Infection-enhancing lipopeptides do not improve intranasal immunization of cotton rats with a delta-G candidate live-attenuated human respiratory syncytial virus vaccine

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Keywords: lipopeptide, adjuvant, live-attenuated virus vaccine, HRSV, paramyxovirus

Development of live-attenuated human respiratory syncytial virus (HRSV) vaccines has proven to be difficult. Several vaccine candidates were found to be over-attenuated and displayed limited immunogenicity. Recently, we identified three synthetic cationic lipopeptides that enhanced paramyxovirus infections *in vitro*. The infection enhancement proved to be mediated by enhanced virus binding to target cells. We hypothesized that these lipopeptides can be used as adjuvants to promote immune responses induced by live-attenuated paramyxovirus vaccines. This hypothesis was tested in a vaccination and challenge model in cotton rats, using a previously described recombinant live-attenuated candidate HRSV vaccine lacking the gene encoding the G glycoprotein (rHRSVΔG). Surprisingly, intranasal vaccination of cotton rats with rHRSVΔG formulated in infection-enhancing lipopeptides resulted in reduced virus loads in nasopharyngeal lavages, reduced seroconversion levels and reduced protection from wild-type HRSV challenge. In conclusion, we were unable to demonstrate the feasibility of lipopeptides as adjuvants for a candidate live-attenuated HRSV vaccine in the cotton rat model.

Introduction

Human respiratory syncytial virus (HRSV) is a major cause of respiratory tract disease in infants, immunocompromised persons and the elderly.^{1,2} In most cases the virus causes a mild and self-limiting upper respiratory tract infection, but in some cases (usually estimated as 1–2%) it may cause a severe lower respiratory tract infection requiring hospitalization.³ Despite the huge global burden of HRSV disease, no licensed vaccines against HRSV are available. Main challenges for the development of HRSV vaccines are that these should be safe and effective at an early age in the presence of maternal antibodies and an immature immune system.⁴

Several different HRSV vaccination approaches have been evaluated over the last few decades, including live-attenuated, inactivated, subunit and vector vaccines.⁵ Studies with formalin-inactivated HRSV vaccines in the 1960s resulted in vaccine-mediated disease enhancement,⁶ an observation which hinders development of non-replicating HRSV vaccines for administration to immunologically naive infants.⁷ Live-attenuated HRSV

vaccines have not been associated with priming of enhanced disease, and therefore constitute a promising approach for intranasal (*i.n.*) vaccination of young infants. However, the vaccine virus needs to achieve sufficient levels of virus replication in the host to induce protective immune responses, but vaccine virus replication should not result in HRSV disease. Despite many attempts, it has proven difficult to find a proper balance between attenuation and immunogenicity.⁸

Previously, we have identified three synthetic cationic lipopeptides that enhanced paramyxovirus infections *in vitro*.⁹ The synthetic bacterial lipopeptide Pam3-Cys-Ser-Lys4 (Pam3CSK4), the prototype ligand for the heterodimeric Toll-like receptor (TLR) 2/1 complex, enhanced HRSV infection in primary epithelial, myeloid and lymphoid cells. Moreover, two structurally related lipopeptides without TLR-signaling capacity, Pam-Cys-SK4 and PHCSK4,^{10,11} were also found to enhance HRSV infection, suggesting that infection enhancement was independent of TLR activation. This was confirmed by the observation that Pam3-Cys-Ser-Pro4 (Pam3CSP4), a synthetic lipopeptide that does stimulate TLR1/2 responses, did not

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Submitted: 02/25/2013; Revised: 07/19/2013; Accepted: 08/09/2013
<http://dx.doi.org/10.4161/hv.26096>

Figure 1. In vitro lipopeptide-mediated enhancement of rHRSV infection. In vitro infection experiments were performed in human EBV-transformed B-lymphoblastic cells (A), well-differentiated normal human bronchial epithelial cells (B), cotton rat lymph node cells, (C) and cotton rat lung slices (D). HRSV was incubated with different lipopeptides (10 µg/ml) or medium as control for 5 min at 37 °C before the virus suspension was added to the cells. In panels A, C and D data are shown as percentages HRSV-infected cells, as determined by flow cytometry. In panel B data are shown as the numbers of GFP⁺ cells per insert. In panel B photographs of representative inserts are shown for each condition. Formulation with Pam3CSK4 or PHCSK4 resulted in significantly increased rHRSV infection percentages. Bars represent means ± SEM of triplicates (A, C), quadruplicates (B) or sextuplicates (D), * $p < 0.05$ in 1-way ANOVA and posthoc Dunnett's correction for multiple comparisons with medium control.

