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Conducting Research on Highly Pathogenic Viruses Using Virus Pseudotypes

The Influence of Ultrapure Water on Data Quality

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Abstract

From 2013 to 2016, more than 11,000 people died of Ebola virus infections in West Africa. This most recent epidemic shows how dangerous so-called emerging viruses can be. Virus pseudotypes can be used to easily investigate the entry pathways of such viruses. Read how important the quality of ultrapure water is, among other factors, for the production of such virus pseudotypes.

Introduction

Novel viruses, termed emerging viruses, pose a threat to human health of a magnitude that should not be underestimated. These viruses have acquired the ability to infect humans either as a consequence of interspecies transmission or of naturally occurring changes in the viral genome [1]. Infections caused by emerging viruses often result in serious diseases or even death as the human immune system may not be capable of combatting an unfamiliar virus, especially of zoonotic origin (see literature for further reading, [2]).

Various factors promote the occurrence and spread of emerging viruses [3]. These include ecological factors, such as deforestation to gain new land for development, and also our lifestyle involving global mobility, as well as international commerce. Thus, the loss of habitat increases the probability that people, pets and farm animals will come in contact with wildlife species originally inhabiting isolated areas and therefore not previously encountered as natural hosts of emerging viruses. Furthermore, the surrounding circumstances of a globalised society involving increased travel also enable dissemination of pathogenic viruses around the world, even before the onset of clinical symptoms.

Within the last 100 years, multiple introductions of emerging viruses into the human population have occurred, which have led to local epidemics or worldwide outbreaks (pandemics; see Table 1). In particular, the Ebola virus most recently raised concerns across the globe, as the West African Ebola virus epidemic, which claimed more than 11,000 lives, devastatingly demonstrated the danger posed by emerging viruses [4].

Research involving emerging viruses is often limited to high-containment laboratories of biosafety levels (BSL) 3 and 4 (see Table 1). Working in BSL-4 laboratories is highly laborious, expensive and permitted only at a few locations so that rapid scientific progress in characterising viral pathogens and developing antiviral drugs is difficult to achieve.

Table 1: Outbreaks of new viruses

Disease	Virus	Outbreak					BSL[e)]
		Year	Location[1)]	Type	Number of Cases	Deaths[2)]	
Spanish flu	Influenza Avirus (H1N1)	1918 to 1920	?	Pandemic	?	>20 mn.[a)]	3
Asian flu	Influenza Avirus (H2N2)	1956 to 1958	Southeast Asia	Pandemic	?	1 – 2 mn.[b)]	3
Severe acute respiratory syndrome	SARS-CoV	2002 to 2004	Southeast Asia	Pandemic	8,096[c)]	774[c)]	3
Middle East respiratory syndrome	MERS-CoV	2012 to present	Arabian Peninsula	Epidemic	1,952[c),d)]	639[c),d)]	3
Ebola virus disease	Ebola virus	2013 to 2016	West Africa	Epidemic	28,616[c)]	11,310[c)]	4

Abbreviations: BSL, biosafety level; mn., million; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus;
1) location of primary infection;
2) numbers based on
a) CDC, (https://www.cdc.gov/) and ProMEDmail (http://www.promedmail.org/);
b) Britannica (https://britannica.com/event/Asian-flu-of-1957);
c) WHO, (//www.who.int/en/);
d) version dated May 12, 2017;
e) classification according to the German Central Committee on Biological Safety,
ZKBS (Zentrale Kommission für die biologische Sicherheit, http://apps2.bvl.bund.de/organismen/organisms.jsf)

Given this situation, virus pseudotypes offer an attractive option for studying the entry of highly pathogenic viruses into cells safely and efficiently. This is possible as not the entire pathogen is analysed, but rather only its components that mediate host cell entry, the envelope proteins. These represent the key to virus entry into cells. In the case of virus pseudotypes, envelope proteins of highly pathogenic viruses are incorporated into a carrier virus (pseudotyping), which cannot replicate autonomously, i.e., are replication-deficient. Commonly used systems for pseudotyping are based on rhabdoviruses (e.g., vesicular stomatitis virus, VSV; Fig. 1) and retroviruses.

