indicates the number of substitutions per site. The analyses based on M-RNA sequences indicate that CCHF viruses are likely to be grouped in six different phylogenetic clades, and tentatively denoted as M1 to 6.

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within the M-RNA segments did not occur during evolution of the CCHF viruses. These data strongly indicate that M-RNA segment reassortment event occur frequently in CCHF viruses. The genetic reassortment may occur in ticks co-infected with different types of CCHF viruses, since the virus persists for long periods in ticks. The reason why M-RNA segment reassortment is more frequently observed is not clear, however, strong interrelation between N protein encoded in the S-RNA segment and RNA polymerase encoded in the L-RNA segment may be required to produce viable virus [67]. In addition, the virus is thought to be highly adapted to a particular species of host ticks in endemic region, and the S- and L-RNA segments may have evolved together in a particular tick. In contrast, the M-RNA segment sequence may not be restricted in a particular tick species, thus the reassortment event is frequently observed in the M-RNA segment. The possible M-RNA segment reassortment events between geographically distant regions are frequently observed as illustrated in Fig 3. Such an M-RNA segment reassortment between most geographically distant regions is observed in Chinese isolates, 7001 and 79 121, which were isolated from a patient in 1970 and from a jerboa in 1979, respectively, in Bachu region of the Xinjiang Autonomous Region in Western China. The M-RNA segments of these isolates are closely related to those of Senegal isolates within the clade M4. Birds are refractory to CCHF virus viremia except ostriches, but many migrating birds are known to be infested by immature ticks, such as *H. marginatum*, thus the virus was likely to be introduced to China by intercontinental migration of birds and then the genetic reassortment of M-RNA segment occurred between the African clade 1 and the Asian clade 2 type viruses.

5. Diagnosis, treatment and vaccine

Early diagnosis is important in terms of treatment of patients and prevention of nosocomial infections. Differential diagnosis is also necessary for other infectious diseases showing similar symptoms. Laboratory diagnosis includes demonstration of virus in the blood specimens and detection of virus-specific antibodies. The demonstration of virus in the specimens are carried out by isolation of the virus in tissue culture or suckling mice, by detection of viral RNA in RT-PCR including more sensitive one-step real-time RT-PCR [39,68–74], and/or by detection of virus antigens in antigen-capture ELISA using a recombinant virus N protein [75]. The CCHF virus specific antibodies were previously detected by complement fixation assay, gel diffusion assay, but recently immunofluorescence assay and ELISA are used to detect virus specific IgM and IgG antibodies including virus antigens or a recombinant virus N protein [72,73,76–82]. In general, viremia is demonstrated in first 9 days from onset of diseases, while antibodies are detected 7 days from onset of disease [2].

Apart from intensive supportive therapy, antiviral drug, ribavirin, is a choice of specific treatment of severe case of CCHF, even though no controlled studies have been performed to confirm its efficacy for CCHF treatment. However, its efficacy was shown in *in vitro* study [83,84] and in mice model [85] and ribavirin treatment was shown to be effective in CCHF patients [41,78,86–92].

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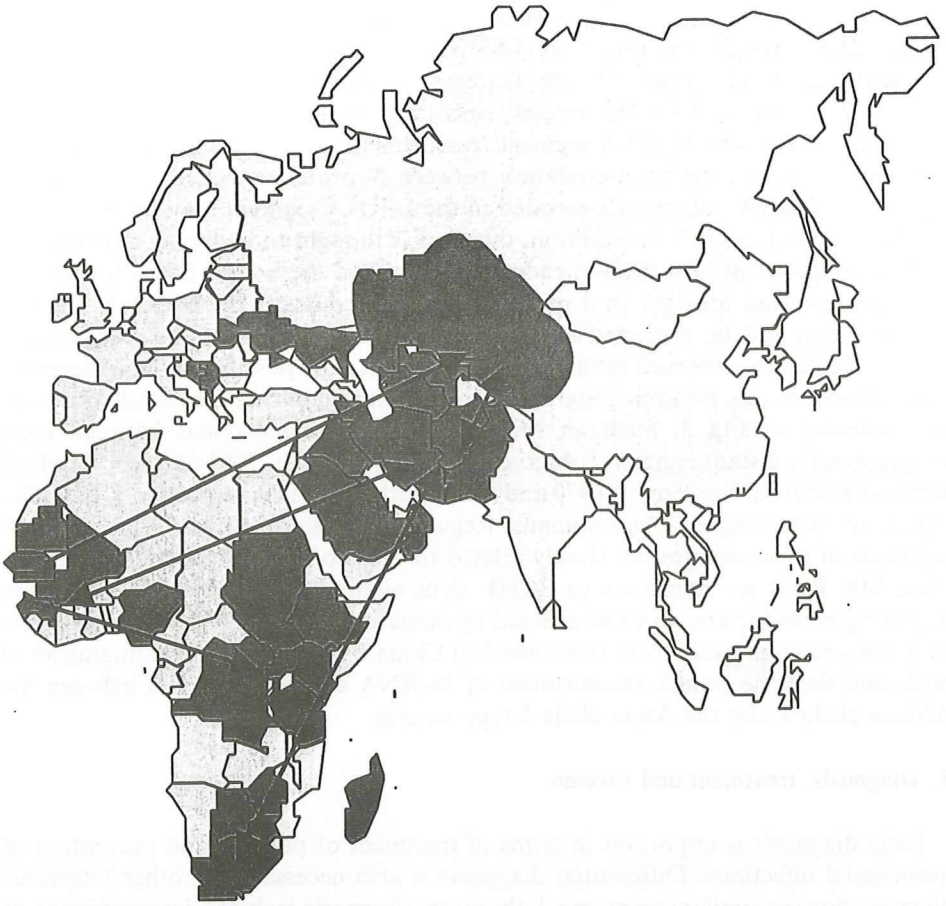

Fig 3. The possible M-RNA segment reassortment events between geographically distant regions. Phylogenetic analyses indicated that M-RNA segment reassortment events occurred frequently between CCHF viruses of geographically distant regions. Genetically distinct CCHF virus is likely to be introduced in geographically distant regions by migrating birds infested with ticks carrying the virus.

In 1970s, CCHF inactivated vaccine was produced from mice brain tissues and used in Russia [5], but it is not known if it is effective and/or safe in humans. Recently, DNA vaccine of CCHF expressing Gn and Gc genes was shown to elicit neutralizing antibodies in mice, however, its protective efficacy is not demonstrated because of a lack of challenge animal model for CCHF virus [93].

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