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Attorneys for Defendants Xlear, Inc. and Nathan Jones

IN THE UNITED STATES DISTRICT COURT FOR THE DISTRICT OF UTAH

UNITED STATES OF AMERICA Plaintiff, v. XLEAR, INC., a corporation, and NATHAN JONES, individually and as an officer of XLEAR, INC., Defendants.	Case No. 2:21-cv-00640-RJS **DEFENDANTS' AMENDED ANSWER TO THE GOVERNMENT'S COMPLAINT FOR CIVIL PENALTIES, PERMANENT INJUNCTION, AND OTHER RELIEF** Chief Judge Robert J. Shelby

Defendants Xlear, Inc. ("Xlear") and Nathan Jones (collectively "Defendants"), by and through counsel, respectfully respond to the allegations in Complaint filed by the United States on October 28, 2021 (Dkt. No. 2) as follows:

NATURE OF THE CASE

1. Defendants admit the allegations of Paragraph 1. Defendants further aver that the Government concedes that Xlear is a "saline nasal spray."

2. Defendants admit that Xlear nasal spray was advertised. Defendants admit they have made limited statements supported by competent and reliable scientific evidence about the use of nasal sprays for the prevention and treatment of COVID-19. Defendants deny the remaining allegations of Paragraph 2.

3. Defendants deny the allegations of Paragraph 3.

4. To the extent that Paragraph 4 alludes to a July 29, 2020 Warning Letter from the FTC to Xlear, Defendants admit that, on or about July 29, 2020, Xlear received a letter from the FTC's Division of Advertising Practices regarding statements that it has made about Xlear nasal spray. The letter speaks for itself, and Defendants deny the remaining allegations of Paragraph 4.

JURISDICTION AND VENUE

5. Paragraph 5 contains a statement of jurisdiction to which no response is required. Defendants, however, admit that this Court has jurisdiction over the subject matter at issue.

6. Paragraph 6 contains a statement of jurisdiction to which no response is required. Defendants, however, admit that this Court has jurisdiction over the parties.

7. Paragraph 7 contains a statement of venue to which no response is required.

PARTIES

8. Paragraph 8 contains the Government's description of its title, to which no response is required. To the extent a response is required, Defendants admit the allegations of Paragraph 8.

9. Defendant Jones admits that he is the founder and president of Xlear and is involved in Xlear's business affairs, the formulation of corporate policy and strategic decisions, press

releases about the efficacy of Xlear nasal sprays, and responding to the FTC's Warning Letter to Xlear. Defendant Jones further admits that he resides in this District and that, through his role with Xlear, transacts and has transacted business in this District and throughout the United States. Defendant Jones denies the remaining allegations of Paragraph 9.

10. Defendant Xlear admits the allegations of Paragraph 10. Defendants admit that Xlear's nasal spray products are available for purchase at national retailers including Rite-Aid, CVS, Walgreens, and Target, and online at Amazon.com. Defendants aver that Xlear has been sold in the United States for over twenty years, to vast numbers of individuals and families, without a single complaint reporting an adverse effect and without any prior action by the United States against the company.

THE FTC ACT

11. Paragraph 11 contains a legal conclusion to which no response is required.

12. Paragraph 12 contains a legal conclusion to which no response is required.

13. Paragraph 13 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny that Xlear nasal spray products are "drugs" as defined in Section 15(c) of the FTC Act, 15 U.S.C. § 55(c). Further, Defendants aver that, for twenty-plus years, pursuant to the Food & Drug Administration, Xlear and its components are not classed as drugs.

THE COVID-19 CONSUMER PROTECTION ACT

14. Paragraph 14 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny the allegations of Paragraph 14. Defendants further aver that Paragraph 14 is based on a misconstruction of the facts and a flawed legal analysis.

15. Paragraph 15 contains a legal conclusion to which no response is required. To the

extent a response is required, Defendants deny the allegations of Paragraph 15. Defendants further aver that Paragraph 15 is based on a misconstruction of the facts and a flawed legal analysis.

16. Paragraph 16 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny the allegations of Paragraph 16. Defendants further aver that Paragraph 16 is based on a misconstruction of the facts and a flawed legal analysis.

17. Paragraph 17 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny the allegations of Paragraph 17. Defendants further aver that Paragraph 17 is based on a misconstruction of the facts and a flawed legal analysis.

DEFENDANTS' UNLAWFUL CONDUCT[1]

18. Defendant Xlear admits the allegations of Paragraph 18. Defendant Xlear further states that Xlear manufactures and sells a high-volume saline irrigation, toothpaste, and mouthwash.

19. Defendants admit that Xlear nasal spray is discussed on xlear.com, YouTube, and other social media posts. Defendants admit they have made limited statements supported by competent and reliable scientific evidence about the use of nasal sprays for the prevention and treatment of SARS-CoV-2 (the virus that causes COVID-19). Defendants deny the remaining allegations of Paragraph 19. Moreover, the so-called "magazine advertorials" are not bought and paid for advertorials but are unpaid media interviews.

20. Defendants deny that a randomized clinical trial is required to support the statements Xlear made and further aver that competent and reliable scientific evidence supports the statements made regarding Xlear nasal spray and other nasal sprays with respect to the SARS-

[1] Defendants deny any factual allegations contained in the headings of the Government's Complaint.

CoV-2 virus.[2] Defendants deny the remaining allegations of Paragraph 20. Moreover, the Defendants aver that in some circumstances involving a novel deadly pathogen (e.g., SARS-CoV-2), the specific sorts of trials claimed as being required by the Government in this case may be precluded under the Government's own regulations; prohibited by medical ethics; logistically unfeasible; and/or may yield less conclusive data than other forms of studies. Numerous medical and scientific experts have opined that random controlled clinical trials ("RCTs") are not the best tools in the face of a global pandemic. *See, e.g.*, Adashek, J.J., Kurzrock, R. Balancing clinical evidence in the context of a pandemic. Nat Biotechnol 39, 270–274 (2021). https://doi.org/10.1038/s41587-021-00834-6 (last viewed Dec. 22, 2021) (content attached hereto as Exhibit A pursuant to DUCivR 7-5). Specifically, to the best of the Defendants' knowledge and belief, in 2020, researchers sought FDA permission to run precisely the sort of RCT the Government seeks to require of the Defendants. In June-July 2020, Dr. Gus Ferrer, an international expert on upper respiratory disease and a frontline doctor treating COVID-19 patients, sought FDA permission to run a human clinical trial using Xlear as a treatment for patients already infected with COVID-19. In August of 2020, the FDA responded and denied permission for the trial. The FDA's stated reason was the Agency does not allow drug action studies to be done on substances classed as cosmetics.

To this end, many of the Government's own actions to combat the pandemic—actions that have altered the lives of an entire nation—have been taken without any RCT data. *See* Peeples, L., Face Masks What the Data Say, Nature, Oct. 6, 2020, available at https://www.nature.com/articles/d41586-020-02801-8 (last viewed Dec. 22, 2021) (content

[2] Moreover, Defendants aver that a well-designed RCT study would not prove or disprove the majority of statements at issue here. For example, statements regarding whether Xlear or any other product blocks the adhesion of the SARS-CoV-2 virus cannot be definitively proven in a clinical setting.

attached hereto as Exhibit B pursuant to DUCivR 7-5) (Reporting that the CDC mask mandate was not backed by a single RCT study at the time) (‘“You can’t do randomized trials for everything — and you shouldn’t.” As clinical researchers are sometimes fond of saying, parachutes have never been tested in a randomized controlled trial, either.”); Xiao J, Shiu E, Gao H, Wong JY, Fong MW, Ryu S, et al. Nonpharmaceutical Measures for Pandemic Influenza in Nonhealthcare Settings—Personal Protective and Environmental Measures. Emerg Infect Dis. 2020;26(5):967-975, available at https://doi.org/10.3201/eid2605.190994 (last viewed Dec. 22, 2021) (content attached hereto as Exhibit C pursuant to DUCivR 7-5) (“In our systematic review, we identified 10 RCTs that reported estimates of the effectiveness of face masks in reducing laboratory-confirmed influenza virus infections in the community . . . In pooled analysis, we found no significant reduction in influenza transmission with the use of face masks.”) (As published by the Centers for Disease Control); Hassad, R., No RCT for Masks? No Problem; Other forms of evidence are available to judge effectiveness of this and other interventions, MedPageToday, Aug. 3, 2020, available at https://www.medpagetoday.com/infectiousdisease/covid19/87870 (last viewed Dec. 22, 2021) (content attached hereto as Exhibit D pursuant to DUCivR 7-5) (“There has been an almost exclusive focus on evidence from experimental studies, specifically the randomized controlled trial (RCT) . . . as it allows for the determination of causality. However, the reason such evidence is still lacking should be obvious—the RCT is neither feasible nor appropriate for determining the effectiveness of mask-wearing in the community in protecting against COVID-19, and moreover, its use will be considered unethical in the context of a deadly pandemic.”).

