Virus Research 173 (2013) 344–349

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Infection and propagation of Crimean-Congo hemorrhagic fever virus in embryonated chicken eggs

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ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 12 January 2013
Accepted 14 January 2013
Available online 23 January 2013

Keywords:
Crimean-Congo hemorrhagic fever virus
Embryonated chicken egg
vRNA
qRT-PCR
Viral load
Virus cultivation

ABSTRACT

The embryonated chicken egg (ECE) provides a convenient, space-saving incubator for the cultivation of many kinds of animal viruses where the egg can be easily observed for viral replication throughout the development of the chicken embryo. Within the family *Bunyaviridae*, the embryonated egg has been used as a host system for many viruses such as *Rift Valley fever virus* and *Akabane virus*. The current study was conducted to determine the cultivation of Crimean-Congo hemorrhagic fever virus (CCHFV) in ECE. Four-day-old eggs were infected with CCHFV via the yolk sac route and harvested embryonic tissues and amino-allantoic fluid (AAF) that were used for virus passage and viral RNA (vRNA) detection. Quantification of vRNA copies was performed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Our study indicated that CCHFV caused the death of the embryonated egg in a dose-dependent manner and the 50% egg infectious dose (EID_{50}) was determined to be 6.47×10^5 copies/egg. CCHFV replicated and passaged well in the egg and high viral loads were detected both in embryonic tissue (10^{9-10} copies/g) and AAF (10^{7-9} copies/ml) of the embryonated egg. Thus, ECE could be used for viral cultivation and preservation, and as a potential host infection model for the study of the pathogenesis of CCHFV.

1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a fatal viral infection described in Africa, Asia, Eastern Europe, and the Middle East, and some new outbreaks have been reported in Kosovo, Afghanistan, Iraq, Kazakhstan, Turkey, Greece, and India (Bajpai and Nadkar, 2011; Ergonul, 2012; Hoogstraal, 1979; Karti et al., 2004; Mishra et al., 2011; Maltezou and Papa, 2011; Zavitsanou et al., 2009). CCHFV is classified as a member of the *Nairovirus* genus within the *Bunyaviridae* family of viruses and World Health Organization (WHO) risk group IV which presents a serious human health risk as a severe disease with a high mortality rate (5–30%) (Ergonul, 2006; Flick and Whitehouse, 2005). There is currently no United States Food and Drug Administration (FDA)-approved vaccine or specific antiviral therapy for CCHF (Ergonul, 2008; Kraus and Mirazimi, 2010). Moreover, the only available and somewhat efficacious vaccine is an inactivated brain tissue preparation from infected newborn laboratory mice that is currently used in Bulgaria (Keshtkar-Jahromi et al., 2011; Mousavi-Jazi et al., 2012; Papa et al., 2011).

CCHFV infection has been demonstrated among domestic and wild vertebrates such as goats, hares, cattle, hedgehogs, and multimammate mice, but there is no evidence that the CCHFV causes disease in animals other than suckling mice (Kraus and Mirazimi, 2010). Passerine birds and domestic chickens experimentally inoculated with CCHFV were refractory to the virus and remained healthy, with no or only a low intensity viremia (Hoogstraal, 1979; Shepherd et al., 1987). Experiments performed in ostriches showed that these birds had a higher prevalence of CCHFV infection compared with other birds, however no signs of illness were observed (Capua, 1998; Swanepoel et al., 1998). CCHFV has been isolated most frequently through the intracranial (i.c.) inoculation of newborn suckling mice, although inoculation of susceptible cell lines may also be used. Viral cultivation in vitro is usually by cell cultivation in Vero, Vero E6, SW13, LLC-MK2, BHK-21 cells, primary chicken embryos cells, and tick cell lines (Bell-Sakyi et al., 2012; Zeller, 2007).

Embryonated chicken eggs (ECEs) are a complex structure comprised of an embryo and its supporting membranes. The developing embryonated egg provides the diversity of cell types needed for successful replication of a wide variety of different microbial pathogens and has been used extensively as an infection model and vaccine production incubator (Anjum et al., 2010; Clavijo et al., 2000; Crespo et al., 2009; Guy, 2008; Jacobsen et al., 2010, 2011).

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http://dx.doi.org/10.1016/j.virusres.2013.01.008

However, little information is currently available on CCHFV isolation or cultivation using ECEs.

Since embryonated eggs are susceptible to infection with the majority of the *Bunyaviridae* viruses, such as *Akabane virus*, *Turlock-Like Bunyavirus*, and *Rift Valley fever virus* (Konno et al., 1988; McPhee et al., 1984; Pepin et al., 2010; Shivaprasad et al., 2002), we investigated the use of ECEs for the cultivation of CCHFV and its use as a potential host model of CCHFV infection.

2. Materials and methods

2.1. Virus and specific-pathogen-free ECE

The CCHF viral strain YL04057 (infected mouse brain stocks) (kindly provided by the Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region) was isolated from ticks (*Hyalomma asiaticum kozlovi*) in Xinjiang in 2004 and were used in our study. Specific-pathogen-free (SPF) ECEs were obtained from Merial Vital Laboratory Animal Technology Company (Beijing).

2.2. Inoculation and collection of specimens from eggs

All operations involving infectious material with CCHFV were performed in a biosafety level (BSL)-3 laboratory facility and complied with the national biosafety regulations.