enhance infection with HRSV, demonstrating the pivotal role of the four cationic lysins in this process. A similar TLR-independent enhancement of in vitro virus infection could be demonstrated for several wild-type HRSV strains, but also for HIV-1, measles virus and human metapneumovirus.⁹ The infection enhancement proved to be mediated by enhanced virus binding to target cells, in which the N-palmitoylated cysteine interacts with the membrane of the virus and/or the host cell and the cationic lysines change the membrane potential.⁹ Previously, other cationic compounds, such as DEAE-dextran, polybrene, RANTES and SEVI, have also been shown to enhance viral infection.^{12–14} We hypothesized that the synthetic cationic lipopeptides could be used as adjuvants to promote immune responses induced by live-attenuated paramyxovirus vaccines.⁹

This hypothesis was recently tested using a canine distemper virus (CDV) vaccination and challenge model in ferrets.¹⁵ Intranasal immunization with a live-attenuated CDV

vaccine¹⁶ formulated in synthetic infection-enhancing cationic lipopeptides indeed resulted in increased vaccine virus loads in nasopharyngeal lavages and peripheral blood mononuclear cells as compared with immunization with non-adjuvanted CDV. In addition, adjuvantation of CDV vaccine with infection-enhancing lipopeptides induced significantly higher CDV neutralizing serum antibody titers as compared with animals vaccinated with non-adjuvanted live-attenuated CDV. Although this study provided proof of principle for in vivo enhancement of replication and immunogenicity of a live-attenuated paramyxovirus vaccine, we sought to reproduce our results in a vaccination and challenge model for HRSV, another member of the *Paramyxoviridae* family.

To this end, we selected a recently described candidate live-attenuated recombinant HRSV vaccine lacking the gene encoding the G glycoprotein.¹⁷ Using reverse genetics, recombinant (r)HRSV-X and an rHRSV lacking the G gene (ΔG) were constructed based on a clinical HRSV isolate (strain

98-25147-X). A single dose immunization with the highly attenuated rHRSVΔG conferred long-term protection in cotton rats, without priming for vaccine-enhanced disease.¹⁷ Moreover, for both viruses molecular clones expressing enhanced green fluorescent protein (EGFP) were also available,¹⁸ facilitating in vitro lipopeptide-mediated infection enhancement studies.

Results

Lipopeptide-mediated enhancement of rHRSV infection in vitro

We have previously shown that the synthetic lipopeptides Pam3CSK4, Pam-Cys-SK4 and PHCSK4 can enhance paramyxovirus infections in vitro. Pam3CSP4, which does not enhance infection in vitro, was used as a negative control.⁹ In order to test if these lipopeptides could also enhance infection of the HRSV strains used in the present study, human Epstein-Barr virus-transformed B-lymphoblastic cell lines (B-LCL) were infected with either rHRSVΔG or HRSV-X in the presence or absence of lipopeptide (10 µg/ml). Pam3CSK4 and PHCSK4 significantly enhanced infection with both viruses as compared with the medium and lipopeptide control (Fig. 1A, $p < 0.05$). To test if the lipopeptides also enhanced infection in natural target cells of HRSV, well-differentiated normal human bronchial epithelial (wd-NHBE) cells were infected and 2 d.p.i. the numbers of infected cells were counted. Again, Pam3CSK4 and PHCSK4 significantly enhanced HRSV infection (Fig. 1B, $p < 0.05$). In order to test whether infection enhancement could also be shown in cotton rat cells, cotton rat lymph node cells or lung slices¹⁹ were infected with HRSV in the presence or absence of lipopeptide. In lymph node cells Pam3CSK4 and PHCSK4 significantly enhanced infection with both viruses as compared with the medium and lipopeptide control (Fig. 1C, $p < 0.05$). In lung slices the intra-assay variation was relatively high, but Pam3CSK4 and PHCSK4 still caused a significant enhancement of HRSV infection (Fig. 1D, $p < 0.05$). Based on these in vitro data, Pam3CSK4 and PHC4SK4 were selected for in vivo testing as potential adjuvants for the rationally attenuated rHRSVΔG.

