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Quantifying the effect of trypsin and elastase on in vitro SARS-CoV infections

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ABSTRACT

The SARS coronavirus (SARS-CoV) has the potential to cause serious disease that can spread rapidly around the world. Much of our understanding of SARS-CoV pathogenesis comes from in vitro experiments. Unfortunately, in vitro experiments cannot replicate all the complexity of the in vivo infection. For example, proteases in the respiratory tract cleave the SARS-CoV surface protein to facilitate viral entry, but these proteases are not present in vitro. Unfortunately, proteases might also have an effect on other parts of the replication cycle. Here, we use mathematical modeling to estimate parameters characterizing viral replication for SARS-CoV in the presence of trypsin or elastase, and in the absence of either. In addition to increasing the infection rate, the addition of trypsin and elastase causes lengthening of the eclipse phase duration and the infectious cell lifespan.

1. Introduction

Severe acute respiratory syndrome (SARS) is a viral respiratory disease similar to pneumonia that was identified in 2002 in China (Drosten et al., 2003). A causative coronavirus, SARS-CoV, can transmit the infection from animals to humans (Drosten et al., 2003; Ksiazek et al., 2003). The 2002 outbreak originated in bats (Hu et al., 2017; Li et al., 2005), recombined in Himalayan palm civets and raccoon dogs, and jumped to humans in an animal market (Rest and Mindell, 2003; Guan et al., 2003; Ksiazek et al., 2003). While the SARS-CoV outbreak was quickly controlled, zoonotic transmission is a severe threat to public health, as evidenced by the recent emergence of SARS-CoV-2 (Chen et al., 2020; Wu et al., 2020), so it is important to understand properties of emerging viruses.

One technique for studying viral replication is the use of in vitro experiments. Unfortunately, in vitro experiments cannot completely replicate the in vivo environment (Mizutani, 2010; Sims et al., 2008). Specifically, not all cell lines used to study respiratory viruses have the proteins necessary to support viral replication (Simmons et al., 2011). In these cases, the missing components are added to the medium to facilitate viral infection (Bertram et al., 2011).

SARS-CoV enters healthy cells by inducing fusion between viral and cellular membranes (Simmons et al., 2004, 2013). Virus-cell fusion is mediated through the S protein that needs to be activated by a host cell protease (Heurich et al., 2014). The localization of this protease determines in which cellular compartment membrane fusion occurs (Kawase et al., 2009; Simmons et al., 2013; Watanabe et al., 2008). The S protein is cleaved into a heterodimer consisting of an extracellular receptor binding subunit, S1, and a membrane-anchored subunit, S2, responsible for mediating membrane fusion (Simmons et al., 2004). Binding of S1 to its cellular receptor induces conformational changes in the S1/S2 complex, allowing viral entry (Song et al., 2018). Thus host cells proteases are indispensable for SARS infectivity (Simmons et al., 2004).

During in vivo infections, the host provides the proteases necessary for cleavage of the S protein (Bertram et al., 2011, 2012; Kam et al., 2009; Zmora et al., 2014). However, during in vitro infections, these proteases need to be added to help the infection proceed. Several possible proteases have been identified (Bertram et al., 2011; Belouzard et al., 2009, 2010; Zmora et al., 2014), but some are more commonly used than others. Trypsin is a prototype serine endopeptidase that has been studied in the context of virus glycoprotein cleavage-activation and has been shown to cleave the SARS-CoV S protein (Kirchdoerfer et al., 2018). Elastase is a protease used to enhance SARS in vitro infections, activating fusion and entry in VeroE6 cells (Matsuyama et al., 2005). Like trypsin, elastase can shift SARS-CoV entry to a low pH-independent route by cleaving the S protein (Belouzard et al., 2010; Matsuyama et al., 2005). Elastase-induced infection is less efficient when compared to trypsin-induced infection (Belouzard et al., 2009, 2010) because elastase has a much smaller S1 cleft than trypsin.

While trypsin and elastase are used in vitro to facilitate viral entry into the cell, it is not clear that their effect is limited to that part of the viral replication cycle. Some of the effects of trypsin and elastase in SARS infections are known; others need to be explored. For example, both elastase and trypsin have been shown to enhance replication of SARS-CoV (Matsuyama et al., 2005). Additionally, activation of the S protein allows not just virus-cell fusion, but cell-cell fusion (Simmons et al., 2011) that can alter the transmission dynamics of SARS-CoV. Cell-cell fusion has been shown to increase in the presence of trypsin (Simmons et al., 2004; Howard et al., 2008). Elastase is produced in the lungs during the inflammatory response by neutrophils (Kawabata et al., 2002) and is known to drive severe pneumonia and lung injury in SARS-CoV infected patients (Ami et al., 2008; Kawabata et al., 2002). It is important to understand how these secondary effects of trypsin and elastase alter the time course of the infection in order to develop a complete understanding of SARS-CoV infection.

In this paper, we analyze and quantify the dynamical changes caused by the addition of trypsin and elastase to SARS infection experiments. We parameterize a within-host mathematical model of respiratory viral infection using experimental data of SARS-CoV growth in VeroE6 cells in the presence and absence of trypsin and elastase in the medium. We find that the addition of trypsin changes all parameter values from untreated infections while the effect of elastase is more limited, significantly changing only the infection rate, duration of the eclipse phase and infectious cell lifespan.