In fact, experts, including those who have run pandemic response for the U.S. Government, believe that RCT’s are no longer the single best source of scientific proof. *See e.g.*, Frieden, T.,

Why the 'gold standard' of medical research is no longer enough, Aug. 2, 2017, available at https://www.statnews.com/2017/08/02/randomized-controlled-trials-medical-research/ (last viewed Dec. 22, 2021) (content attached hereto as Exhibit E pursuant to DUCivR 7-5) (Dr. Frieden served as Director of the Centers for Disease Control and Prevention (CDC), the Government's lead agency in pandemic prevention and response, from 2009 to 2017). Dr. Frieden's expert view on RCT's:

> Despite their strengths, RCTs have substantial limitations. They can be very expensive to run. They can take many years to complete, and even then may not last long enough to assess the long-term effect of an intervention such as vaccine immunity, or to detect rare or long-term adverse effects. Findings from RCTs may not be valid beyond the study population — a trial that included a high-risk population in order to maximize the possibility of detecting an effect, for example, may not be relevant to a low-risk population. RCTs may not be practical for population-wide interventions and often aren't relevant for urgent health issues such as infectious disease outbreaks, for which public health decisions must be made quickly.
>
> . . . .
>
> Glorifying RCTs above other approaches, even when these other approaches may be either superior or the only practical way to get an answer, relegates patients to receiving treatments that aren't based on the best available evidence.
>
> An approach that uses all appropriate evidence types and builds on the existing evidence base using proven best practices is the one most likely to result in clinical and public health action that will save lives.

Id. (emphasis added); *see also* Frieden T.R. Evidence for health decision making — beyond randomized, controlled trials. N Engl J Med. 2017;377(5):465–75. https://doi.org/10.1056/NEJMra1614394. Further other studies underscore why these experts advise against over-reliance on RCT data. For example, a reanalysis of RCT's, published in the highly regarded Journal of the American Medical Association and republished by the NIH, led by Stanford professor John Iaonnides, concluded 35 percent of RCT study conclusions could not be

replicated (supported) based on their own underlying data. Ebrahim S, Sohani ZN, Montoya L, et al. Reanalyses of Randomized Clinical Trial Data. JAMA. 2014;312(10):1024–1032. doi:10.1001/jama.2014.9646, published at https://jamanetwork.com/journals/jama/fullarticle/1902230, republished at https://pubmed.ncbi.nlm.nih.gov/25203082/.

Further, *inter alia*, the Defendants aver that the available, relevant RCTs support the statements made by Xlear. The Government's complaint states that Xlear is "a saline nasal spray." Multiple clinical trials, including at least two that the Government specifically knows of, have shown that saline nasal cleansing shows benefits in reducing the duration and severity of the illness in individuals with moderate to high risk, who are already sick with COVID-19.

- A peer-reviewed, published Randomized Clinical Trial (RCT) conducted at Vanderbilt University in 2020, found that the use of nasal sprays significantly reduced the severity and duration of symptoms among non-hospitalized COVID-19 patients (the Vanderbilt University Study): "The effect of nasal irrigation on symptom resolution was substantial, with nasal congestion and headache resolving a median of 7 to 9 days earlier in the intervention groups. Our analysis suggests that nasal irrigations may shorten symptom duration and may have potential as a widely available and inexpensive intervention to reduce disease burden among those affected. In the interim, we would advocate the use of hypertonic nasal saline irrigations in non-hospitalized COVID-19 patients as a safe and inexpensive intervention to reduce symptom burden." Kimura, K., et al., Interim analysis of an open-label randomized controlled trial evaluating nasal irrigations in nonhospitalized patients with coronavirus disease 2019. Int Forum Allergy Rhinol. 2020; 10: 1325– 1328, available

at https://pubmed.ncbi.nlm.nih.gov/32914928/ (last viewed Dec. 22, 2021) (content attached hereto as Exhibit F pursuant to DUCivR 7-5). The Vanderbilt University RCT was funded by the Government's own National Institutes of Health (NIH) and is republished as authority on the NIH's own website. Defendants have specifically made the FTC aware of the findings of this RCT.

- A more recent RCT study, conducted at Augusta University in Georgia, found that nasal irrigation significantly reduces the risk of hospitalization among COVID-19 infected people: "The total risk of hospitalization or death (10.6%) was 8.4 times that of enrolled patients (SE=2.74; P=.006) . . . . There were no significant differences by additive." Amy Baxter, et al., Rapid initiation of nasal saline irrigation to reduce morbidity and mortality in COVID+ outpatients: a randomized clinical trial compared to a national dataset, medRxiv 2021.08.16.21262044, doi:https://doi.org/10.1101/2021.08.16.21262044 available at https://www.medrxiv.org/content/10.1101/2021.08.16.21262044v2 (last viewed Dec. 22, 2021) (content attached hereto as Exhibit G pursuant to DUCivR 7-5). It should be stressed that a reduction of 8.4 times (not percent) is a vastly greater impact than a host of other measures now being used to combat the pandemic with the Government's full approval.
- A third clinical trial case study, peer-reviewed, and published, that studied the efficacy of Xlear nasal spray specifically found (a clinical trial in humans already infected with COVID-19) that Xlear spray significantly reduced the severity and duration of the illness in COVID-19 patients—all with co-morbidities (the Larkin Hospital Study). The Larkin Hospital Study found Xlear nasal spray showed "remarkable results" in

helping treat COVID-19 patients. The Larkin Hospital Study "noted improvement of symptoms as early as day 4. Furthermore, on day 7, patients tested negative on repeat RT-PCR nasopharyngeal swab instead of the average 14-day period of negativization of COVID-19. By using xylitol plus GSE in the form of an intranasal spray (Xlear nasal spray), as an adjunct to the ongoing treatment, the time to negativization was reduced by 50%." The Larkin Hospital Study noted no complications or adverse effects. Go et al., Intranasal Therapy and COVID-19: A Comprehensive Literature Review, J Allergy Infect Dis, 2021; 2(1):9-16, citing Go et al., Potential Role of Xylitol Plus Grapefruit Seed Extract Nasal Spray Solution in COVID-19: Case Series, Cureus, 2020 Nov; 12(11): e11315, available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7645297/ (last viewed Dec. 22, 2021) (content attached hereto as Exhibit H pursuant to DUCivR 7-5). The Larkin Hospital Study is republished as authority on the Government's (NIH's) website. In order to comply with ethics and Food & Drug Administration rules, the Larkin Hospital Trial Study presents a series of case studies, not a larger-scale trial. As such, the reported results discuss only three of the patients in the Study. Nevertheless, the Larkin Study is a peer-reviewed, published RCT.

- A fourth clinical trial found nasal washing is effective in reducing viral load in the nose. (Multiple experts, including the Government's own Dr. Fauci, have linked viral load in the nose with COVID-19 infection and transmission). Hendley JO, Gwaltney JM, Viral titers in nasal lining fluid compared to viral titers in nasal washes during experimental rhinovirus infection, J Clin Virol. 2004;30(4):326–328, available at https://pubmed.ncbi.nlm.nih.gov/15163422/. This trial looked at viral load of

rhinovirus. Rhinovirus, like SARS-CoV-2, is an upper respiratory viral illness that most commonly starts in the nose. These two diseases are so closely associated that another clinical trial has found that the prevalence of rhinovirus in the nose is a strong indicator that an intervention is effective against COVID-19. Kitanovski, S., Horemheb-Rubio, G., Adams, O. et al. Rhinovirus prevalence as indicator for efficacy of measures against SARS-CoV-2. BMC Public Health 21, 1178 (2021). https://doi.org/10.1186/s12889-021-11178-w, available at https://bmcpublichealth.biomedcentral.com/articles/10.1186/s12889-021-11178-w (last viewed Dec. 22, 2021) (content attached hereto as Exhibit I pursuant to DUCivR 7-5).

21. Defendants admit that Xlear made certain statements, including those set forth in Paragraph 20. Defendants further aver that the statements are supported by competent and reliable scientific evidence. Specifically, a number of independent, published *in vitro* studies have found that Xlear specifically is virucidal against SARS-CoV2 (it kills and/or deactivates the COVID-19 virus), and antiviral against SARS-CoV-2 (it blocks the COVID-19 virus from adhering to and infecting tissue). These studies include the following:

- Ferrer, Gustavo, et al, A Nasal Spray Solution of Grapefruit Seed Extract plus Xylitol Displays Virucidal Activity Against SARS-Cov-2 In Vitro, BioRxiv (Nov. 25, 2020), available at https://www.biorxiv.org/content/10.1101/2020.11.23.394114v1.full (last viewed Dec. 22, 2021) (content attached hereto as Exhibit J pursuant to DUCivR 7-5);
- Cannon, Mark, et al, In Vitro Analysis of the Anti-viral Potential of nasal spray constituents against SARS-CoV-2, bioRxiv 2020.12.02.408575, available at https://www.biorxiv.org/content/10.1101/2020.12.02.408575v2.full.pdf+html (last

viewed Dec. 22, 2021) (content attached hereto as Exhibit K pursuant to DUCivR 7-5);

- Institute for Antiviral Research, Utah State University, Study Report; Antiviral Efficacy Against Virus Infections in Human-Derived Tracheal/Bronchial Epithelial Cells, Dec. 1, 2021 (content attached hereto as Exhibit L pursuant to DUCivR 7-5). This new in vitro study tested the antiviral efficacy of each of the compounds in Xlear (namely grapefruit seed extract (GSE) and xylitol) against three viruses, most importantly the Delta strain of SARS-CoV-2 (B.1.617.2). Most notably this study found both xylitol and GSE had significant antiviral efficacy against the Delta strain. In fact, both of these compounds, which are Xlear ingredients, had greater antiviral efficacy than did Remdesivir, which the FDA has approved as a treatment for COVID-19—to great fanfare. *See* FDA, FDA News Release; FDA Approves First Treatment for COVID-19, Oct. 22, 2020, available at https://www.fda.gov/news-events/press-announcements/fda-approves-first-treatment-covid-19 (last viewed Dec. 22, 2021) (content attached hereto as Exhibit M pursuant to DUCivR 7-5). Further, this study used the human tissue found in the nasal passages, where the vast majority of COVID-19 infections begin.