Vaccination—virus loads and VN titers

Twenty three cotton rats were vaccinated i.n. with a dose of 10^4 TCID₅₀ rHRSVΔG in a volume of 10 µl. In previous animal experiments this dose was found to induce suboptimal immune responses. The first group ($n = 7$) was rHRSVΔG-vaccinated without lipopeptide and served as a control in the challenge experiment. In groups 2 and 3 (both $n = 8$) the virus was mixed with Pam3CSK4 or PHCSK4 (final concentration 30 µg/ml), respectively. To determine the effect of lipopeptides on rHRSVΔG infection in vivo, vaccine virus loads in nasopharyngeal lavages and throat swabs were analyzed by virus isolation. No virus was detected in throat swabs in any of the groups. Low amounts of virus were detected in nasopharyngeal lavages of 1 and 3 animals of the control group at 2 and 4 d post-vaccination (d.p.v.), respectively. However, no virus was detected in the nose of the animals vaccinated with rHRSVΔG formulated in lipopeptide (Fig. 2A).

VN antibody levels were measured 29 and 63 d.p.v. (Fig. 2B). At 29 d.p.v. VN antibodies were detected in 5/7, 3/8 and 3/8

Figure 2. Vaccination period—virus loads and VN antibodies. (A) Vaccine virus loads in nasopharyngeal lavages as measured by virus isolation. Virus loads in the non-adjuvanted control group ($n = 7$) were statistically significantly higher than those of the lipopeptide-adjuvanted groups ($n = 8$) (lines represent geometric mean $\pm$ geometric standard deviation [GSD], * $p < 0.05$). (B) Virus neutralizing (VN) antibody titers were measured at days -4, 29, and 63 d post vaccination. In the control group more animals seroconverted than in the adjuvanted groups (lines represent geometric mean $\pm$ GSD, * $p < 0.05$, area under the curve [AUC]).

animals of groups 1 (non-adjuvanted control), 2 (Pam3CSK4) and 3 (PHCSK4), respectively.

Challenge—virus loads

Animals were challenged i.n. with a high dose of HRSV-X 67 d.p.v. In all animals challenge virus was isolated from nasopharyngeal lavages, throat swabs and lung homogenates 5 d post challenge (Fig. 3). The virus loads in group 1 (non-adjuvanted) tended to be lower than, but were not statistically significant from those in groups 2 (Pam3CSK4-adjuvanted) and 3 (PHCSK4-adjuvanted).

Discussion

In the current study, infection-enhancing lipopeptides⁹ were evaluated in the cotton rat model as potential adjuvants for a live-attenuated recombinant HRSV vaccine lacking the G glycoprotein. This candidate vaccine was previously shown

Figure 3. Challenge period—virus loads. Challenge virus was isolated from all animals in all specimens tested. The virus loads in group 1 (n = 7) were not statistically significantly different from those in groups 2 (n = 8) and 3 (n = 8). Bars represent means $\pm$ SEM, virus loads in lung homogenates are shown per gram lung tissue.

to induce protective immunity in cotton rats.¹⁷ However, in the current study we selected a suboptimal vaccine virus dose, with the aim of demonstrating enhancement of infectivity and immunogenicity following intranasal infection in the presence of infection-enhancing lipopeptides. Unfortunately, vaccination in the presence of infection-enhancing lipopeptides was inferior to vaccination in absence of lipopeptides, as evidenced by reduced virus loads in nasopharyngeal lavages, a tendency of reduced seroconversion rates and reduced protection upon HRSV-X challenge infection.

These observations are in contrast with our previously reported data using a canine distemper virus vaccination and challenge model in ferrets.¹⁵ A number of potential explanations for this observation were evaluated. Firstly, the lipopeptides could have induced a strong innate immune response, thereby accelerating clearance of the vaccine virus. However, there were no differences in intensities of RT-PCR products detecting mRNA encoding innate immunity genes (IFN- α and Mx1) in nasopharyngeal lavages between animals vaccinated with or without lipopeptides (data not shown). Secondly, in comparison to our ferret study the i.n. inoculation volume used in cotton rats was smaller, avoiding direct deposition of vaccine virus in the lungs. As a consequence, it may have been more difficult for the virus to cross the mucociliary barrier in the nose of cotton rats. However, in vitro enhancement of rHRSV Δ G infections of human epithelial or lymphoid cells proved to be unaffected by the presence or absence of mucus-containing washings obtained from wd-NHBE or of the volume of the virus inoculum (data not shown). Thirdly, formulation of the vaccine virus in cationic lipopeptides may have resulted in a positive membrane potential of the vaccine virus particles, resulting in electrostatic interactions with negatively charged cotton rat mucin polymers on the respiratory mucosa.²⁰ As shown in Figure 1, ex vivo HRSV infection of well-differentiated