2.2.1. Preparation of samples for egg inoculation

Tissue samples were homogenized in 10% (w/v) phosphate buffered saline (PBS, pH 7.0–7.4) using sterile plastic pestles in 1.5 ml microcentrifuge tubes. Homogenates were then centrifuged at 1000–1500 × *g* for 15 min in a refrigerated centrifuge and the supernatant collected. Fluid samples were centrifuged at 1000–1500 × *g* for 15 min and the supernatants collected. The supernatants (100 μl) were used for inoculation or determination of infectious dose.

2.2.2. Yolk sac inoculation and incubation of eggs

The current study used 4-day-old eggs for yolk sack inoculation (McPhee et al., 1984). Eggs were placed in an egg flat with the air cell in the upright position. Eggs were candled to ensure viability and the edge of the air cell marked using a pencil. The marked area was disinfected and a small hole was drill just above the mark. A 1 ml syringe was used to inoculate the eggs and inject 100 μl of inoculum into the yolk sack. The holes were sealed and the eggs returned to a 37 °C incubator.

2.2.3. Collection of embryonic tissues and amino-allantoic fluid (AAF) from eggs

Eggs were candled once daily after inoculation. Embryonated eggs that had died in the first 24 h p.i. were considered due to nonspecific causes and discarded. Eggs were refrigerated (4 °C) overnight and then placed in an egg flat with the air cell up. The portion of the eggshell that covered the air cell was disinfected and sterile forceps were used to crack and remove the egg shell over the air cell. Forceps were used to enter through the shell membrane and chorioallantoic membrane (CAM). For embryonic tissue collection, the embryo was grasped with sterile forceps and gently removed from the egg and then transferred into sterile snap-cap tubes. For the collection of AAF, a pipette was used to aspirate the fluid in sterile snap-cap tubes. Selected tissues and AAF were collected for the quantitation of CCHFV vRNA, and the remaining samples were stored at −80 °C.

2.3. qRT-PCR for the detection CCHFV vRNA

Viral RNA was prepared from 100 μl or 10 mg sample using an RNase mini kit (Qiagen) and dissolved in 25 μl Dnase- and RNase-free distilled water. First strand cDNA synthesis was performed using random primers with 12 μl of the RNA from each sample in 20 μl reaction using the RevertAid First Strand cDNA synthesis Kit (Fermentas) following the manufacturer's instructions.

Infectious dose was determined as copies/egg and the viral load defined as copies/g tissue and copies/ml fluid. A SYBR green quantitative real-time RT-PCR (qRT-PCR) assay was developed for the detection and quantification of CCHFV vRNA copies. The primers were designed to target the S segment of CCHFV: S299F (nt position at the YL04057 strain: 299–318): 5′-TGGGTTAGCTCCACTGGTAT-3′; S484R (nt: 465–484): 5′-TGCATTGACACGGAAACCTA-3′ (Duh et al., 2006). A segment (186-bp S genomic region) of CCHFV YL04057 RNA was amplified using S299F/S484R, and the amplicon was cloned into the pGEM-T vector (Promega). This plasmid, named CCHF SQ, was used for the CCHFV quantification assay. The assay was performed in a LightCycler 2.0 (Roche) system using the SYBR green master mix kit (Toyobo). Amplifications were carried out in 20 μl reaction mixtures containing 2 μl of template and the cycling conditions were: one cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 58 °C for 10 s and 72 °C for 15 s. Ten-fold serial dilutions (6.3×10^8 to 6.3×10^2 copies/ml) of CCHF SQ were used to determine the dynamic range of quantification. The calculation of standards (sFig. 1a and b), dissociation curves (sFig. 1c) and the analysis of samples (sFig. 2) were performed by using the LightCycler 4.1 software (Roche).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.01.008.

2.4. Primary inoculation of eggs

The CCHFV infected mouse brain tissue inoculum suspension was prepared according to Section 2.2.1. The suspension was 10-fold serially-diluted in PBS (pH 7.0–7.4) to obtain the desired concentration. Determination of infectious dose was performed using 100 μl of each virus dilution and calculation of CCHFV vRNA copies.

Seven groups of twenty 4-days-old eggs were used in this experiment. Six groups of eggs were inoculated with 100 μl of different doses of CCHFV suspension via the yolk sac. The remaining group was inoculated with 100 μl PBS alone used as a negative control. Mortality was recorded 1–14 days post inoculation (p.i.). Dead eggs at 2–14 day p.i. and survived embryos at day 15 p.i. were harvested and stored at −80 °C.

2.5. Passage of CCHFV in eggs

To demonstrate that CCHFV could be passaged in eggs, three dead embryonated eggs (first passage) from Section 2.4 were used to determine viral loads and then used as the inoculation material for the second passage, each in three 4-day-old embryonated eggs. Three dead embryonated eggs from the second passage were used to determine viral load and were used for the third passage as previous. Eggs inoculated with PBS (pH 7.0–7.4) were used as controls. Inoculated eggs were incubated at 37 °C and candled once daily, from day 2 to 7. Entire embryos and AAF were harvested from dead infected embryonated eggs and stored at −80 °C. The viral load of each sample was determined by qRT-PCR.

2.5.1. Passage using embryonic tissue

Entire dead embryos were harvested and the embryo suspension was used as the inoculum, as previously described in Section