Likewise, a new *in vitro* study found that use of a simple saline solution sops SAR-CoV2 viral replication in human lung tissue. Rafael R. G. Machado, Talita Glaser, Danielle B. Araujo, Lyvia Lintzmaier Petiz, Danielle B. L. Oliveira, Giuliana S. Durigon, Alessandra L. Leal, João Renato R. Pinho, Luis C. S. Ferreira, Henning Ulrich, Edison L. Durigon, and Cristiane Rodrigues Guzzo, ACS Pharmacology & Translational Science 2021 4 (5), 1514-1527, available at https://pubmed.ncbi.nlm.nih.gov/34651104/. As the Government agrees, Xlear is a saline nasal solution.

Further, new in silico (computer modeling) research has determined that compounds of

grapefruit seed extract (GSE) were effective inhibitors of the SARS-CoV-2 virus. Belmina Saric, et al., In silico analysis of selected components of grapefruit seed extract against SARS-CoV-2 main protease, EuroBiotech J., 5 (special issue 1); 2021, 5. DOI: https://doi.org/10.2478/ebtj-2021-0015. Most importantly, these experts concluded natural GSE (which is a component of Xlear) was more effective than synthetic versions of the compound.

Moreover, as this pandemic surges yet again, and with waning vaccine durability and efficacy, growing numbers of breakthrough cases, and an increasing percentage of breakthrough cases resulting in serious illness and/or death, the COVID-19 medical expert community is increasingly focusing on the need to combat this virus in the nose and nasal cavity. *See, e.g.*, Pilicheva, B.; Boyuklieva, R. Can the Nasal Cavity Help Tackle COVID-19? Pharmaceutics 2021, 13, 1612. https://doi.org/10.3390/pharmaceutics13101612; Akiko Iwasaki, The Answer to Stopping the Coronavirus May Be Up Your Nose, N.Y. Times, May 16, 2022, available at https://www.nytimes.com/2022/05/16/opinion/covid-nasal-vaccine.html; NIH, Intranasal proteins could protect against COVID-19 variants, May 10, 2022, available at https://www.nih.gov/news-events/nih-research-matters/intranasal-proteins-could-protect-against-covid-19-variants.

Further numerous other studies and published expert medical reviews have called for the use of nasal cleansing and nasal hygiene, generally, to combat COVID-19 infections. Collectively, these studies, a non-exhaustive list included below, are compelling and reliable scientific evidence substantiating Xlear's statements:

- Lipworth B, Chan R, RuiWen Kuo C. COVID-19: Start with the nose. J Allergy Clin Immunol. 2020;146(5):1214. doi:10.1016/j.jaci.2020.06.038;
- Spinelli, M. et al., Importance of non-pharmaceutical interventions in lowering the viral inoculum to reduce susceptibility to infection by SARS-CoV-2 and potentially disease

severity, The Lancet, Feb. 22, 2020, available at https://www.thelancet.com/journals/laninf/article/PIIS1473-3099(20)30982-8/fulltext (last viewed Dec. 22, 2021) (content attached hereto as Exhibit N pursuant to DUCivR 7-5).

- Cegolon L, Javanbakht M, Mastrangelo G. Nasal disinfection for the prevention and control of COVID-19: A scoping review on potential chemo-preventive agents [published online ahead of print, 2020 Aug 18]. Int J Hyg Environ Health. 2020;230:113605. doi:10.1016/j.ijheh.2020.113605.
- Ferrer, G, Sanchez-Gonzalez, M., Effective Nasal Disinfection as an Overlooked Strategy in Our Fight against COVID-19, Ear Nose Throat J., 2021 Mar 26;1455613211002929, doi: 10.1177/01455613211002929, available at https://pubmed.ncbi.nlm.nih.gov/33765853/ (last viewed Dec. 22, 2021) (content attached hereto as Exhibit O pursuant to DUCivR 7-5).

Further, nasal hygiene (to include the use of saline nasal cleansing, which includes Xlear as a modality) is already indicated for other strains of coronavirus (which SARS-CoC-2 is classed as). Common colds are caused by a range of upper respiratory tract infectious viruses, including several variants of coronavirus as supported by the following:

- CDC, Common Human Coronavirus, available at https://www.cdc.gov/coronavirus/general-information.html, last retrieved May 19, 2022 ("Common human coronaviruses, including types 229E, NL63, OC43, and HKU1, usually cause mild to moderate upper-respiratory tract illnesses, like the common cold.").
- Abbasi J. COVID-19 and the Common Cold—Preexisting Coronavirus Antibodies May Hinder SARS-CoV-2 Immunity. JAMA. 2022;327(7):609–610.

doi:10.1001/jama.2022.0326 available at https://jamanetwork.com/journals/jama/fullarticle/2788621 ("Betacoronaviruses, like SARS-CoV-2—cause about 30% of common colds . . . .").

- Mesel-Lemoine, M., Millet, J., Vidalain, P. O., Law, H., Vabret, A., Lorin, V., Escriou, N., Albert, M. L., Nal, B., & Tangy, F. (2012). A human coronavirus responsible for the common cold massively kills dendritic cells but not monocytes. Journal of virology, 86(14), 7577–7587, available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3416289/. ("Epidemiological studies suggest that [Human Coronaviruses account for 15 to 30% of common colds . . . .").

As a rule, countermeasures—prevention methods and therapeutics (for example, over the counter drugs like cold and flu remedies)—are not variant specific—they are used against the class of colds (which includes those resulting from a number of human coronavirus variants). (As opposed to viral vaccines, which are variant specific, which is problematic.) One countermeasure that experts, including the US Government, recommend to fight the common cold, including coronavirus-infections, is nasal hygiene. *See, e.g.*, NIH, Flu & Colds in Depth, available at https://www.nccih.nih.gov/health/flu-and-colds-in-depth ("For colds: Complementary approaches that have shown some promise include . . . rinsing the nose and sinuses (with a neti pot or other device) . . . ."); DeGeorge KC, Ring DJ, Dalrymple SN. Treatment of the Common Cold. Am Fam Physician. 2019 Sep 1;100(5):281-289. PMID: 31478634, reprinted by NIH as authority at https://pubmed.ncbi.nlm.nih.gov/31478634/ ("Treatments with proven effectiveness for cold symptoms in adults include over-the-counter analgesics, zinc, nasal decongestants . . . The only established safe and effective treatments for children are . . . nasal saline irrigation . . . .").

Defendants aver that the entire body of scientific evidence here meets and exceeds the

competent and reliable scientific evidence standard. *See Fed. Trade Comm'n v. QT, Inc.*, 512 F.3d 858, 861 (7th Cir. 2008) ("Nothing in the Federal Trade Commission Act…requires placebo-controlled, double-blind studies…. The burden is on the Commission to prove that the statements are false…. Placebo-controlled, double-blind testing is not a legal requirement for consumer products."); *United States v. Bayer Corp.*, No. CV 07-01(JLL), 2015 WL 5822595, at *3–4 (D.N.J. Sept. 24, 2015) (citing FTC Guidance) ("The court should look to 'the totality of the evidence' because 'the surrounding body of evidence will have a significant impact both on what type, amount and quality of evidence is required to substantiate a claim and on how that claim is presented.'").

Moreover, Defendants contend that Exhibit D to the Government's Complaint has been selectively edited as to mislead the Court. The full version of this post, attached hereto as Exhibit B, includes a prominent disclaimer that Xlear is not intended to treat anything. And, Defendants aver that this post has long since been removed.

22. Defendants deny that there is no competent and reliable scientific evidence to support Xlear's claims, and further aver that competent and reliable scientific evidence supports the statements made regarding Xlear nasal spray and other nasal sprays with respect to the SARS-CoV-2 virus. Defendants deny the remaining allegations of Paragraph 22.

23. Defendants deny the allegations of Paragraph 23.

24. Defendants admit that an "Education" page on Xlear's website discusses various studies. Defendants admit they have made limited statements supported by competent and reliable scientific evidence about the use of nasal hygiene (including sprays) for the prevention and treatment of COVID-19. Defendants deny the remaining allegations of Paragraph 24. Defendants admit that the study conducted at the University of Tennessee and referenced by Xlear says that

"New Studies Conclude Xlear Kills and/or Deactivates SARS-CoV-2. . . .. Xlear's components are antiviral—they block viral adhesion in the nose. See, for example, . . . [t]his Univ. of Tennessee study[.]" Defendants admit that the study conducted at the University of North Carolina at Chapel Hill and referenced by Xlear says that "[M]any researchers are looking for a good vaccine or treatment to use for COVID-19. [T]here are options that are inexpensive and also effective against Sars-CoV-2. This article reviews three studies that support the idea of using a simple nasal spray like Xlear with xylitol to combat illness…This [UNC Chapel Hill] study shows that administering treatment through the nose is the best way to treat COVID-19, especially in its early stages." Moreover, the magazine statements are not bought and paid for advertorials but are unpaid media interviews.