The objective of this study was to verify whether the envelope protein-mediated entry of virus pseudotypes reflects host cell entry of intact viruses. In addition, this investigation was designed to determine which influence the level of purity of the reagents used, in this case laboratory water, has on the production of virus pseudotypes.

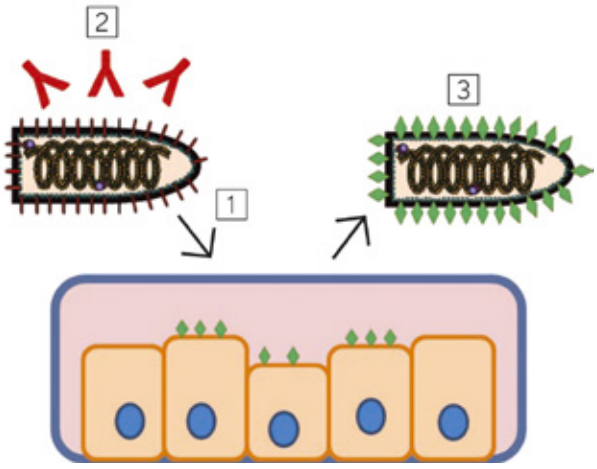


Fig. 1: Production of VSV pseudotypes: 1) Entry of VSV G-trans-complemented VSV into envelope protein-expressing cells. 2) Neutralisation of excess virus. 3) Harvesting of VSV pseudotypes with the envelope protein to be investigated embedded in their membranes.

Materials and Methods

Cell culture, expression plasmids and transfection:
HEK-293T, MDCKII and Vero E6 cells were incubated in Dulbecco's-modified Eagle's medium supplemented with 10% foetal calf serum and 1% penicillin/streptomycin solution at 37°C and 5% CO_2. For passaging and seeding, the cells were washed with phosphate-buffered saline (PBS) and detached by incubating with trypsin | EDTA.

To produce VSV pseudotypes, plasmids of the following viral envelope proteins were used as expression vectors: VSV glycoprotein (VSV G), Ebola virus glycoprotein (EBOV GP), spike glycoprotein of the Middle East respiratory syndrome coronavirus (MERS-CoV S), haemagglutinin (HA) and neuraminidase (NA) of the influenza A virus responsible for the 'Spanish flu' pandemic, H1N1 (1918). In addition, an expression plasmid for dipeptidyl peptidase 4 (DPP4) was employed. Empty expression plasmid was used as a control. HEK-293T cells were transfected using calcium | phosphate precipitation, where all buffers and solutions were prepared using either demineralised water or Sartorius Arium® Pro VF ultrapure water.

Production of ultrapure water
The Arium® Pro VF ultrapure water (the system is shown on the previous page) used was produced as described by Nitzki and Herbig (2013) [5]. This water has a TOC content (total organic carbon, i.e., organically bound carbon) of down to < 2 ppb and a conductivity of 0.055 µS/cm (corresponds to a resistivity of 18.2 MΩ × cm), compensated to 25°C.

Production of VSV pseudotypes
For the generation of VSV pseudotypes, a replication-deficient VSV vector was employed in which the genomic information for the VSV glycoprotein, VSV G, was replaced by two reporter genes, i.e., open reading frames (ORFs) coding for green fluorescent protein (GFP) and firefly luciferase (fLUC). To propagate this virus (VSV*ΔG-fLUC), the VSV G must be provided in trans (e.g., by transfection of an expression plasmid). VSV pseudotypes were produced as described in Hoffmann et al. (2016) [6].

Pretreatment of target cells with sialidase or inhibitors: MDCKII cells were treated with 200 mU of recombinant sialidase to remove terminal sialic acids from glycoproteins and glycolipids of the plasma membrane. To test whether envelope protein-mediated entry of VSV pseudotypes into cells depends on an acidic pH and the activity of cellular cysteine proteases, Vero E6 cells were incubated with 50 nM bafilomycin A1 or 50 µM E-64d. The chemicals employed were diluted in cell culture medium; incubation was carried out for 2 hours at 37°C and 5% CO_2. Untreated cells served as controls.