normal human bronchial epithelial cells (wd-NHBE) with tight junctions, moving cilia and goblet cells was enhanced by these cationic lipopeptides. However, it is important to note that in these primary epithelial cells enhancement was not observed in every experiment: in some experiments the addition of lipopeptides to the virus appeared to have little or no effect on the efficiency of HRSV infection, and in one experiment we actually observed lipopeptide-mediated inhibition of HRSV infection. In this particular experiment the epithelial layer contained a relatively large number of mucus-producing cells. Thus, we hypothesize that under some conditions the cationic lipopeptides can promote HRSV binding to negatively charged mucin polymers, resulting in immobilization of the virus and inhibition of infection. This may also have occurred in our vaccination study.

In conclusion, we were unable to demonstrate the feasibility of infection-enhancing lipopeptides as adjuvants for a candidate live-attenuated HRSV vaccine in the cotton rat model.

Materials and Methods

Ethical statement

The animal protocol was approved by an independent animal experimentation ethics committee. Experiments were performed in compliance with European guidelines for animal experimentation. Animals were monitored daily, and animal care was in compliance with institutional guidelines.

Animals

Four- to eight-week-old cotton rats (*Sigmodon hispidus*) were obtained from an in-house specific pathogen-free breeding colony. All animals were housed individually in filter-top cages supplemented with paper as cage enrichment and received food and water ad libitum. One animal of the control group died three days before vaccination of unknown causes.

Cells, viruses, and lipopeptides

Vero cells (ATCC CCL-81) were cultured in M199 medium (Invitrogen) with 5% FBS and supplements. HEp-2 cells (ATCC CCL-23) were grown in DMEM (Lonza) with 10% FBS and supplements. Epstein-Barr virus (EBV)-transformed human B-lymphoblastic cells (B-LCL) were grown in RPMI-1640 medium (Lonza) with 10% fetal bovine serum (FBS). Well-differentiated human normal bronchial epithelial cells (wd-NHBE) were grown on transwell filters with 0.4 μ m pore size (Corning) as described elsewhere.²¹ Lymph nodes were collected from cotton rats of an unrelated approved study, and stored in phosphate-buffered saline. Single cell suspensions were prepared using cell strainers with a 100 μ m pore size (BD Biosciences), and resuspended in RPMI-1640 medium (Lonza) with 10% fetal bovine serum (FBS) and supplements as described before.¹⁵ Cotton rat lung slices were prepared by inflation with low melting point agarose as described elsewhere.¹⁹

Stocks of the live-attenuated recombinant HRSV lacking the G gene (rHRSV Δ G) and the challenge virus (HRSV-X) were prepared in Vero cells and purified as described previously.¹⁷ Infection studies in wd-NHBE cells were also performed using rgRSV, a recombinant HRSV strain A2 virus expressing GFP²² (kind gift of Dr ME Peebles and Dr PL Collins). Stocks of rgRSV

were prepared in HEp-2 cells. Pam3CSK4, PHCSK4, Pam-Cys-SK4, and Pam3CSP4⁹ were a kind gift of Dr KH Wiesmüller (EMC Microcollections)

In vitro and ex vivo HRSV infections

The lipopeptides were added to the viruses to a final concentration of 10 µg/ml, and the mixtures were incubated for 5 min at 37 °C. Cells were infected at a multiplicity of infection (MOI) 2 (rHRSVΔG) or 20 (HRSV-X and rgRSV). One day (B-LCL, LN cells) or two days (wd-NHBE, lung slices) post infection cells were analyzed by automated counting (wd-NHBE) or flow cytometry.