25. Defendants deny that its cited studies do not support Xlear's claims and further aver, as set out above, that competent and reliable scientific evidence supports the statements made regarding Xlear nasal spray and other nasal sprays, and nasal hygiene generally, with respect to the SARS-CoV-2 virus. Defendants deny the remaining allegations of Paragraph 25.

26. Defendants admit that the *in vitro* study conducted at the University of Tennessee and referenced by Xlear tested the combined effects of two ingredients, iota-carrageenan and xylitol, on monkey kidney cell cultures infected with SARS-CoV-2. Defendants admit that Xlear nasal spray does not contain iota-carrageenan and further aver that the omission of iota-carrageenan is immaterial to the relevance of this study. Specifically, the Government conveniently fails to inform this Court that the trial specifically addressed the efficacy of xylitol independent of any other compound:

> The other remarkably interesting result is that xylitol exhibits antiviral activity on SARS-CoV-2 based on the results obtained with sample P3. Xylitol has been demonstrated to reduce titers of Human Respiratory Syncytial Virus in Hep-2 cells culture and in infected mice [references omitted].

Bansal S, Jonsson CB, Taylor SL, Figueroa JM, Dugour AV, Palacios C, Vega JC. Iota-carrageenan and xylitol inhibit SARS-CoV-2 in Vero cell culture. PLoS One. 2021 Nov 19;16(11):e0259943. doi: 10.1371/journal.pone.0259943, available at: https://pubmed.ncbi.nlm.nih.gov/34797868/ (last viewed Dec. 22, 2021) (content attached hereto as Exhibit P pursuant to DUCivR 7-5). (Xlear contains xylitol above the concentration used in this Study.). Defendants further aver that the use of *in vitro* kidney cells is industry standard. As a result, the Utah study and several other studies use these kidney cells. Defendants deny the remaining allegations of Paragraph 26.

27. Defendants admit that the study conducted at the University of North Carolina at Chapel Hill and referenced by Xlear says that nasal surfaces might be the dominant initial site for SARS-CoV-2 respiratory tract infection and, therefore, "complementary therapeutic strategies that reduce viral titer in the nose early in the disease, e.g., nasal lavages, topical antivirals, or immune modulation, might be beneficial." Defendants deny the remaining allegations of Paragraph 27.

28. Defendants admit that Xlear nasal spray is discussed on the websites xlear.com, dontgetsickclub.com, and commonsensemedicine.org. Dontgetsickclub.com was created by Xlear, has no marketing information, and is not a sales website. Rather, this website provides links to scientific studies, which was done in response to discussions with the FTC. Defendants deny the remaining allegations of Paragraph 28.

29. Defendants deny that Xlear made certain statements on Commonsensemedicine.org. Commonsensemedicine.org is owned by a nonprofit run by Dr. Lon Jones, a physician who invented the Xlear formula and is an occasional advisor to Xlear. Defendants have no ownership or control over Commonsensemedicine.org. As such, statements on Commonsensemedicine.org are not made by Defendants nor attributable to Defendants.

Defendants deny that Dr. Lon Jones is an owner or director of Xlear.

30. Defendants deny that its cited studies do not support Xlear's claims. Defendants further aver that competent and reliable scientific evidence supports the statements made regarding nasal hygiene generally, and Xlear nasal spray and other nasal sprays specifically, with respect to the SARS-CoV-2 virus. Defendants admit that Xlear has made statements based on competent and reliable scientific evidence that: "study shows that administering treatment through the nose is the best way to treat COVID-19, especially in its early stages." Defendants deny the remaining allegations of Paragraph 30.

31. Defendants deny the allegations of Paragraph 31. Contrary to the Government's unsupported assertion that Xlear and Mr. Jones have made money from the statements cited as problematic by the Government, domestic sales of Xlear's nasal spray dropped, not increased, since the onset of COVID-19, during the period of time during which the Government alleges Xlear's violations.

32. Defendants deny the allegations of Paragraph 32. To the extent a response is required, Defendants state that the alleged quotes speak for themselves. Defendants note that Xlear's Facebook page has a disclaimer on the front page. Defendants state that Xlear has no control over third-party reviews posted on the website of an independent, third-party retailer's website. Moreover, contrary to the Government's contention, none of the third-party reviews discuss or describe any statements made by Xlear. For example, example "32.b" clearly recounts the reviewer's own personal experience: "I personally had a bad case of COVID . . . then saw the studies and results on Xlear. Knowing it was affordable, accessible and safe, I bought some. I can tell you this, with in [sic] 24 hours I started to feel so much better." The scientific studies regarding Xlear and COVID-19 are independently done and published in journals. It is counter-intuitive for

the Government to use this person's experience—using Xlear to feel better while sick with COVID-19—to allege consumer injury.

33. Paragraph 33 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny the allegations of Paragraph 33. Defendants admit that Xlear has made statements based on competent and reliable scientific evidence.

34. Defendants admit that, on or about July 29, 2020, Xlear received a letter from the FTC's Division of Advertising Practices regarding statements that it has made about Xlear nasal spray. The letter speaks for itself, but Defendants admit that the letter contains the language excerpted in Paragraph 34. Defendants deny the remaining allegations in Paragraph 34.

35. Defendants admit that Xlear responded to the July 29, 2020 FTC letter and subsequent FTC staff communications and admit Xlear removed statements that the FTC alleged were unsupported. Defendants' efforts to appease the Government were based solely on Defendants' desire to avoid costly and protracted litigation. Defendants deny the remaining allegations of Paragraph 35.

36. Defendants admit that counsel spoke with the FTC staff in early March 2021. Paragraph 36 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny the remaining allegations of Paragraph 36.

37. Paragraph 37 contains a legal conclusion to which no response is required. To the extent a response is required, Defendants deny the allegations of Paragraph 37. Defendants further aver that these allegations are stale and relate to conduct more than a year and a half ago.

COUNT ONE
FTC Act Section 5(a) and Section 12 Violations

38. Defendants incorporate their responses to paragraphs 1-37 as if fully set forth herein.

39. Defendants admit that Xlear has made statements based on competent and reliable scientific evidence that the use of nasal hygiene, specifically Xlear nasal spray products, is effective in treating or preventing COVID-19, and that the results of scientific studies show that Xlear is effective in treating or preventing COVID-19 in humans. Defendants deny the remaining allegations of Paragraph 39.

40. Defendants deny the allegations of Paragraph 40.

41. Defendants deny the allegations of Paragraph 41.

42. Defendants deny the allegations of Paragraph 42.

43. Defendants deny the allegations of Paragraph 43.

COUNT TWO
COVID-19 Consumer Protection Act Violations

44. Defendants incorporate their responses to paragraphs 1-43 as if fully set forth herein.

45. Defendants admit that Xlear has made statements based on competent and reliable scientific evidence that the use of Xlear nasal spray products are effective in treating or preventing COVID-19, and that the results of scientific studies show that Xlear is effective in treating or preventing COVID-19 in humans. Defendants deny the remaining allegations of Paragraph 45.

46. Defendants admit that Xlear has made statements based on competent and reliable scientific evidence that:

a. The use of Xlear nasal spray is proven to provide four hours of protection against infection with the SARS-CoV-2 virus.

b. "With the pandemic raging worldwide, we must use every tool we can to fight it. Failing that needlessly risks millions of lives. Weighing our 20-year safety record,

against the risks of this deadly virus, it's clear Xlear needs to be in widespread use."

c. "People should be using Xlear as part of a layered defense to prevent getting COVID-19. If everyone used Xlear, in addition to taking other steps recommended by public health officials, we believe we could help the nation defeat COVID-19 faster."

Defendants deny the remaining allegations of Paragraph 46.

47. Defendants deny the allegations of Paragraph 47.

48. Defendants deny the allegations of Paragraph 48.

49. Defendants deny the allegations of Paragraph 49.

50. Defendants deny the allegations of Paragraph 50.

51. Defendants deny the allegations of Paragraph 51.

52. Defendants deny the allegations of Paragraph 52.

53. Defendants deny the allegations of Paragraph 53.

CONSUMER INJURY

54. Defendants deny the allegations of Paragraph 54.

55. To the extent there are any remaining factual allegations that have not been expressly denied, those allegations are denied. Moreover, as set out more fully below, Defendants aver that the greater harm to consumers—in fact the American public generally—comes from the Government's refusal to adopt scientifically-substantiated countermeasures to COVID-19, and the Government's efforts to silence those who seek to educate the public about these countermeasures, of which this lawsuit is part and parcel.

PRAYER FOR RELIEF

56. The Government's prayer for relief characterizes the relief the Government seeks to which no response is required. To the extent a response is required, Defendants deny that they have engaged in any of the unlawful acts or omissions alleged against them and deny that the Government is entitled to any of the relief prayed for in the prayer for relief or any other relief. The Government's sought after injunction will constitute a significant burden on Defendants' First Amendment Rights.