Analysis of envelope protein-mediated host cell entry: Target cells were incubated with VSV pseudotypes for 1 hour before they were washed with PBS and further incubated with cell culture medium for 16–20 hours. Afterwards, the cells were lysed using luciferase lysis buffer, and the luciferase activity in the cell lysates was measured in a chemiluminometer using commercially available assay kits to assess the efficiency of transduction (i.e., host cell entry of pseudotypes) mediated by envelope proteins.

Results

It is known that the Middle East respiratory syndrome coronavirus (MERS-CoV, formerly called human coronavirus, EMC = hCoV-EMC), binds to the cell surface through the interaction between the viral spike glycoprotein (S) and the cellular membrane protein dipeptidyl peptidase 4 (DPP4), thus enabling cell entry [7]. To confirm whether this also applies in the context of VSV pseudotypes, target cells were transfected with an expression vector for DPP4 or an empty expression plasmid (no receptor). As expected, the directed expression of DPP4 led to a significant increase in the host cell entry of VSV pseudotypes if the pseudotypes had MERS-CoV S embedded in their envelope (see Fig. 2A).

Influenza A viruses, the causative agents of influenza disease, require terminal sugar structures, so-called sialic acids occurring as natural modifications on cellular membrane glycoproteins and glycolipids, as receptors to mediate entry into target cells (see literature for further reading [8]). To study whether the incorporation of influenza A viral envelope proteins in VSV pseudotypes also results in sialic acid-dependent cell entry, VSV pseudotypes with H1N1 (1918) HA/NA were employed, and the sialic acids were enzymatically removed from the surfaces of target cells. As expected, removal of sialic acids resulted in a dramatic decrease in the host cell entry of VSV pseudotypes that had H1N1 (1918) HA/NA embedded in their envelope (see Fig. 2B). This finding confirms that virus pseudotypes reflect the entry mechanism of authentic influenza A viruses into cells.