In vivo HRSV infection experiment

An amount of 23 cotton rats were vaccinated i.n. at day 0 with a suboptimal dose of 10⁴ TCID₅₀ rHRSVΔG in a volume of 10 µl. The first group (n = 7) was rHRSVΔG -vaccinated without lipopeptide and served as a control in the challenge experiment. In groups 2 and 3 (both n = 8) the virus was mixed with Pam3CSK4 or PHCSK4 (final concentration 30 µg/ml), respectively. The mixture was incubated for 5 min at 37 °C.

Animals were challenged i.n. with 100 µl 3 × 10⁵ TCID₅₀ HRSV-X 67 d post vaccination (d.p.v.), and euthanized by exsanguination five days post challenge. All procedures were performed under 4% isoflurane anesthesia.

Samples and assays

Virus isolation from nasopharyngeal lavages and throat swabs was performed as previously described.^{17,15} Right lungs were homogenized with a FastPrep-24[®] (MP Biomedicals) in 600 µl virus transport medium¹⁵ and centrifuged at 640 × g for 15 min. The lower limit of detection of the assay was 40 TCID₅₀/ml. For calculation of group averages, samples with undetectable virus loads were given a value of 40.

Virus neutralizing (VN) antibody levels were determined using a plaque reduction neutralization assay as described.¹⁸ The lower limit of detection of the assay was a serum dilution of 1:10. For calculation of group averages, samples with undetectable antibody levels were given a value of 10.

Statistical analyses were performed as described previously.¹⁵ Statistical analyses were performed with ANOVA with Dunnett's correction of Mann-Whitney U test for AUC where appropriate.^{9,15} A *p*-value of < 0.05 was considered statistically significant. All in vitro and ex vivo experiments were performed at least three times, with the exception of the cotton rat lung slice experiment which was performed once.

Disclosure of Potential Conflicts of Interest

ADMEO wishes to declare, for the avoidance of any misunderstanding on competing interests, that he co-founded and is chief scientific officer of Viroclinics Biosciences, a company set up in collaboration with Erasmus MC. However, for clarification, no materials or support were received from the company, and no agreements were in place concerning the execution or publication of this work.

Acknowledgments

We thank all zoo technicians at the isolator facility of the Netherlands Vaccine Institute in Bilthoven, the Netherlands, for their contributions to this study. This study was supported by the Virgo consortium, funded by the Dutch government project number FES0908. SY was funded by ZonMw (grant# 91208012).