ADDITIONAL DEFENSES

Pursuant to Fed. R. Civ. P. 8 and 12(b), Defendants assert the following additional defenses. By listing the following defenses, Defendants do not concede, explicitly or implicitly, that any or all of the listed defenses are affirmative defenses under applicable law or that Defendants bear the burden of proof thereon. Defendants also do not by listing their defenses in this Answer limit their ability to present any defense that does not need to be identified by Answer.

FIRST DEFENSE

The Government fails to state a claim upon which relief may be granted pursuant to Fed. R. Civ. P. 12(b)(6).

Most notably, the crux of this case is the Government's allegation that the Defendants lacked adequate competent and reliable scientific evidence to reasonably satisfy experts in the field. The field here is COVID-19 medicine and nasal hygiene as a disease prevention tool. The Defendants aver a host of studies that support the statements made.

In contrast, the Government fails to offer a single scientific study, RCT or otherwise, that counters or refutes the Defendants' studies. In fact, the Government does not refute the Defendants' science in anyway. Instead, the Government baldly concludes inadequacy.

The Supreme Court held in *Ashcroft v Iqbal*, a complaint is inadequate if "it tenders 'naked assertion[s]' devoid of 'further factual enhancement' . . . . Threadbare recitals of the elements of a cause of action, supported by mere conclusory statements, do not suffice." *Ashcroft v. Iqbal*, 556 U.S. 662, 678 (2009) (citing and quoting *Bell Atl. Corp. v. Twombly*, 550 U.S. 544, 557 (2007)) (internal quotations omitted).

The Government offers no facts to support its allegations that reasonable experts in the field of COVID-19 medicine would find the Defendants' substantiation inadequate. The Government presents not a single expert's opinion in the field of COVID-19 medicine and/or nasal hygiene disease prevention that Defendants' substantiation is inadequate. All the Government pleads is a series conclusory statements that the substantiation requirement is not met. This is precisely the sort of threadbare element supported only by conclusory statements that the Supreme Court rejected in *Iqbal*.

SECOND DEFENSE

The statements identified are not false or misleading but are truthful and accurate. Each statement is supported by competent and reliable scientific evidence, including various studies. The Government's contention that RCTs are required by law is wholly without merit; RCTs are not always necessary to substantiate claims about the health benefits of foods and nutrients. Moreover, under certain circumstances with respect to a novel, deadly pathogen (e.g., SARS-CoV-2 (COVID-19)) the specific types of RCTs the Government seeks to mandate from whole cloth here can be: precluded under the Government's own regulations; prohibited by medical ethics; logistically unfeasible; and/or yield less conclusive data than other forms of studies. The statements at issue in this litigation are substantiated by medical evidence, include effective disclaimers disclosing the limitations of the supporting research, and are based on the type of

scientific evidence that could have been regulatorily and ethically obtained.

As discussed above, Defendants aver that there is substantial competent and reliable scientific evidence to substantiate their statements.

THIRD DEFENSE

Defendants' conduct is not "egregious," does not constitute significant consumer harm, provides a benefit to consumers, and there has not been any justifiable reliance on the part of consumers.

FOURTH DEFENSE

The statements at issue in the Complaint did not result from any misconduct or deceitfulness on the part of the Defendants, and the Government will be unable to demonstrate that Defendants had knowledge that any such statement was conveying a "misleading claim."

FIFTH DEFENSE

At all relevant times, Defendants acted in good faith and in a lawful manner toward consumers and in conformity with all applicable laws and regulations.

SIXTH DEFENSE

Defendants allege that any and all damages and injury alleged in the Complaint, if any, were caused, if at all, in whole or in part, by the conduct, fault and/or negligence of persons or entities other than Defendants.

SEVENTH DEFENSE

Xlear is not violating or imminently about to violate laws enforced by the FTC.

EIGHTH DEFENSE

Xlear has remedied any concerns the FTC may have regarding its statements and

business practices, and there will be no proof of any purported ongoing consumer harm such that this action is moot.

NINTH DEFENSE

The Government's actions here constitute a violation of the Defendants' First Amendment Rights, and the injunctive relief sought is barred by the First Amendment because it would have the effect of prohibiting speech that is neither false nor misleading.

Throughout this matter, the Government has routinely sought to restrain Defendants from raising public awareness—in a time of a deadly global pandemic. Government's efforts here with regard to Defendants' commercial speech fail the test set out by the Court in *Central Hudson. See Pom Wonderful v. Fed'l Trade Comm'n.*, 777 F.3d 478 (D.C. Cir. 2015), *citing Cent. Hudson Gas & Elec. Corp. v. Pub. Serv. Comm'n*, 447 U.S. 557, 566, 100 S.Ct. 2343, 65 L.Ed.2d 341 (1980); *see also Edenfield v. Fane*, 507 U.S. 761,766 (1993) ("The commercial marketplace, like other spheres of our social and cultural life, provides a forum where ideas and information flourish. Some of the ideas and information are vital, some of slight worth. But the general rule is that the speaker and the audience, not the government, assess the value of the information presented. Thus, even a communication that does no more than propose a commercial transaction is entitled to the coverage of the First Amendment . . . .").

The Government's systemic restraint on Defendants' speech goes well beyond the four-corners of the Government's complaint. For example, the Defendants have sought to promote a petition initially signed by healthcare researchers and front-line healthcare workers (doctors and others) to the Centers for Disease Control (CDC). The petition calls for the CDC to issue guidance, much like other CDC Guidance on masking and social distancing, on the use of nasal cleansing to combat COVID-19. That petition now has over 6,400 individual signatories. During the course

of Defendants' interactions with the Government, the Government demanded that the Defendants cease efforts to promote the petition of the CDC.

The Act of petitioning the Government for a change in Government policy with the intent of saving American lives is pure protected speech. *See Cent. Hudson Gas & Elec. v. Public Svc. Comm'n*, 447 U.S. 557, 562 (1980) ("commercial speech is 'speech that proposes a commercial transaction.'") A petition for a change in Government policy does not propose a transaction—it proposes the Government actually take action to safeguard American lives. As the Supreme Court held in *United Mineworkers*, "the rights to assemble peaceably and to petition for a redress of grievances are among the most precious of the liberties safeguarded by the Bill of Rights." *United Mineworkers of America v Illinois State Bar Assoc.*, 389 U.S. 217, 222 (1967). However, here the Government has deliberately and unlawfully sought to preclude Defendants from exercising that right.

The Government does not raise its longstanding efforts to restrain Defendants' First Amendment Rights in its complaint for obvious reasons. However, that does not negate the fact of the Government's actions. These actions provide vital context and motive to all the Government's actions and allegations.

Moreover, the Government's actions here have already had a chilling effect on Defendants' First Amendment Rights. For example, when the Augusta University, Georgia, RCT Study noted above first came to Defendants' attention, Defendants wanted to inform the public about the study. (That study found that nasal cleansing with a saline irrigant can reduce the rate of COVID-19 hospitalization among people already sick with COVID-19 and at moderate to high risk by 8.4 times.) People facing a deadly pandemic have a vital interest in that information. However, the Defendants did not do so because they feared Government reprisals.

When the Defendants received the results of the Institute for Antiviral Research, Utah State University, Study (attached hereto at Exhibit L), finding the components of Xlear have greater antiviral efficacy (in vitro) than the FDA-approved COVID-19 treatment Remdesivir, the Defendants thought the public had a right to know this information. However, the Defendants have not publicized these study results out of fear of Government reprisals. The Delta strain remains, at the time of this writing, the strain causing the vast majority of new COVID cases in the U.S. with the Omicron variant rapidly spreading as well. The Defendants have yet to run testing against the Omicron strain. However, lab tests have already found efficacy against no less than three strains and there is no data to suggest it would not have similar effect on Omicron. The public would have been well, and better, served to know this information.

TENTH DEFENSE

The Government is pursuing this enforcement action in order to retaliate against Xlear and Nathan Jones for their criticisms of the federal government's response to the COVID-19 pandemic in violation of Defendants' First Amendment rights.

As a company, Xlear is committed to improving the United States' medical response through proactive health and staying healthy through transformational hygiene. Nathan Jones has spoken publicly on the health benefits of natural, preventative efforts, including nasal hygiene (including using sprays).

Nathan Jones and Xlear have publicly criticized the Government's handling of COVID-19, including its myopic focus on vaccines as the favored tool to combat COVID-19 through statements to the media and on public health podcasts, lobbying members of Congress, and working to publicize efforts to petition the CDC to tell the American people about the benefits of nasal sprays. Nathan Jones and Xlear have been particularly critical of the Government's

relationship with pharmaceutical companies – a relationship that is detrimental to the health and welfare of the American people.