2. Materials and methods

2.1. Mathematical model

We use a within host model of viral infection with the method of stages to generate gamma distributions for the cell transitions from eclipse to infectious and infectious to dead. This model has previously been used to analyse other respiratory infections such as influenza (Pinilla et al., 2012; Paradis et al., 2015; Beggs and Dobrovolny, 2015; González-Parra et al., 2018), and RSV (González-Parra et al., 2018; González-Parra and Dobrovolny, 2018). The system of differential equations is

$$\dot{T} = -\beta TV \tag{1}$$

$$\dot{E}_1 = \beta TV - \frac{n_E}{\tau_E}E_1 \tag{2}$$

$$\dot{E}_j = \frac{n_E}{\tau_E}E_{j-1} - \frac{n_E}{\tau_E}E_j \quad \text{for } j = 2, \dots, n_E \tag{3}$$

$$\dot{I}_1 = \frac{n_E}{\tau_E}E_{n_E} - \frac{n_I}{\tau_I}I_1 \tag{4}$$

$$\dot{I}_j = \frac{n_I}{\tau_I}I_{j-1} - \frac{n_I}{\tau_I}I_j \quad \text{for } j = 2, \dots, n_I \tag{5}$$

$$\dot{V} = p\sum_{j=1}^{n_I} I_j - cV, \tag{6}$$

where the target cells T are infected by the virus V at an infection rate β. Then the infected cells enter an eclipse phase E and pass through all the compartments E_j with mean eclipse duration τ_E before becoming infectious. Next, cells in the infectious compartments, I_j, produce new virus particles at a rate p and at the same time the virus loses infectivity at a rate c. Infectious cells die after a mean time of τ_I. The model is depicted in Fig. 1. In addition to the model parameters, we also calculate the infecting time $t_{inf} = \sqrt{2/(p\beta)}$ that represents the average time between virus leaving a cell and entering the next cell (González-Parra et al., 2018; Holder and Beauchemin, 2011).

2.2. Fitting experimental data

We used experimental data of SARS in vitro infections in the presence and absence of the proteases elastase and trypsin. We extracted the data from Matsuyama et al. (Matsuyama et al., 2005) using WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/). In this experiment, VeroE6 cells were infected with 10 pfu of SARS-CoV Frankfurt 1 strain (multiplicity of infection (MOI) of 0.0001). After 30 min adsorption, cells were cultured in the presence of low-concentration proteases: 62.5 µg/mL trypsin or 125 µg/mL elastase, or without proteases for 20 h. Virus in the supernatant is measured via RT-PCR and calibrated to pfu.

We fit the mathematical model of viral infection (described by Eq. (1)) to experimental data of the viral infections in the presence and absence of the proteases separately to the infection model using least-squares minimization. We assumed that infections in the presence of different proteases were described by different infection parameters (β, p, τ_E, τ_I, n_E and n_I). Since the data does not include the viral decay phase, we set the viral clearance rate to c=0.9/d found for in vitro infections of SARS HKU39849 (Rowell and Dobrovolny, 2020). We set the initial conditions as the target cells $T(0) = 1$, and cells in the eclipse and infectious compartment (E_j, I_j) equal to 0, so all the cells were uninfected when the virus was added. We used the L-BFGS-B method from the Scipy library of Python to calculate the minimum sum of squared residuals (SSR).

2.3. Bootstrapping and statistical analysis

We produced distributions for each parameter in the presence and absence of trypsin and elastase using bootstrapping. Bootstrapping is a general method of statistical inference based on building a sampling distribution for a model by resampling from the data at hand (Efron and
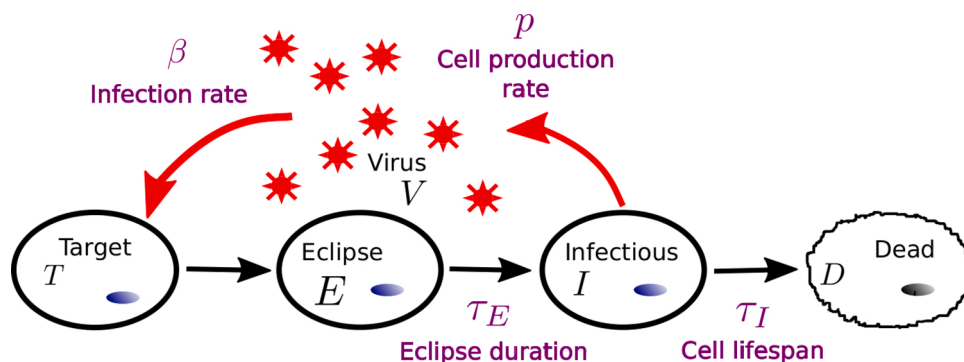

Fig. 1. Schematic of the mathematical model. Target cells are infected by virus at rate β. They remain in the eclipse phase for a time τ_E and actively release virus at rate p for a time τ_I.

Tibshirani, 1993; Davison and Hinkley, 1997). We generate 1000 surrogate data sets for the elastase, the trypsin, and untreated data, and then fit each surrogate data set as described above to generate a distribution for each parameter.