References

- Collins PL. Human respiratory syncytial virus. In: Samal, S. K. ed. *The biology of paramyxoviruses*. Norfolk (UK): Caister Academic Press, 2011:341-410.
- Collins PL, Melero JA. Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years. *Virus Res* 2011; 162:80-99; PMID:21963675; <http://dx.doi.org/10.1016/j.virusres.2011.09.020>
- Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev* 2010; 23:74-98; PMID:20065326; <http://dx.doi.org/10.1128/CMR.00032-09>
- Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. *Immunol Rev* 2011; 239:149-66; PMID:21198670; <http://dx.doi.org/10.1111/j.1600-065X.2010.00972.x>
- Anderson LJ, Dormitzer PR, Nokes DJ, Rappuoli R, Roca A, Graham BS. Strategic priorities for respiratory syncytial virus (RSV) vaccine development. *Vaccine* 2013; 31(Suppl 2):B209-15; PMID:23598484; <http://dx.doi.org/10.1016/j.vaccine.2012.11.106>
- Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol* 1969; 89:422-34; PMID:4305198
- Borchers AT, Chang C, Gershwin ME, Gershwin LJ. Respiratory Syncytial Virus-A Comprehensive Review. *Clin Rev Allergy Immunol* 2013; (Forthcoming); PMID:23575961; <http://dx.doi.org/10.1007/s12016-013-8368-9>
- Power UF. Respiratory syncytial virus (RSV) vaccines—two steps back for one leap forward. *J Clin Virol* 2008; 41:38-44; PMID:18340669; <http://dx.doi.org/10.1016/j.jcv.2007.10.024>
- Nguyen DT, de Witte L, Ludlow M, Yüksel S, Wiesmüller K-H, Geijtenbeek TBH, Osterhaus ADME, de Swart RL. The synthetic bacterial lipopeptide Pam3CSK4 modulates respiratory syncytial virus infection independent of TLR activation. *PLoS Pathog* 2010; 6:e1001049; PMID:20808895; <http://dx.doi.org/10.1371/journal.ppat.1001049>
- Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, Schwandner R, Luppa PB, Ebel F, Busch DH, Bauer S, et al. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. *J Clin Invest* 2004; 113:1473-81; PMID:15146245
- Parcina M, Wendt C, Goetz F, Zawatzky R, Zähringer U, Heeg K, Bekeredjian-Ding I. Staphylococcus aureus-induced plasmacytoid dendritic cell activation is based on an IgG-mediated memory response. *J Immunol* 2008; 181:3823-33; PMID:18768836
- Rossi CR, Kiesel GK. Bovine respiratory syncytial virus infection of bovine embryonic lung cultures: enhancement of infectivity with diethylaminoethyl-dextran and virus-infected cells. *Arch Virol* 1978; 56:227-36; PMID:565198; <http://dx.doi.org/10.1007/BF01317851>
- Münch J, Rücker E, Ständker L, Adermann K, Goffinet C, Schindler M, Wildum S, Chinnadurai R, Rajan D, Specht A, et al. Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* 2007; 131:1059-71; PMID:18083097; <http://dx.doi.org/10.1016/j.cell.2007.10.014>
- Roan NR, Münch J, Arhel N, Mothes W, Neidleman J, Kobayashi A, Smith-McCune K, Kirchhoff F, Greene WC. The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. *J Virol* 2009; 83:73-80; PMID:18945786; <http://dx.doi.org/10.1128/JVI.01366-08>
- Nguyen DT, Ludlow M, van Amerongen G, de Vries RD, Yüksel S, Verburgh RJ, Osterhaus ADME, Duprex WP, de Swart RL. Evaluation of synthetic infection-enhancing lipopeptides as adjuvants for a live-attenuated canine distemper virus vaccine administered intra-nasally to ferrets. *Vaccine* 2012; 30:5073-80; PMID:22705079; <http://dx.doi.org/10.1016/j.vaccine.2012.05.079>
- Silin D, Lyubomska O, Ludlow M, Duprex WP, Rima BK. Development of a challenge-protective vaccine concept by modification of the viral RNA-dependent RNA polymerase of canine distemper virus. *J Virol* 2007; 81:13649-58; PMID:17898047; <http://dx.doi.org/10.1128/JVI.01385-07>
- Widjojoatmodjo MN, Boes J, van Bers M, van Remmerden Y, Roholl PJM, Luytjes W. A highly attenuated recombinant human respiratory syncytial virus lacking the G protein induces long-lasting protection in cotton rats. *Virol J* 2010; 7:114; PMID:20525213; <http://dx.doi.org/10.1186/1743-422X-7-114>
- van Remmerden Y, Xu F, van Eldik M, Heldens JGM, Huisman W, Widjojoatmodjo MN. An improved respiratory syncytial virus neutralization assay based on the detection of green fluorescent protein expression and automated plaque counting. *Virol J* 2012; 9:253; PMID:23114196; <http://dx.doi.org/10.1186/1743-422X-9-253>

19. Nguyen DT, de Vries RD, Ludlow M, van den Hoogen BG, Lemon K, van Amerongen G, Osterhaus AD, de Swart RL, Duprex WP. Paramyxovirus infections in ex vivo lung slice cultures of different host species. *J Virol Methods* 2013; 193:159-65; PMID:23777750; <http://dx.doi.org/10.1016/j.jviromet.2013.06.016>
20. Lieleg O, Vladescu I, Ribbeck K. Characterization of particle translocation through mucin hydrogels. *Biophys J* 2010; 98:1782-9; PMID:20441741; <http://dx.doi.org/10.1016/j.bpj.2010.01.012>
21. Verkaik NJ, Nguyen DT, de Vogel CP, Moll HA, Verbrugh HA, Jaddoe VWV, Hofman A, van Wamel WJB, van den Hoogen BG, Buijs-Offerman RMGB, et al. *Streptococcus pneumoniae* exposure is associated with human metapneumovirus seroconversion and increased susceptibility to *in vitro* HMPV infection. *Clin Microbiol Infect* 2011; 17:1840-4; PMID:21883660; <http://dx.doi.org/10.1111/j.1469-0691.2011.03480.x>
22. Hallak LK, Spillmann D, Collins PL, Peebles ME. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. *J Virol* 2000; 74:10508-13; PMID:11044095; <http://dx.doi.org/10.1128/JVI.74.22.10508-10513.2000>