Unfortunately, and to the Government's chagrin, Mr. Jones' criticisms have been borne out. The following facts show this to be true:

- Years into this pandemic, the United States is facing yet another spike in cases. *See* Fernit Nirappil, et al., How big is the latest U.S. coronavirus wave? No one really knows., Wash. Post, May 17, 2022, available at https://www.washingtonpost.com/health/2022/05/17/latest-coronavirus-wave-us/. An increasing number of COVID deaths are now occurring among those vaccinated (and even boosted). *See* Arielle Mitropoulos, Arielle Mitropoulos, Growing proportion of COVID deaths occurring among vaccinated: ABC News analysis, ABC News, May 12, 2022, available at https://abc11.com/covid-deaths-breakthrough-cases-vaccine-vaccinated/11841569/. This latest surge is occurring despite billions of dollars in Federal spending to develop, procure and promote vaccines as the answer to the pandemic.
- The current variants are increasingly vaccine resistant. Breakthrough case rates are so high, that Government officials no longer are encouraging vaccinations to prevent infections—the new pablum is vaccinations are necessary to reduce more serious cases. *See, e.g.*, The White House, Press Briefing by White House COVID-19 Response Team and Public Health Officials, May 18, 2022, (statement of Dr. Rochelle Walensky), available at: https://www.whitehouse.gov/briefing-room/press-briefings/2022/05/18/press-briefing-by-white-house-covid-19-response-team-and-public-health-officials-may-18-2022/; Andrew Jeong, et al.,

Virus may infect most, Fauci says, but risk of severe illness 'very, very low' for vaccinated, Wash. Post, Jan. 12, 2022, available at https://www.washingtonpost.com/nation/2022/01/12/covid-omicron-variant-live-updates/.

- The duration and efficacy of protection of the current vaccines and boosters continues to wane. *See, e.g.*, Thompson MG, Natarajan K, Irving SA, et al. Effectiveness of a Third Dose of mRNA Vaccines Against COVID-19–Associated Emergency Department and Urgent Care Encounters and Hospitalizations Among Adults During Periods of Delta and Omicron Variant Predominance — VISION Network, 10 States, August 2021–January 2022. MMWR Morb Mortal Wkly Rep 2022;71:139–145. DOI: https://pubmed.ncbi.nlm.nih.gov/35085224/.
- Waning vaccine durability has reached such a critical point that US officials are now privately calling for massive Federal spending to develop yet another vaccine, in hopes it will be more durable.
- Health experts, including US government officials, increasingly realize that effectively combatting the virus requires nasal interventions. *See, e.g.*, Pilicheva, B.; Boyuklieva, R. Can the Nasal Cavity Help Tackle COVID-19? Pharmaceutics 2021, 13, 1612. https://doi.org/10.3390/pharmaceutics13101612; Akiko Iwasaki, The Answer to Stopping the Coronavirus May Be Up Your Nose, N.Y. Times, May 16, 2022, available at https://www.nytimes.com/2022/05/16/opinion/covid-nasal-vaccine.html; NIH, Intranasal proteins could protect against COVID-19 variants, May 10,2022, available at https://www.nih.gov/news-events/nih-research-matters/intranasal-proteins-could-protect-against-covid-19-variants.

This is constitutionally-protected speech that became more prominent in early 2021, nearly a year into the pandemic. By that time, Xlear had removed any statements the FTC identified as problematic to the agency from its website and social media pages. Despite this, the FTC ramped up its enforcement efforts against Xlear and Mr. Jones following their increased public criticism of the Government's response to the COVID-19 pandemic. An enforcement action like this would chill a person of ordinary firmness from continuing to engage in a protected activity and has chilled Xlear and Nathan Jones' speech.

The statements that form the basis of the Government's Complaint are similar to those that Xlear has made for more than 20 years, without ever receiving a Warning Letter or facing an enforcement action. Xlear's competitors have made similar statements on their websites, and they have not received an FTC Warning Letter, nor have they faced an enforcement action. In fact, on or about February 16, 2021, Xlear provided the FTC staff a list of competitor statements that were vastly more aggressive concerning COVID-19 benefits. For example, Halodine, which received a FDA warning letter, but never a FTC warning letter, had as a banner at the top of the company website "as seen on CDC.gov." These statements were made known to the FTC and, to the best of our knowledge and information, no action was ever taken by the agency.

This selective prosecution, as well as the timing of the FTC's enforcement actions, demonstrates that the Government's enforcement action was filed without probable cause and with an intent to retaliate for Xlear and Mr. Jones' criticisms. In fact, the Government's actions -- including filing this Complaint -- conveniently coincide with criticisms leveled by the Defendants regarding the Government's pandemic response. It is apparent, then, that the Government's actions were "*substantially motivated*" as a response to Defendants' criticisms. *See Worrell v. Henry*, 219 F.3d 1197, 1212 (10th Cir. 2000)(emphasis added).

The Government violated the First Amendment by initiating an enforcement against Xlear and Nate Jones in retaliation for their exercise of their First Amendment rights and used its official authority to punish Xlear and Nate Jones for criticizing the Government's response to the COVID-19 pandemic in order to influence Defendants' speech decisions going forward.

ELEVENTH DEFENSE

Defendants hereby give notice that they intend to rely upon any other defense that may become available or appear during the course of discovery proceedings in this case.

PRAYER FOR RELIEF ON THE GOVERNMENT'S COMPLAINT

WHEREFORE, Defendants pray that judgment be entered against the Government to include that:

A. The Government takes nothing from the Complaint;

B. The Government's Complaint be dismissed with prejudice;

C. Defendants be awarded their costs and attorneys' fees; and

D. For such other and further relief as the Court deems just and proper.

Respectfully submitted,

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CERTIFICATE OF SERVICE

I hereby certify that, on this 16th day of July, 2022, a true and correct copy of the foregoing **DEFENDANTS' AMENDED ANSWER TO THE GOVERNMENT'S COMPLAINT FOR CIVIL PENALTIES, PERMANENT INJUNCTION, AND OTHER RELIEF** has been filed with the Clerk of Court, by using the CM/ECF system to deliver a true and correct copy of the foregoing to the following:

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Exhibit A

Balancing clinical evidence in the context of a pandemic

- Correspondence

- Published: 05 February 2021

Balancing clinical evidence in the context of a pandemic

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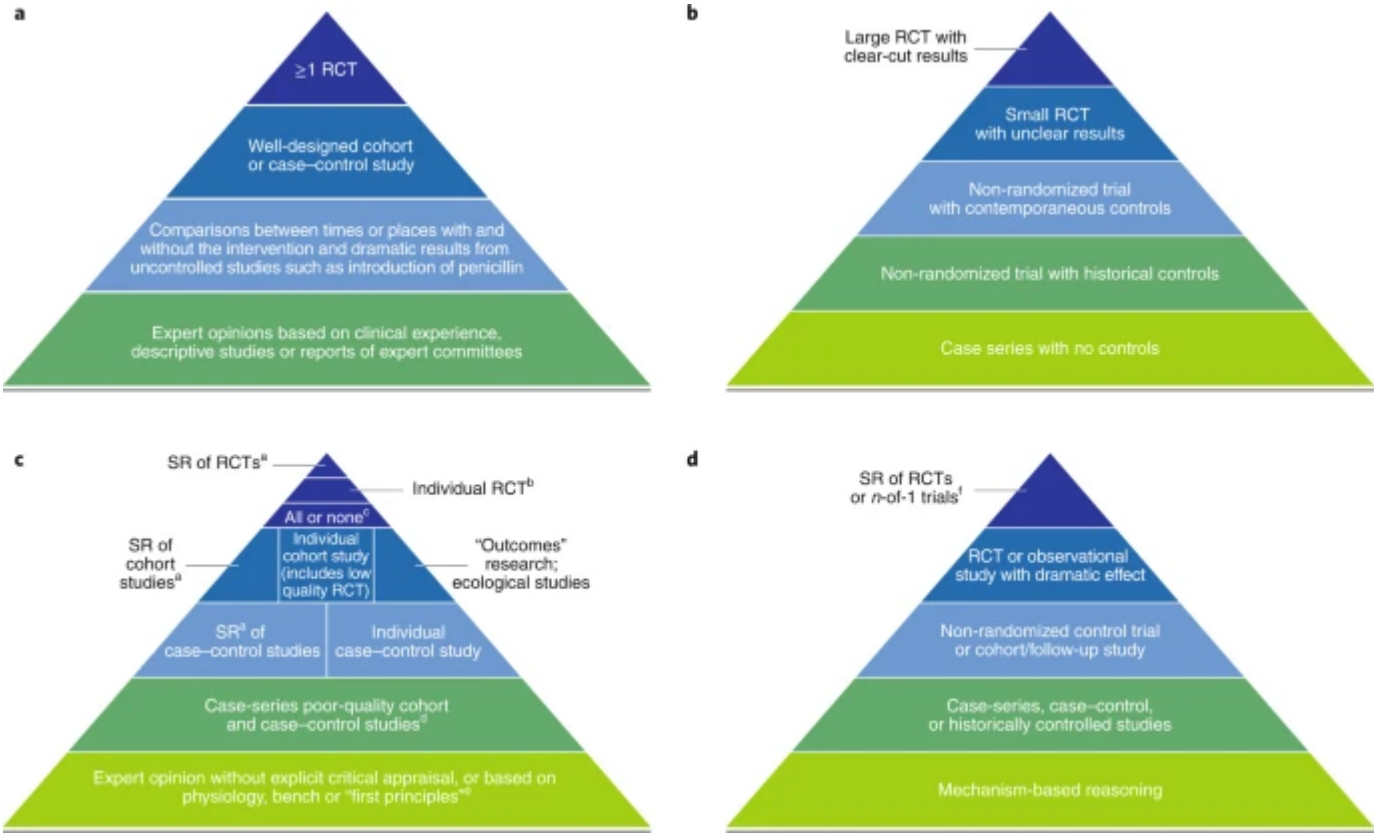
To the Editor — The levels-of-evidence hierarchy stratifies the quality of medical research for evidence-based medicine (EBM) therapeutic decisions (Fig. 1)[1]. Physicians and policy makers are encouraged to find the highest level of evidence to solve clinical questions. An important issue that remains poorly addressed is how to balance levels of evidence and urgency of medical need. This issue is crucial to the discovery of treatments for terminal cancers, as well as other lethal diseases, but has been most recently highlighted by the global turmoil caused by the COVID-19 pandemic and the search for countermeasures[2]. Here, we provide our perspective on updated established levels of evidence[3,4] and discuss the debate regarding a properly balanced strategy for identifying and accessing new treatments for medical emergencies and disease outbreaks.