Statistical comparisons are performed using the Mann-Whitney (Wilcoxon rank-sum) test, which is used to compare two distributions without assuming normality. To avoid over-powered analysis, we use 100 random samples of 10 estimates from each distribution to perform the Mann-Whitney test and report the mean *p* value. A mean *p* value of less than 0.01 is considered statistically significant.

3. Results

3.1. Model fits to data

We fit a mathematical model of viral infection (described by Eq. (1)) to experimental data of SARS infection in the presence of trypsin, or elastase, or in the absence of both. Fitting of the model to data allows estimation of a number of parameters characterizing different phases of the viral replication cycle such as duration of the eclipse phase (τ_E), infectious cell lifespan (τ_I), infection rate (β), production rate (p), and infecting time (t_{inf}). Model fits to experimental data are shown in Fig. 2 and best fit parameters are given in Table 1. Parameter correlation plots are included in the supplemental material.

The addition of proteases leads to a higher infection rate with the addition of trypsin leading to a 40-fold increase in the infection rate from the untreated infection and the addition of elastase leading to a 30-fold increase from the untreated infection. Increase in the infection rate is offset slightly by a reduced viral production rate when proteases are added. The addition of elastase reduces production by about half and the addition of trypsin reduces production to about one third of the untreated value. The eclipse phase duration (τ_E) is markedly higher in the presence of proteases; 7.83 h in the presence of trypsin and 20.3 h in the presence of elastase, compared to 1.31 h for the untreated infection. The infectious lifespan (τ_I) is also longer in the presence of proteases; 79.2 h in the presence of trypsin and 67.9 h in the presence of elastase, compared to 1.21 h for the untreated infection. Finally, the infecting time is lower in the presence of proteases with both trypsin and elastase estimated to reduce infecting time to about half an hour compared to almost two hours in the untreated infection.

3.2. Statistical analysis

While parameter estimates suggest that there are differences in dynamics between protease-mediated infections and untreated infections, we need to assess whether these differences are statistically significant. Fig. 3 shows the bootstrapped parameter estimates for untreated infections, trypsin-treated infections, and elastase-treated infections. For some parameters, like infectious cell lifespan and infection rate, there is a clear separation between parameter distributions for untreated and protease-treated infections. To check for statistical significance in differences between parameter distributions, we used the Mann–Whitney *U*-test; *p*-values from the test are given in Table 2. All parameter values

differ significantly for trypsin and untreated data. The addition of elastase does not significantly alter the production rate or the infecting time, although it does significantly change the infection rate, eclipse duration, and infectious cell lifespan. For all parameters, the addition of trypsin or elastase results in statistically indistinguishable parameter values.

4. Discussion

Since trypsin and elastase are added to SARS-CoV in vitro infections to facilitate viral entry, we expected to observe changes in the infection rate and both proteases substantially increased the infection rate (43-fold for trypsin and 30-fold for elastase). SARS-CoV can enter cells via an endosomal pathway that does not require extracellular proteases (Kawase et al., 2009; Bertram et al., 2013), so this is the likely entry pathway during untreated infections. Our results suggest that both elastase and trypsin activate the cell membrane entry pathway allowing for easier and quicker entry into the cell. While cell entry is more efficient in protease-mediated infections, we observe compensating delays in other parts of the replication cycle. The addition of trypsin and elastase both lead to longer eclipse phase durations and infectious lifespans, resulting in viral titer time courses that do not appear drastically different from protease-free infections.

More generally, our findings indicate that proteases affect more of the viral replication cycle than just cell entry and that different proteases do not affect the replication cycle in the same manner. This is important for correct interpretation and extrapolation of in vitro experiments since proteases used in vitro are not necessarily the proteases that mediate the infection in vivo. Several proteases have been identified as possible mediators of SARS-CoV infection in vivo including MSPL (Zmora et al., 2014), DESC1 (Zmora et al., 2014), cathepsin L (Huang et al., 2006; Simmons et al., 2005), HAT (Bertram et al., 2011), and TMPRSS2 (Glowacka et al., 2011; Matsuyama et al., 2010; Shulla et al., 2011). It will be crucial to determine whether these proteases also affect more than just cell entry to get a thorough understanding of SARS-CoV infection in vivo. This is particularly important since researchers have considered using fusion protease inhibitors as possible antivirals for SARS-CoV (Shen et al., 2017; Zhou et al., 2015; Laporte and Naesens, 2017), so understanding fully the role of proteases in the infection can help prevent unintended side effects.

Additionally, some proteases can alter the kinetics of the cells being used in the experiment. For example, trypsin is known to alter Vero cell growth kinetics (Rourou et al., 2013) as do a number of other proteases (Rourou et al., 2009). This could alter the number of target cells available for infection. Trypsin is also know to interact with components of the innate immune response (Seitz et al., 2011; Indalao et al., 2017; Kido, 2015) generated by infected cells, also potentially contributing to the observed changes in the viral replication cycle.

