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Short communication

Stability of infectious human coronavirus NL63

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The human coronavirus NL63 was identified in 2004 and subsequent studies showed its worldwide distribution. Infection with this pathogen is associated with upper and lower respiratory tract diseases of mild to moderate severity. Furthermore, HCoV-NL63 is the main cause of croup in children. Within this study an optimal protocol for freeze-drying that allows safe and effective preservation of HCoV-NL63 infectious material was developed. Lyophilized virus preparations can be stored either at ambient temperature or at +4 °C. In the latter case samples may be stored for at least two months. Surprisingly, conducted analysis showed that HCoV-NL63 virions are exquisitely stable in liquid media and can be stored also without preservatives at ambient temperature for up to 14 days.

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Coronaviruses (CoVs) belong to the family *Coronaviridae*, order *Nidovirales*. This subfamily is currently divided into 4 groups based on phylogenetic relationship (International Committee on Taxonomy of Viruses and King, 2012). Groups alpha and beta consist of mammalian CoVs, whereas gamma and delta viruses infect birds and marine mammals (Woo et al., 2010). Human CoVs for years were considered relatively harmless common cold viruses outside on the mainstream research on viral pathogens. That has changed after the outbreak of the SARS-CoV (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003) which brought CoVs to the limelight. An intense of CoVs research resulted better understanding of their pathogenesis and biology. Furthermore, two novel human CoVs were discovered: human coronavirus NL63 (van der Hoek et al., 2004) and human coronavirus HKU1 (Pyrc et al., 2010; Woo et al., 2005). Their detailed investigations revealed great variability of coronavirus species infecting animals and the potential threat of zoonotic transmission to human community. Very recently this viral family again drew attention, as another animal coronavirus – Middle

East respiratory syndrome coronavirus (MERS-CoV) – crossed the species border, proving that the emergence of SARS-CoV was not an isolated case (Centers for Disease and Prevention, 2013; Zaki et al., 2012).

Intensified research on coronaviruses and development of a novel research tool based on a coronaviral backbone (e.g., vectors, vaccines) requires a method for safe storage and shipment of infectious material. Coronavirus particles are relatively stable, but previous reports show that this stability is insufficient for storage and transport of coronavirus-based vaccines, vectors and for the material exchange between laboratories (Lamarre and Talbot, 1989; Muller et al., 2008). Lyophilization is the method of choice for cost-effective stabilization of infectious material. It is a process, where a sample is frozen and subsequently the solvent is removed by sublimation (primary drying) and then by desorption (secondary drying) to the level that inhibits biological growth and chemical reactions (Jennings, 1999). To improve the stability of a sample during lyophilization and storage stabilizing substances, including lyoprotectants (Townsend and DeLuca, 1988) and bulking agents (Jennings, 1999), are added. There are numerous reports describing the freeze-drying of viruses, mainly for vaccine production (Amorij et al., 2008; Audouy et al., 2011; Bieganski et al., 1998; Burger et al., 2008; Croyle et al., 1998; de Jonge et al., 2007; Furuya et al., 2010; Geeraedts et al., 2010; Gupta et al., 2010; Kang et al., 2010; Lang et al., 2009; Levy and Fieldsteel, 1982; Yannarell et al., 2002). These methods differ in terms of process parameters and buffer content and stability of preparations ranging from 12 weeks

Abbreviations: CoVs, coronaviruses; HBS, HEPES Buffered Saline; HCoV, human coronavirus; IBV, Infectious Bronchitis Virus; MERS-CoV, Middle East respiratory syndrome coronavirus; PBS, phosphate buffered saline; RT, room temperature; SARS-CoV, Severe Acute Respiratory Syndrome coronavirus.