Fig. 1: Levels of evidence by time period.



a, Canadian Task Force on the Periodic Health Examination's Levels of Evidence (1979)[1]. **b**, Levels of evidence from Sackett (1989)[5]. **c**, OCEBM Levels of Evidence Working Group (March 2009); levels of evidence shown were for therapy[4]. **d**, OCEBM Levels of Evidence Working Group (2011). Levels of evidence shown were for therapy[3]. [a]Systematic review (SR) with homogeneity, meaning one that is free of concerning variations in the directions and degrees of the results. [b]Individual RCTs should have narrow confidence interval. [c]All or none means that all patients died before treatment became available but some now survive on it, or some patients died before the treatment became available but none now die on it. [d]Poor quality means a study that failed to clearly define comparison groups and/or measure exposures and outcomes in the same (preferably blinded) objective way in controls and cases, and/or failed to identify and control for confounders. [e]A first principle is a basic assumption that cannot be deduced from any other proposition or assumption. [f]An *n*-of-1 trial was defined as a type of randomized controlled trial in which a sequence of alternative therapies is randomly given to a patient. The outcomes of regimens are compared, with the aim of deciding on the

optimum treatment for the patient. The OCEBM also clarified that the intended interpretation of the first tier was not either systematic reviews of randomized trials or systematic reviews of *n*-of-1 trials, but rather was "either *n*-of-1 randomized trials or systematic reviews of randomized trials"[3].

The gold standard

Because they attenuate bias by allocating people to arms on the basis of chance alone, randomized controlled trials (RCTs) are always assigned the top rung on the levels-of-evidence ladder[1,3,4,5]. However, not all RCTs are conducted properly, and the conclusions should therefore be carefully scrutinized in the context of the power of studies and of estimating types of errors. Even so, RCTs rightly remain a gold standard for EBM.

Notably, RCTs can also take years to perform. While there were several published RCTs regarding treatments for COVID-19 in 2020, in general these trials take time[6,7,8,9,10]. But serious diseases require more urgent answers, and we may need to weigh the life years lost waiting for conclusions from RCTs versus those of the possible deaths from therapies derived from other types of evidence, should the therapies turn out to be ineffective with longer follow up. In this regard, a survey of all 31 anticancer drugs approved in the United States over 34 years on the basis of the survival surrogate endpoints (response rate or progression-free survival), without a RCT, demonstrated that these agents fared well, with all showing long-term safety and efficacy[11].

Importantly, in the time since the original levels-of-evidence hierarchy was created in 1979 and expanded in 1989 (Fig. 1a,b)[1,5], there has been an evolution in thinking about levels of evidence, as depicted in the updated hierarchies from the Oxford Centre for Evidence-Based Medicine (OCEBM) (Fig. 1c,d)[3,4]. Indeed, in these modernized guidelines, some types of observational studies with striking effects now occupy the first or second tier of the levels-of-evidence pyramid.

In recent years, the digital world has also made remarkable developments. Computerized medical records now give us access to dense data on millions of patients, with the possibility of using real-world observations for drug discovery and even approvals. Indeed, the US Food and Drug Administration (FDA) has already set a precedent by approving two drugs for advanced cancers on the basis of high response rates noted in part or in whole by retrospective data mining or real-world data[12,13]. These considerations are important because of the urgency of certain diseases and in crises, such as the present pandemic.

Moving to next-generation evidence

Increasing adoption of levels of evidence occurred after publications on this topic in 1979 and updates in 1989 (Fig. 1a,b)[1,5]. However, these first-generation levels of evidence have since been extensively reappraised by expert committees[3,4]. Notably, in 2009 and 2011, the OCEBM hierarchies for therapeutic studies (Fig. 1c,d)[3,4] changed the equation for levels of evidence by moving all-or-none studies to the top tier in 2009 (with all-or-none studies implying that all patients perished before treatment existed but some now survive, or that some patients died before the treatment existed, but none now succumbs). Observational studies with dramatic results were raised to the second tier in 2011, thereby upgrading "dramatic results from uncontrolled studies such as introduction of penicillin" from a very low tier in the 1979 levels-of-evidence pyramid (Fig. 1a)[1]. Effectively, these changes established the significance of certain types of high-impact observational studies and recognized a paradigm shift in trial design[14] driven by a variety of rapidly emerging platform technologies, biological tools and digital technologies, while keeping RCTs at the top of the hierarchy.

The earliest levels-of-evidence hierarchies were conceived more than 40 years ago[1], before the advent of modern computer technology. To put this into context, there was no Internet or Google; computers were the size of a large room, used paper punch cards and had only 1 kilobyte of memory. In contrast, a contemporary iPhone may have 4 gigabytes (4 million kilobytes) of random-access memory and 512 gigabytes of storage. Today's most powerful computers have 160 terabytes of random-access memory (160 billion kilobytes), which was unimaginable in the era when levels of evidence were first

developed. This type of digital power provides fertile soil for the growth of new and powerful types of evidence[15].

Structured trials have undergone major changes with the advent of adaptive designs that exploit advanced statistical methodology to optimize understanding of response and toxicity with many fewer patients than in classic RCTs. Modernized evidence now also includes that derived from mining clinical trial databases or real-world electronic medical or insurance records, as well as data from downloadable apps (including rapidly developed direct-to-patient apps to collect self-reported information on COVID-19) processed via machine learning (https://covid.joinzoe.com/us; Fig. 2)[14].

Fig. 2: Emerging technologies fuel new types of trials and evidence.

Advances in powerful genomic sequencing, digital technologies and machine learning have enabled novel trial design. Adaptive trials refer to studies in which data collection and analysis are ongoing throughout the life of the trial, and the number of patients in each arm or other characteristics of the arms are adapted in real time on the basis of that

data. *n*-of-one individualized trial design in this context refers to trials in which each patient receives a different treatment on the basis of that patient's characteristics; the success of the trial is judged by the effectiveness of the strategy to determine treatments, rather than on the effectiveness of any one type of treatment. Real-world data can now be collected from millions of computerized electronic medical or insurance or other similar records and analyzed. A master observation protocol also collects real-world data, but the data may follow a certain preconceived structure for consistency.

Adaptive designs augment clinical trial flexibility by continuously reassessing results accumulating in the trial to modify the trial's course in accordance with prespecified guidelines[16]. Adaptive designs for exploratory clinical trials deal mostly with dose–response modeling and/or with identifying safe and effective doses. In confirmatory trials, the adaptive lexicon encompasses telescoped or seamless phase 1–3 designs, trials with ongoing sample size re-estimation, biomarker-driven adaptive population enrichment studies (allocating a larger proportion of the participants to treatment groups that are performing well and hence minimizing the number of participants in treatment groups that are doing poorly), and adaptive group sequential design (which permits alteration of sample size and/or endpoints during the course of the trial). Adaptive trials can often allow accurate conclusions to be drawn quickly and with much smaller numbers of patients than are needed for standard RCTs, which is particularly important in the case of COVID-19.

Large-scale, rapid evaluation of real-world data has also become a reality, leading to regulatory approvals. For example, the anti-programmed cell death 1 (PD-1) human IgG4 monoclonal antibody Keytruda (pembrolizumab) received FDA approval, in part, from a retrospective, pooled analysis and data mining of five single-arm trials in various tumor types showing an objective response rate of ~40%)[12]. Another example of using real-world data and digital technology for regulatory purposes is the FDA approval of Ibrance (palbociclib), a small-molecule inhibitor of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) for men with breast cancer[13]. The data included information on 2,675 patients that was collected over six years, including analysis from the PALOMA-2 and PALOMA-3 clinical studies, insurance claims, and electronic health records. This was the first oncology approval,

to our knowledge, to have been derived largely or in whole from real-world data without a trial[13]. Going forward, the question is, in health emergencies like COVID-19, can rapid collection and analysis of massive amounts of data find clinically meaningful benefits, without the lengthy process required for a prospective trial?