Other viruses are also known to use proteases to facilitate viral entry in vitro. Trypsin in particular has been used in vitro for several viruses including influenza virus (Klenk et al., 1875), pseudorabies virus (Riteaum et al., 2006), rotavirus (Benureau et al., 2005; Arias et al., 1996), reovirus (Yeung et al., 1989), and the coronavirus responsible for
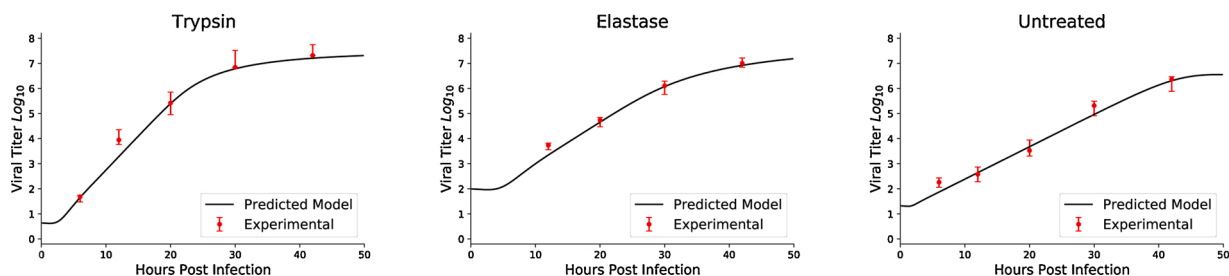

Fig. 2. Fits for SARS-CoV data in the presence of trypsin (left) or elastase (center), and in the absence of either (right).

Table 1

Best fit parameter values, SSRs, and 95% confidence intervals (CI) for SARS in vitro infections with trypsin, with elastase, or without protease.

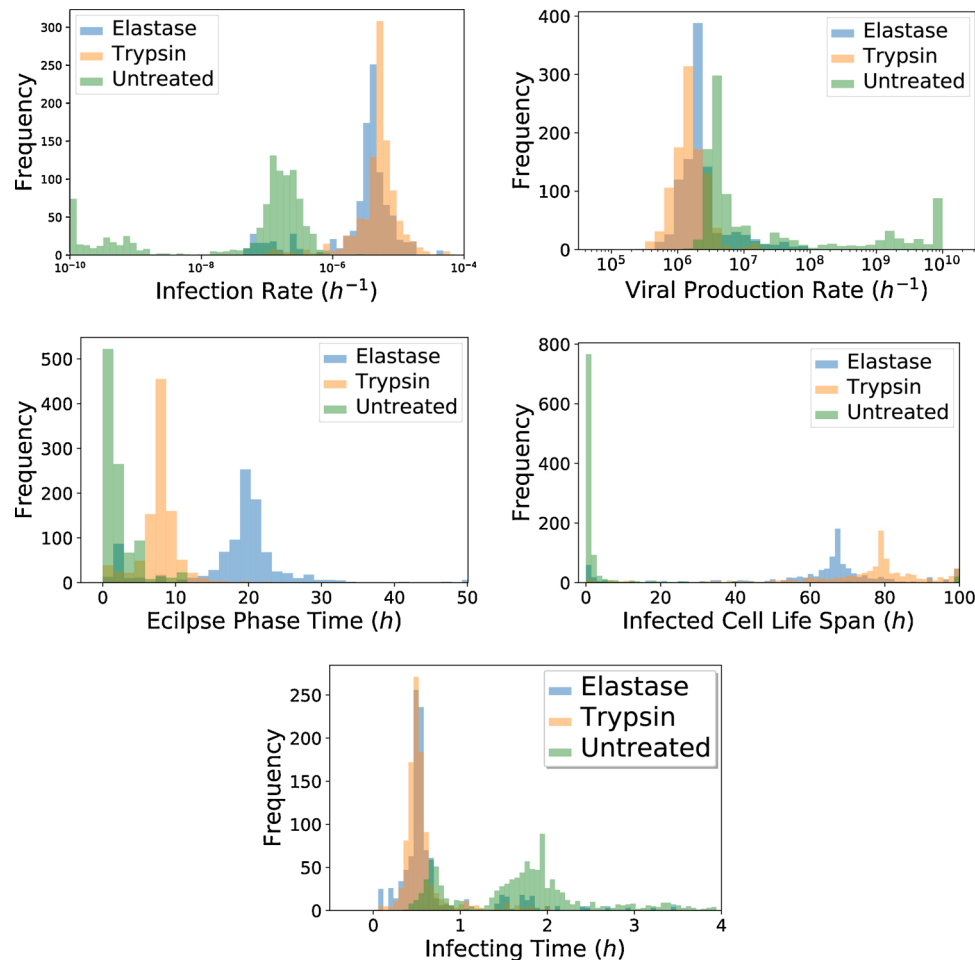| Parameter	Trypsin	95% CI	Untreated	95% CI	Elastase	95% CI
β (h^{-1})	5.46×10^{-6}	$(0.361–19.1)\times10^{-6}$	1.26×10^{-7}	$(0.00100–4.88)\times10^{-7}$	3.77×10^{-6}	$(0.0640–13.9)\times10^{-6}$
p (h^{-1})	1.38×10^{6}	$(0.541–4.43)\times10^{6}$	4.13×10^{6}	$(2.44–1000)\times10^{6}$	1.97×10^{6}	$(0.698–33.3)\times10^{6}$
τ_E (h)	7.83	1.22–13.2	1.31	0.537–9.32	20.3	1.60–71.0
τ_I (h)	79.2	6.64–100	1.21	0.181–70.3	67.9	0.935–100
t_{inf} (h)	0.515	0.289–1.53	1.96	0.535–3.59	0.520	0.174–2.43
SSR	0.266	0.0268–0.705	0.238	0.0238–0.247	0.0623	0.0264–0.296

Fig. 3. Parameter distributions for SARS-CoV infecting VeroE6 cells in the presence of elastase, trypsin or absence of both. Parameters are: infection rate (top left), viral production rate (top right), eclipse phase duration (center left), infectious cell life span (center right), and infecting time (bottom).