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at +4 °C (de Jonge et al., 2007) to one year at ambient temperature (Geeraedts et al., 2010; Levy and Fieldsteel, 1982). Precausta et al. developed a protocol that allowed storage of the Infectious Bronchitis Virus (IBV) for 12 months at +6 °C using 40 mg/ml mannitol as the lyoprotectant (Precausta et al., 1980). Roseto et al. lyophilized Bovine Enteric Coronavirus without addition of any stabilizing formulation for structural studies using electron microscopy (Roseto et al., 1982). Virus stability during storage was not tested. No studies on freeze-drying preservation of other coronaviruses, including human coronaviruses were previously reported.

Human coronavirus NL63 belongs to the genus alphacoronavirus (International Committee on Taxonomy of Viruses and King, 2012; Pyrc et al., 2006, 2007; van der Hoek et al., 2004). This pathogen is spread worldwide and is responsible for 1–10% of cases of viral respiratory tracts diseases (Bastien et al., 2005; Cabeca et al., 2012; Gaunt et al., 2010; Kon et al., 2012; Moes et al., 2005). Symptoms of HCoV-NL63 upper respiratory tract infection include rhinitis, cough, fever and sore throat, while infection of the lower respiratory tract manifests in bronchitis, bronchiolitis and pneumonia (Pyrc et al., 2007). Furthermore, HCoV-NL63 was proved to be the main cause of croup in children (Sung et al., 2010; van der Hoek et al., 2005).

The aim of this study was the development of a safe method for freeze-drying preservation of infectious HCoV-NL63. To this end LLC-MK2 cells (ATCC: CCL-7; *Macaca mulatta* kidney epithelial cell line) were used for *in vitro* culture of HCoV-NL63 (Schildgen et al., 2006). The cells were cultured on T25 flasks (TPP, Germany) at +37 °C with 5% CO₂ in minimal essential medium (MEM), containing 2 parts of Hank's MEM and 1 part of Earle's MEM (Life Technologies, Poland) supplemented with 3% heat-inactivated fetal bovine serum (Life Technologies, Poland), penicillin (100 U/ml), and streptomycin (100 µg/ml) (H/E medium). HCoV-NL63 (Amsterdam I strain) stock was generated by infecting LLC-MK2 cells. Infected cells were lysed 6 days post-infection by two freeze-thaw cycles. The virus-containing fluid was cleared by centrifugation, aliquoted and stored at –80 °C. A control from mock infected cells was prepared in the same manner as the virus stocks. Virus yield was assessed by virus titration on fully confluent LLC-MK2 cells, according to Reed and Muench formula (Reed and Muench, 1938). All formulations used during lyophilization are presented in Table 1 and were prepared as 2× concentrated solutions in distilled water. Five times concentrated HBS buffer was prepared in distilled water. All samples were sterilized by filtration. Samples were prepared as follows: formulations 1–7: 80 µl 5× HBS, 20 µl sterile water, 200 µl 2× concentrated stabilizing formulation, 100 µl HCoV-NL63 stock (TCID₅₀ = 1.4 million) in medium H/E 3% P/S; formulations 8–13: 100 µl sterile water, 200 µl of 2× concentrated stabilizing formulation, 100 µl

HCoV-NL63 stock (TCID₅₀ = 1.4 million) in medium H/E 3% P/S; HBS control: 80 µl 5× HBS, 220 µl sterile water, 100 µl HCoV-NL63 stock (TCID₅₀ = 1.4 million) in medium H/E 3% P/S; PBS control: 300 µl 1× PBS (NaCl 8.0 g/l, KCl 0.2 g/l, Na₂HPO₄ 1.44 g/l, KH₂PO₄ 0.24 g/l, pH 7.4), 100 µl HCoV-NL63 stock (TCID₅₀ = 1.4 million) in medium H/E 3% P/S; –H/E 3% P/S. The solutions were prepared in 2 ml tubes (Sarstedt, Germany), snap frozen in liquid nitrogen and positioned within the sterile, pre-cooled lyophilization container with open caps.