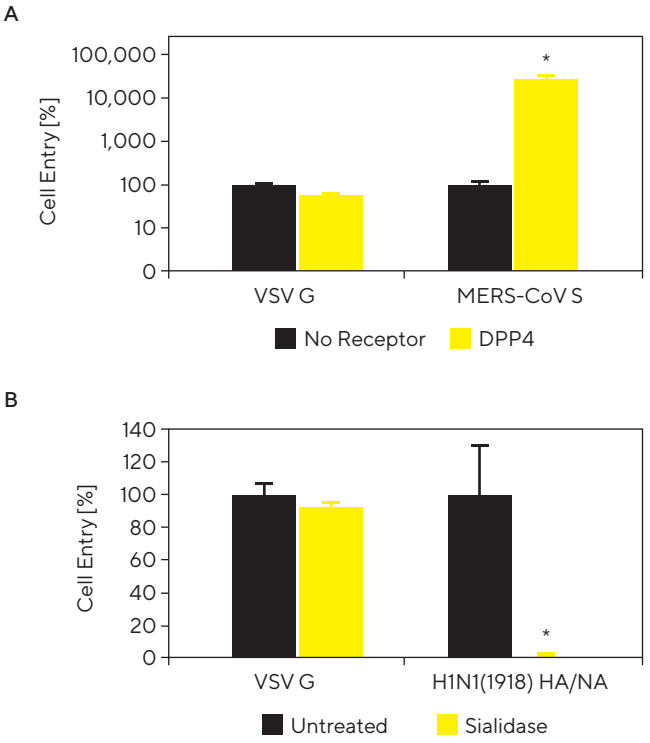


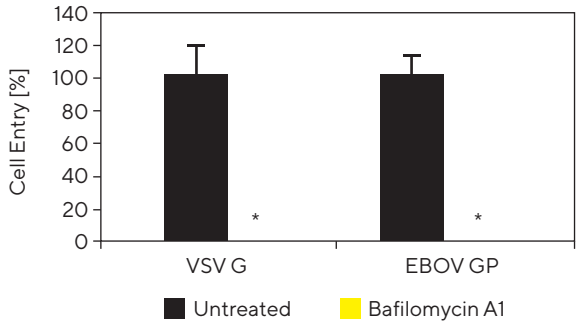
Fig. 2. Receptor-dependent entry of virus pseudotypes into cells: (A) VSV pseudotypes with VSV G or MERS-CoV S were used for inoculation of HEK-293T cells, which had previously been transfected either with an empty vector (no receptor) or an expression plasmid for human dipeptidyl peptidase 4 (DPP4). (B) VSV pseudotypes with VSV G or H1N1 (1918) HA/NA envelope proteins were employed for inoculation of MDCKII cells, which either had been pretreated with sialidase or were untreated. The transduction efficiency, i.e., entry efficiency, was determined by the activity of virally encoded luciferase and normalised. In addition, the statistical significance of the data was confirmed by a t-test (*: $p < 0.05$).

Activation of the Ebola virus glycoprotein during cell entry is pH-dependent and requires the activity of cysteine proteases

The Ebola virus glycoprotein (EBOV GP) is post-translationally modified to contain multiple oligosaccharides, and this dense clustering of glycans is believed to shield the virus from efficient detection by the human immune system [9]. However, during host cell entry, part of the EBOV GP bearing the majority of sugar modifications must be removed [10]. This functional priming is mediated by cellular cysteine proteases [11] that are present in endosomal vesicles and are active only at low endosomal pH.

In the following, we investigated whether EBOV GP-mediated entry of VSV pseudotypes also depends on the activity of cellular cysteine proteases and a low pH. For this purpose, VSV pseudotypes with EBOV GP embedded in their envelope were produced and subsequently used to inoculate target cells that had previously been incubated with bafilomycin A1 (prevents acidification within endosomes by proton pump blockage) or a cysteine protease inhibitor (E-64d). This showed that EBOV GP-mediated cell entry in the context of VSV pseudotypes also depends on an acidic environment (see Fig. 3A) and the activity of cysteine proteases (see Fig. 3B). In addition, we could also demonstrate that treatment with E-64d specifically blocks EBOV GP-driven cell entry, as VSV-G-mediated pseudotype is independent of cysteine proteases, although it is dependent on low endosomal pH.

A



B

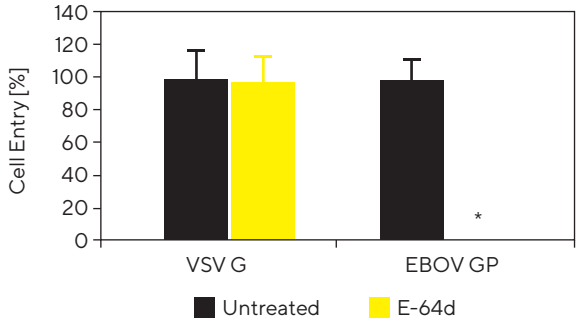


Fig. 3: pH-dependent entry of virus pseudotypes into cells:
VSV pseudotypes with VSV G or EBOV GP were used to inoculate Vero E6 cells that had previously been treated with bafilomycin A1 (A) or E-64d (B) (untreated cells were used as controls). The transduction efficiency, i.e., entry efficiency, of the virus pseudotypes was determined by the activity of virally encoded luciferase, and the resulting data were normalised. In addition, the statistical significance of the data was confirmed by a t-test (*: $p < 0.05$).

The purity level of the laboratory water used influences the quality of VSV pseudotypes

After it could be shown in the previous trials that VSV pseudotypes are suitable models for studying the host cell entry of highly pathogenic viruses, the next issue to be clarified was the particular influence that the purity level of the laboratory water used has on the quality of VSV pseudotypes. During production of VSV pseudotypes, various buffers and solutions are employed that are all prepared with water. However, it must be noted that not just any type of water suffices for preparation of these reagents. Rather, the right choice of purity level must be made for laboratory use.