Table 2

Mann–Whitney p values for comparing parameter distributions. Numbers in bold indicate statistically significant differences.

Parameter	Trypsin/Untreated	Elastase/Untreated	Trypsin/Elastase
β	**3.27** $\times10^{-4}$	**6.16** $\times10^{-3}$	0.217
p	**1.64** $\times10^{-3}$	0.0130	0.351
τ_E	**7.44** $\times10^{-3}$	**1.65** $\times10^{-3}$	0.0340
τ_I	**2.44** $\times10^{-3}$	**2.50** $\times10^{-3}$	0.209
t_{inf}	**3.58** $\times10^{-3}$	0.0142	0.453

Middle East respiratory syndrome (MERS) (Qian et al., 2013a). A recent study even suggests that the barrier for some coronaviruses in jumping species is the availability of proteases to cleave surface proteins (Menachery et al., 2020). In all cases, trypsin helps with fusion of the virus with the cell membrane, so there should be some similarities in the effect on at least the entry phase of the replication cycle. Understanding the

similarities and differences between trypsin's (or other proteases') effect on different viruses can help expedite experiments when a new virus, such as MERS-CoV or SARS-CoV-2, develops.

The limitations of this study are driven by the data. Given the small number of data points, not all the parameters are identifiable (Miao et al., 2011). More experiments, with data taken over a longer time period including more of the decay phase, will help ensure parameter identifiability and can help reduce spread of the distributions, perhaps allowing identification of other protease-induced parameter changes. The model also leaves out some details of the infection. For example, the cell–cell fusion often induced by the presence of proteases (Zmora et al., 2014; Bertram et al., 2011; Simmons et al., 2011) is not included in the model. While immune response is limited in vitro, some elements of the innate response are present (Fehr et al., 2016; Qian et al., 2013b), but are also not explicitly included in the model. While not explicitly included, these effects are captured implicitly in changes in parameter values, as exemplified by the increased clearance rate of

elastase-induced infections that can be attributed to an elastase-enhanced immune response.

The use of in vitro experiments is necessary for the development of antivirals and vaccines; proteases are used to facilitate viral infections in cell lines that lack the necessary proteins. Understanding the effect of proteases on the phases of the replication cycle is crucial for development of these therapies. Our study showed that trypsin-induced infections differed from elastase-induced infections not just in the infection rate, but also in the infecting time and viral clearance rate.

Credit statement

Conceptualization, TR; methodology, TR; software, TR; validation, TR and HMD; formal analysis, TR; writing – original draft preparation, TR; writing – review and editing, TR and HMD; supervision, HMD; project administration, HMD. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.virusres.2021.198423.

References

Ami, Y., Nagata, N., Shirato, K., Watanabe, R., Iwata, N., Nakagaki, K., Fukushi, S., Saijo, M., Morikawa, S., Taguchi, F., 2008. Co-infection of respiratory bacterium with severe acute respiratory syndrome coronavirus induces an exacerbated pneumonia in mice. Microbiol. Immunol. 52 (2), 118–127. https://doi.org/10.1111/j.1348-0421.2008.00011.x.

Arias, C., Romero, P., Alvarez, V., Lopez, S., 1996. Trypsin activation pathway of rotavirus infectivity. J. Virol. 70 (9), 5832–5839.

Beggs, N.F., Dobrovolny, H.M., 2015. Determining drug efficacy parameters for mathematical models of influenza. J. Biol. Dyn. 9 (S1), 332–346. https://doi.org/10.1080/17513758.2015.1052764.

Belouzard, S., Chu, V.C., Whittaker, G.R., 2009. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. Proc. Natl. Acad. Sci. USA 106 (14), 5871–5876.

Belouzard, S., Madu, I., Whittaker, G.R., 2010. Elastase-mediated activation of the severe acute respiratory syndrome coronavirus spike protein at discrete sites within the S2 domain. J. Biol. Chem. 285 (30), 22758–22763.

Benureau, Y., Huet, J., Charpilienne, A., Poncet, D., Cohen, J., 2005. Trypsin is associated with the rotavirus capsid and is activated by solubilization of outer capsid proteins. J. Gen. Virol. 86, 3143–3151. https://doi.org/10.1099/vir.081045-0.

Bertram, S., Glowacka, I., Müller, M.A., Lavender, H., Gnirss, K., Nehlmeier, I., Niemeyer, D., He, Y., Simmons, G., Drosten, C., et al., 2011. Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. J. Virol. 85 (24), 13363–13372.

Bertram, S., Heurich, A., Lavender, H., Gierer, S., Danisch, S., Perin, P., Lucas, J.M., Nelson, P.S., Pohlmann, S., Soilleux, E.J., 2012. Influenza and SARS-Coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. PLoS ONE 7 (4), e35876. https://doi.org/10.1371/journal.pone.0035876.