The drying process was carried out in the shelf lyophilizer – TMFreeZone Triad 7400030 (Labconco) with attached vacuum pump 195 (Labconco). To ensure safety, the sealed box was prepared from poly(methyl methacrylate) with a rubber seal. The container was designed for a standard probe stand. For sterilization, the box was disinfected with 70% ethanol and exposed to ultraviolet radiation. The gas exchange between the container and the lyophilizer was possible via the vent protected with a 0.1 µm air filter (Sartorius stedim, Germany). Before the lyophilization the container was pre-cooled at –20 °C. Process parameters were chosen based on literature data. In order to maintain stable temperature, the apparatus was stabilized for 3–4 h before the process (shelf temperature –30 °C, collector temperature –80 °C, vacuum off). The first drying step was carried out for 40 h at –30 °C and the pressure of 0.22 mBar. After this time the temperature was increased to –10 °C and the process was carried out for another 24 h. Subsequently, samples in the container were transferred to the laminar flow hood and capped. All samples were stored at –20 °C, +4 °C or at room temperature for stability testing.

The freeze-dried virus was dissolved in 400 µl of sterile water and incubated at +37 °C with mixing for 15 min to facilitate the dissolving of the cake. Obtained solution was diluted 5 times in H/E medium. LLC-MK2 cells were seeded on the 96-well culture plate and incubated for 48 h at +37 °C with 5% CO₂ to obtain fully confluent cell monolayer. Medium was removed from the cells and fresh medium containing serially diluted virus or control samples reconstituted from lyophilized material were added. Two hours post-inoculation medium was removed and fresh H/E medium was applied. Six days post infection the cytopathic effect on the LLC-MK2 cells was assessed. Obtained data were re-calculated to TCID₅₀ according to Reed and Muench formula (Reed and Muench, 1938).

The primary screening of different buffer composition revealed that already during the freeze-drying process some formulations do not provide sufficient protection. First, 1× HBS buffer was selected over the 1× PBS buffer, as PBS exhibits significant pH instability during the freezing process (Amorij et al., 2008). Second, the requirement for a bulking agent was confirmed, in line with observations made by Jennings (1999). Lack of a bulking

Table 1

Composition of stabilizing formulations used during freeze-drying of HCoV NL63 samples. Composition of different formulations including lyoprotectants, bulking agents, additional excipients and references to original works are included in the table. All formulas are presented for 1× concentrated solutions. Formulation names are used throughout the manuscript.

Lp.	Name	Lyoprotectant	Bulking agent	Reference
1.	In25	Inulin 25 g/l	–	Audouy et al. (2011), de Jonge et al. (2007), Geeraedts et al. (2010), Amorij et al. (2008)
2.	In40	Inulin 40 g/l	–	Croyle et al. (1998), Bieganski et al. (1998), Amorij et al. (2008)
3.	Treh100	Trehalose 100 g/l	–	Croyle et al. (1998)
4.	Sucr0.5	Sucrose 0.5 M (171 g/l)	–	Levy and Fieldsteel (1982)
5.	Sucr74.6	Sucrose 74.6 g/l	Gelatin 2%	Croyle et al. (1998)
6.	Sor10	Sorbitol 10%	Gelatin 0.4%	Kang et al. (2010)
7.	Sor2	Sorbitol 2%	Gelatin 0.5%	Burger et al. (2008)
8.	50Mi ^a	Myo-inositol 50 g/l	–	
9.	35Mi15Mann ^a	Myo-inositol 35 g/l	15 g/l Mannitol	
10.	25Mi25Mann ^a	Myo-inositol 25 g/l	25 g/l Mannitol	
11.	35Mi15Sor ^a	Myo-inositol 35 g/l	15 g/l Sorbitol	
12.	50Mi2Leu ^a	Myo-inositol 50 g/l	2 g/l Leucine	
13.	30Mi15Gel ^a	Myo-inositol 30 g/l	15 g/l Gelatin	

^a Samples supplemented with gelatin (25 g/l; with the exception of formulation 13), L-arginine (16 g/l), alanine (1 g/l), and histidine (2.1 g/l).

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