To investigate whether the use of ultrapure water yields higher quality VSV pseudotypes, two batches of VSV pseudotypes were generated in a parallel experiment: One batch was prepared using demineralised water from a central lab water supply source (conductivity of 3.7 – 4.1 µS/cm at 19°C) as the basic solvent for all solutions and buffers, while a further batch utilising solutions and buffers based on Arium® Pro VF ultrapure water (conductivity 0.055 µS/cm compensated to 25°C) was generated. EBOV GP and MERS-CoV S as envelope proteins were examined. Following parallel production of VSV pseudotypes by maintaining identical incubation conditions, the target cells were inoculated and the envelope protein-mediated entry of VSV pseudotypes was quantified.

This experimental setup demonstrated that host entry (as a parameter for the degree of quality) of the VSV pseudotypes produced using Arium® Pro VF ultrapure water as the basic solvent for all buffers and solutions was significantly higher compared with that of pseudotypes for which demineralised water was employed in their production (Fig. 4).

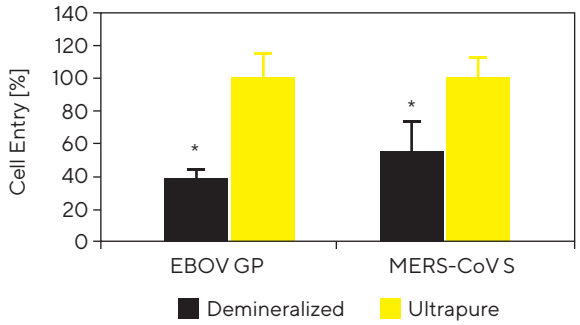


Fig. 4: The purity level of the laboratory water used influences the quality of virus pseudotypes: VSV pseudotypes with EBOV GP or MERS-CoV S were prepared with buffers and solutions based on demineralised water (shown under 'Demineralised') and Arium® Pro VF ultrapure water (shown under 'Ultrapure') and used to inoculate Vero E6 cells. The transduction efficiency, i.e., entry efficiency, of the virus pseudotypes was determined by quantifying virally encoded luciferase, and the resulting data were normalised. In addition, the statistical significance of the data was confirmed by a t-test (*: $p < 0.05$).

Discussion

During this study, virus pseudotypes were produced on the basis of a replication-deficient vesicular stomatitis virus (VSV), and the envelope proteins of various highly pathogenic viruses were studied. We showed that host cell entry relied on identical receptor molecules and biochemical processes as described for authentic viruses. Furthermore, we demonstrated that the use of ultrapure water for production of VSV pseudotypes resulted in an optimisation of this process. Future investigations should be designed to clarify what this observation is based upon: Is this due to an increased quantity of VSV pseudotypes produced, more efficient cell entry or to improved stability of the pseudotypes? For instance, we can speculate that the absence of salts, proteinases or lipases when ultrapure water is used increases the stability of VSV pseudotypes.

In conclusion, it can be stated that virus pseudotypes are important tools for investigating host cell entry of highly pathogenic viruses. As these virus pseudotypes do not restrict research on such highly pathogenic viruses to BLS-3 or BLS-4 laboratories, this enables a larger number of scientific facilities to conduct such research.

As a result, the cell entry of emerging viruses can be characterised and suitable detection procedures and antiviral strategies (medications, vaccines) can be developed faster. The use of pseudotypes also reduces the considerable labour intensity involved in high-containment laboratories requiring whole-body protection suits, as well as the considerable costs and the limitations entailed by such lab work (e.g., no access to equipment that is not directly located inside the high-containment laboratory). Furthermore, compared with authentic, highly pathogenic viruses, virus pseudotypes minimise the risk of infection of lab personnel following unintentional exposure, thus representing a significant safety aspect.

Optimisation of the production process, for example, through the use of highly pure reagents, particularly those based on water, can additionally contribute to improving the sensitivity of subsequent test procedures, increasing production quantities and thus further lowering production costs.

Acknowledgements

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