Bertram, S., Dijkman, R., Habjan, M., Heurich, A., Gierer, S., Glowacka, I., Welsch, K., Winkler, M., Schneider, H., Hofmann-Winkler, H., Thiel, V., Pohlmann, S., 2013. TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. J. Virol. 87 (11), 6150–6160. https://doi.org/10.1128/JVI.03372-12.

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., Xia, J., Yu, T., Zhang, X., Zhang, L., 2020. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet 395 (10223), 507–513. https://doi.org/10.1016/S0140-6736(20)30211-7.

Davison, A.C., Hinkley, D.V., 1997. Bootstrap Methods and Their Application. Cambridge University Press, Cambridge; New York, NY, USA reprinted with corrections 1998, 1999, 2000, 2003.

Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R., Berger, A., Burguiere, A., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J., Muller, S., Rickerts, V., Sturmer, M., Vieth, S., Klenk, H., Osterhaus, A., Schmitz, H., Doerr, H., 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. 348 (20), 1967–1976. https://doi.org/10.1056/NEJMoa030747.

Efron, B., Tibshirani, R., 1993. An Introduction to the Bootstrap. Chapman and Hall, New York includes indexes.

Fehr, A.R., Channappanavar, R., Jankevicius, G., Fett, G., Zhao, J., Athmer, J., Meyerholz, D.K., Ahel, I., Perlman, S., 2016. The conserved coronavirus macrodomain promotes virulence and suppresses the innate immune response during severe acute respiratory syndrome coronavirus infection. MBIO 7 (6), e01721. https://doi.org/10.1128/mBio.01721-16.

Glowacka, I., Bertram, S., Müller, M.A., Allen, P., Soilleux, E., Pfefferle, S., Steffen, I., Tsegaye, T.S., He, Y., Gnirss, K., et al., 2011. Evidence that TMPRSS2 activates the severe acute respiratory syndrome coronavirus spike protein for membrane fusion and reduces viral control by the humoral immune response. J. Virol. 85 (9), 4122–4134.

González-Parra, G., Dobrovolny, H.M., 2018. A quantitative assessment of dynamical differences of RSV infections in vitro and in vivo. Virol 523. https://doi.org/10.1016/j.virol.2018.07.027.

González-Parra, G., De Ridder, F., Huntjens, D., Roymans, D., Ispas, G., Dobrovolny, H.M., 2018. A comparison of RSV and influenza in vitro kinetic parameters reveals differences in infecting time. PLOS ONE 13 (2), e0192645. https://doi.org/10.1371/journal.pone.0192645.

Guan, Y., Zheng, B., He, Y., Liu, X., Zhuang, Z., Cheung, C., Luo, S., Li, P., Zhang, L., Guan, Y., et al., 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. Science 302 (5643), 276–278.

Heurich, A., Hofmann-Winkler, H., Gierer, S., Liepold, T., Jahn, O., Pohlmann, S., 2014. TMPRSS2 and ADAM17 cleave ACE2 differentially and only proteolysis by TMPRSS2 augments entry driven by the severe acute respiratory syndrome coronavirus spike protein. J. Virol. 28 (2), 1293–1307. https://doi.org/10.1128/JVI.02202-13.

Holder, B.P., Beauchemin, C.A., 2011. Exploring the effect of biological delays in kinetic models of influenza within a host or cell culture. BMC Public Health 11 (S1), S10. https://doi.org/10.1186/1471-2458-11-S1-S10.

Howard, M.W., Travanty, E.A., Jeffers, S.A., Smith, M., Wennier, S.T., Thackray, L.B., Holmes, K.V., 2008. Aromatic amino acids in the juxtamembrane domain of severe acute respiratory syndrome coronavirus spike glycoprotein are important for receptor-dependent virus entry and cell-cell fusion. J. Virol. 82 (6), 2883–2894. https://doi.org/10.1128/JVI.01805-07.

Hu, B., Zeng, L.-P., Yang, X.-L., Ge, X.-Y., Zhang, W., Li, B., Xie, J.-Z., Shen, X.-R., Zhang, Y.-Z., Wang, N., et al., 2017. Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. PLoS Pathogens 13 (11), e1006698.

Huang, I., Bosch, B., Li, F., Li, W., Lee, K., Ghiran, S., Vasilieva, N., Dermody, T., Harrison, P., Dormitzer, P., Farzan, M., Rottier, P., Choe, H., 2006. SARS coronavirus, but not human coronavirus NL63, utilizes cathepsin L to infect ACE2-expressing cells. J. Biol. Chem. 281 (6), 3198–3203. https://doi.org/10.1074/jbc.M508381200.

Indalao, I., Sawabuchi, T., Takahashi, E., Kido, H., 2017. IL-1 beta is a key cytokine that induces trypsin upregulation in the influenza virus-cytokine-trypsin cycle. Arch. Virol. 162 (1), 201–211. https://doi.org/10.1007/s00705-016-3093-3.

Kam, Y.-W., Okumura, Y., Kido, H., Ng, L.F., Bruzzone, R., Altmeyer, R., 2009. Cleavage of the SARS coronavirus spike glycoprotein by airway proteases enhances virus entry into human bronchial epithelial cells in vitro. PLoS ONE 4 (11), e7870. https://doi.org/10.1371/journal.pone.0007870.

Kawabata, K., Hagio, T., Matsuoka, S., 2002. The role of neutrophil elastase in acute lung injury. Eur. J. Pharmacol. 451 (1), 1–10. https://doi.org/10.1016/S0014-2999(02)02182-9.

Kawase, M., Shirato, K., Matsuyama, S., Taguchi, F., 2009. Protease-mediated entry via the endosome of human coronavirus 229E. J. Virol. 83 (2), 712–721. https://doi.org/10.1128/JVI.01933-08.

Kido, H., 2015. Influenza virus pathogenicity regulated by host cellular proteases, cytokines and metabolites, and its therapeutic options. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 91 (8), 351–368. https://doi.org/10.2183/pjab.91.351.

Kirchdoerfer, R.N., Wang, N., Pallesen, J., Wrapp, D., Turner, H.L., Cottrell, C.A., Corbett, K.S., Graham, B.S., McLellan, J.S., Ward, A.B., 2018. Stabilized coronavirus spikes are resistant to conformational changes induced by receptor recognition or proteolysis. Sci. Rep. 8, 17823. https://doi.org/10.1038/s41598-018-36918-8.

Klenk, H., Rott, R., Orlich, M., Blodorn, J., 1875. Activation of influenza-A viruses by trypsin treatment. Virology 68 (2), 426–439. https://doi.org/10.1016/0042-6822(75)90284-6.

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., et al., 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348 (20), 1953–1966.

Laporte, M., Naesens, L., 2017. Airway proteases: an emerging drug target for influenza and other respiratory virus infections. Curr. Opin. Virol. 24, 16–24. https://doi.org/10.1016/j.coviro.2017.03.018.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J.H., Wang, H., Crameri, G., Hu, Z., Zhang, H., et al., 2005. Bats are natural reservoirs of SARS-like coronaviruses. Science 310 (5748), 676–679.

Matsuyama, S., Ujike, M., Morikawa, S., Tashiro, M., Taguchi, F., 2005. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. Proc. Natl. Acad. Sci. USA 102 (35), 12543–12547.

Matsuyama, S., Nagata, N., Shirato, K., Kawase, M., Takeda, M., Taguchi, F., 2010. Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. J. Virol. 84 (24), 12658–12664. https://doi.org/10.1128/JVI.01542-10.

Menachery, V.D., Dinnon, K.H., Yount, B.L., McAnarney, E.T., Gralinski, L.E., Hale, A., Graham, R.L., Scobey, T., Anthony, S.J., Wang, L., Graham, B., Randell, S.H., Lipkin, W.I., Baric, R.S., 2020. Trypsin treatment unlocks barrier for zoonotic bat

coronavirus infection. J. Virol. 94 (5), e01774–e1819. https://doi.org/10.1128/JVI.01774-19.

Miao, H., Xia, X., Perelson, A.S., Wu, H., 2011. On identifiability of nonlinear ODE models and applications in viral dynamics. SIAM Rev. 53 (1), 3–39. https://doi.org/10.1137/090757009.

Mizutani, T., 2010. Signaling pathways of SARS-CoV in vitro and in vivo. In: Lal, S. (Ed.), Molecular Biology of the SARS Coronavirus. Springer, pp. 305–322. https://doi.org/10.1007/978-3-642-03683-5_19.

Paradis, E.G., Pinilla, L.T., Holder, B.P., Abed, Y., Boivin, G., Beauchemin, C.A.A., 2015. Impact of the H275Y and I223V mutations in the neuraminidase of the 2009 pandemic influenza virus in vitro and evaluating experimental reproducibility. PLOS ONE 10 (5), e0126115. https://doi.org/10.1371/journal.pone.0126115.

Pinilla, L.T., Holder, B.P., Abed, Y., Boivin, G., Beauchemin, C.A.A., 2012. The H275Y neuraminidase mutation of the pandemic A/H1N1 influenza virus lengthens the eclipse phase and reduces viral output of infected cells, potentially compromising fitness in ferrets. J. Virol. 86 (19), 10651–10660. https://doi.org/10.1128/?JVI.07244-11.

Qian, Z., Dominguez, S.R., Holmes, K.V., 2013a. Role of the spike glycoprotein of human middle east respiratory syndrome coronavirus (MERS-CoV) in virus entry and syncytia formation. PLOS ONE 8 (10), e76469. https://doi.org/10.1371/journal.pone.0076469.

Qian, Z., Travanty, E.A., Oko, L., Edeen, K., Berglund, A., Wang, J., Ito, Y., Holmes, K.V., Mason, R.J., 2013b. Innate immune response of human alveolar type II cells infected with severe acute respiratory syndrome-coronavirus. Am. J. Respir. Cell Mol. Biol. 48 (6), 742–748. https://doi.org/10.1165/rcmb.2012-0339OC.

Rest, J.S., Mindell, D.P., 2003. SARS associated coronavirus has a recombinant polymerase and coronaviruses have a history of host-shifting, Infection. Genet. Evol. 3 (3), 219–225.

Riteaum, B., de Vaureix, C., Lefevre, F., 2006. Trypsin increases pseudorabies virus production through activation of the ERK signalling pathway. J. Gen. Virol. 87, 1109–1112. https://doi.org/10.1099/vir.0.81609-0.

Rourou, S., van der Ark, A., van der Velden, T., Kallel, H., 2009. Development of an animal-component free medium for vero cells culture. Biotechnol. Prog. 25 (6), 1752–1761. https://doi.org/10.1002/btpr.279.

Rourou, S., Riahi, N., Majoul, S., Trabelsi, K., Kallel, H., 2013. Development of an in situ detachment protocol of vero cells grown on cytodex1 microcarriers under animal component-free conditions in stirred bioreactor. Appl. Biochem. Biotechnol. 170 (7), 1724–1737. https://doi.org/10.1007/s12010-013-0307-y.

Rowell, C.E., Dobrovolny, H.M., 2020. Energy requirements for loss of viral infectivity. Food Environ. Virol. 12, 281–294. https://doi.org/10.1007/s12560-020-09439-9.

Seitz, C., Isken, B., Heynisch, B., Rettkowski, M., Frensing, T., Reichl, U., 2011. Trypsin promotes efficient influenza vaccine production in MDCK cells by interfering with the antiviral host response. Appl. Microbiol. Biotechnol. 93, 601–611.

Shen, L.W., Mao, H.J., Wu, Y.L., Tanaka, T., Zhang, W., 2017. TMPRSS2: a potential target for treatment of influenza virus and coronavirus infections. Biochimie 142, 1–10. https://doi.org/10.1016/j.biochi.2017.07.016.

Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., Gallagher, T., 2011. A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. J. Virol. 85 (2), 873–882. https://doi.org/10.1128/JVI.02062-10.

Simmons, G., Reeves, J.D., Rennekamp, A.J., Amberg, S.M., Piefer, A.J., Bates, P., 2004. Characterization of severe acute respiratory syndrome-associated coronavirus (sars-cov) spike glycoprotein-mediated viral entry. Proc. Natl. Acad. Sci. USA 101 (12), 4240–4245.

Simmons, G., Gosalia, D.N., Rennekamp, A.J., Reeves, J.D., Diamond, S.L., Bates, P., 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. USA 102 (33), 11876–11881.

Simmons, G., Bertram, S., Glowacka, I., Steffen, I., Chaipan, C., Agudelo, J., Lu, K., Rennekamp, A.J., Hofmann, H., Bates, P., et al., 2011. Different host cell proteases activate the SARS-coronavirus spike-protein for cell-cell and virus-cell fusion. Virology 413 (2), 265–274.

Simmons, G., Zmora, P., Gierer, S., Heurich, A., Pöhlmann, S., 2013. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. Antivir. Res. 100 (3), 605–614.

Sims, A.C., Burkett, S.E., Yount, B., Pickles, R.J., 2008. SARS-CoV replication and pathogenesis in an in vitro model of the human conducting airway epithelium. Virus Res. 133 (1), 33–44. https://doi.org/10.1016/j.virusres.2007.03.013.

Song, W., Gui, M., Wang, X., Xiang, Y., 2018. Cryo-EM structure of the SARS coronavirus spike glycoprotein in complex with its host cell receptor ACE2. PLOS Pathogens 14 (8), e1007236. https://doi.org/10.1371/journal.ppat.1007236.

Watanabe, R., Matsuyama, S., Shirato, K., Maejima, M., Fukushi, S., Morikawa, S., Taguchi, F., 2008. Entry from the cell surface of severe acute respiratory syndrome coronavirus with cleaved S protein as revealed by pseudotype virus bearing cleaved S protein. J. Virol. 82 (23), 11985–11991. https://doi.org/10.1128/JVI.01412-08.

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H., Pei, Y.-Y., Yuan, M.-L., Zhang, Y.-L., Dai, F.-H., Liu, Y., Wang, Q.-M., Zheng, J.-J., Xu, L., Holmes, E.C., Zhang, Y.-Z., 2020. A new coronavirus associated with human respiratory disease in China. Nature 579 (7798), 265–271. https://doi.org/10.1038/s41586-020-2008-3.

Yeung, M., Lim, D., Duncan, R., Shahrabadi, M., Cashdollar, L., Lee, P., 1989. The cell attachment proteins of type-1 and type-3 reovirus are differentially susceptible to trypsin and chymotrypsin. Virology 170 (1), 62–70. https://doi.org/10.1016/0042-6822(89)90352-8.

Zhou, Y., Vedantham, P., Lu, K., Agudelo, J., Carrion, R., Nunneley, J.W., Barnard, D., Pohlmann, S., McKerrow, J.H., Renslo, A.R., Simmons, G., 2015. Protease inhibitors targeting coronavirus and filovirus entry. Antivir. Res. 116, 76–84. https://doi.org/10.1016/j.antiviral.2015.01.011.

Zmora, P., Blazejewska, P., Moldenhauer, A.-S., Welsch, K., Nehlmeier, I., Wu, Q., Schneider, H., Pohlmann, S., Bertram, S., 2014. DESC1 and MSPL activate influenza A viruses and emerging coronaviruses for host cell entry. J. Virol. 88 (20), 12087–12097. https://doi.org/10.1128/JVI.01427-14.