Recent years have seen the emergence of yet another type of data collection: the master observational trial; for example, the local PREDICT trial at the University of California[17], the IMPACT trial at MD Anderson Cancer Center[18] or the national Master Registry of Oncology Outcomes Associated With Testing and Treatment (ROOT) trial (NCT04028479). The ROOT study, as an example, plans to prospectively follow patients for data collection and allows analysis of biological as well as clinical information[14,19]. The ROOT master observational trial differs from real-world data collection in that the former prospectively structures the data, whereas real-world data collection involves the downloading of information from medical records or other databases.

A related development to the above is the use of smart phone apps for self-reporting by patients in the community. This has been exploited for COVID-19, with the launch on March last year of a free smartphone downloadable app for symptom tracking (https://covid.joinzoe.com/us-2) developed by Zoe Global in collaboration with the Massachusetts General Hospital, King's College London and the University of Nottingham. In a few weeks (from launch until 21 April 2020), an astounding 2,618,862 people (including 2,450,569 from the United Kingdom and 168,293 from the United States) used the app to report COVID-19-relevant symptoms. The app gathers data and tracks, in real time, how the disease progresses by recording self-reported health information on a daily basis: demographics, symptoms, hospitalization, test outcomes and pre-existing medical conditions. The results showed that, among 18,401 individuals who had undergone a SARS-CoV-2 test, the proportion of participants who reported loss of smell and taste was higher in those with a positive test result (4,668 of 7,178 individuals; 65.03%) than in those with a negative test result (2,436 of 11,223 participants; 21.71%) (odds ratio = 6.74; 95% confidence interval = 6.31–7.21)[20]. The model was able to predict COVID-19 infection without patients having to be tested. Using machine learning, the mobile application will also offer data on geographical hot spots, risk factors, harbinger symptoms and clinical outcomes. It

represents a proof of concept for exploiting digital approaches to scale epidemiologic data collection at a remarkable pace[21].

Interestingly, in the most recent version of the OCEBM (Fig. 1d), *n*-of-1 trials, in which there is randomization of treatment in the individual patient, shares the highest and same level of evidence as systematic review of RCTs[3]. The most common form of *n*-of-1 trials uses a multiple-crossover design; multiple exposures to reversible treatments are given in a random order, and the patient's response to each treatment can be compared with each of his or her other responses. These *n*-of-1 studies have been carried out in chronic fatigue, sleep disturbances, reflux disease and depression, for example, but are rarely to never carried out in oncology[22]. Indeed, the classic *n*-of-1 trial typically cannot be applied to aggressive or to acute illnesses because randomizing patients with lethal diseases to multiple treatments, some of which may be ineffective, may result in permanent disability or death.

The classic type of *n*-of-1 trial described above should be differentiated from a distinct new terminology wherein *n*-of-1 refers to individualizing therapy in the precision medicine setting[23]. Using cancer as an example, these types of *n*-of-1 studies acknowledge that metastatic tumors are genomically complex and distinct from each other, indicating that each patient needs a customized combination therapy solution. Thus, the classic analysis that determines how well a drug regimen works in a group of people is not applicable (because each patient receives a different regimen). For these types of *n*-of-1 precision studies, the efficacy of the matching strategy is assessed, rather than the efficacy of any drug or combination of drugs. Effective genomic-sequencing-based matching approaches demonstrate improved outcomes for *n*-of-1 precision medicine studies in patients with lethal malignancies[23] and might be translatable to other complicated diseases that require individualized treatment tactics. In this context, COVID-19 may illustrate the need to individualize the clinical approach on the basis of patient age, type and number or comorbidities, and presenting symptoms, as well as host immune response and genetic background predisposition when data on the latter become available.

Emerging lessons from COVID-19

From when the pandemic was declared on 11 March 2020 to five weeks later, there were 142 studies registered on https://clinicaltrials.gov that were “Recruiting, Active, not recruiting Studies | Interventional Studies | COVID”. For context, in the case of metastatic lung cancer, one of the most lethal cancers, there were 345 studies that were “Recruiting, Active, not recruiting Studies | Interventional Studies | metastatic lung cancer” at that time — but these studies had been opened over a period of years, rather than just a few weeks. The number of deaths per year of lung cancer is ~150,000 in the USA alone[24] and ~2 million globally[25]; COVID-19 has killed >400,000 people in one year in the United States. In essence, in just over a one-month span after the pandemic was declared, the number of active recruiting and non-recruiting interventional studies begun or activated for COVID-19 was already at almost one half that for metastatic lung cancer. Furthermore, these trials are being rapidly published. Indeed, the *New England Journal of Medicine* published three COVID-19 therapeutic trials in the eight weeks after the pandemic was declared (only one of which was a RCT; Table 1)[7,26,27].

Table 1 Clinical trials on COVID-19 treatments published in the *New England Journal of Medicine* from 12 March through 17 July 2020

The need for more speed and efficiency in clinical trial development and completion has been long recognized in the cancer field, with median times to opening trials often being anywhere from 6 months to over 1.5 years and requiring hundreds of administrative steps[28]. The COVID-19 pandemic clearly demonstrates that a road to rapid trial activation, completion and reporting exists. In the wake of the pandemic, this road should be traversable for the benefit of lethal diseases, such as cancer.

Although mechanism-based reasoning occupies the lowest tier of the levels-of-evidence pyramid (Fig. 1d) and is often inadequate by itself to establish a new therapy, such reasoning was the foundation on which clinical trials to advance COVID-19 care was built. From preclinical studies, it was hoped that human immunodeficiency virus (HIV) protease inhibitors like Kaletra (lopinavir and ritonavir) would be efficacious against SARS-CoV-2. In the first reported RCT for a COVID-19 treatment, Kaletra was found, however, to yield no benefit[7]. Preclinical studies[29] also suggested that the endosomal inhibitor

hydroxychloroquine (HCQ) — a drug that also decreases viral budding in vitro and has known anti-inflammatory properties — inhibits viral replication of COVID-19. These observations provided the rationale for trials in the setting of pre-exposure (NCT04334148) and post-exposure (NCT04308668) prophylaxis in healthcare workers. Arguments were also made for interleukin-6 (IL-6) inhibitors (Actemra (tocilizumab), Kevzara (sarilumab) and Sylvant (siltuximab)) on the basis of their ability to suppress cytokine storm in severe COVID-19. The race to find treatments for COVID-19 led to the FDA's decision to grant Emergency Use Authorizations for HCQ in April, although it was revoked in June. As yet the antiviral Veklury (remdesivir) is the only drug to receive a full approval for COVID-19, on the basis of three RCTs.

Data are also being curated at record pace and prepublished before peer review, as well as having been published after peer review in prominent medical journals within weeks of the start of the pandemic. Rapid review should not, however, mean a compromise of reproducibility and transparency standards, an issue that arose when *The Lancet* and *The New England Journal of Medicine* published COVID-19 papers that required retraction. On the other hand, many rapidly published papers provide urgently needed data. For instance, observations from compassionate use of Veklury showed that 57% (17 of 30) of previously mechanically ventilated patients were extubated[26]. An observational study on 1,376 patients, also published in *The New England Journal of Medicine* (less than 2 months after the pandemic was announced), showed that there was no significant association between HCQ use and intubation[27]. Ultimately, these preliminary observations must be explored in RCTs, but they still provide important evidence in a pressing situation.

The pursuit of a COVID-19 therapy is unveiling the capability to rapidly investigate and deploy medications, which could be a lasting positive legacy of the pandemic. Indeed, several aspects of the COVID-19 reaction highlight how the slow processes for conceiving and activating clinical trials, as well as evaluating and approving drugs, can become immensely more efficient during a public health crisis. These clinical trials and publications, with 142 interventional studies registered on clinicaltrials.gov within five weeks of the declaration of the pandemic and three therapeutic studies published in *The New England Journal of Medicine* within eight weeks (Table 1), have been fast-tracked on the basis of the perceived emergency generated by the COVID-19 situation. The search for

a COVID-19 treatment has been fueled by a mechanism-based understanding of COVID-19 biology, as well as anecdotal reports (ironically, the lowest tiers in the OCEBM levels-of-evidence pyramid for therapies; Fig. 1d)[3,4].

Newer forms of evidence are also being interrogated. For instance, in a program providing rapid access to compassionate use of the antiviral Veklury, ~60% of patients hospitalized for severe COVID-19 demonstrated improvement, a finding that was quickly disseminated by publication[26]. These findings also raise the possibility of implementing master observational studies for COVID-19, as has been proposed for cancer with clinical trials such as ROOT that plan large-scale structured data acquisition in an observational setting[14,19]. In addition, acquisition of real-world data by exploiting digital technology to download medical or insurance records or to mine clinical trial databases has also led to approvals in cancer[12,13] and may provide rapid access to important information related to COVID-19 therapeutic effectiveness. It is understood that some of the studies that are ongoing or proposed for COVID-19 are not RCTs and, therefore, while providing proof of concept, may still need to be confirmed by RCTs. Still, it is critical to appreciate how our response to COVID-19 has demonstrated that we do not need to become mired in old or misinterpreted dogma concerning levels-of-evidence rankings to advance a field where there is urgency.

It is also important to recognize that levels-of-evidence hierarchies have been extensively updated since their earliest renditions, 30 to 40 years ago[1,5]. Indeed, in 2009 and 2011, the OCEBM levels-of-evidence pyramid for treatment studies (Fig. 1c,d)[3,4] raised several forms of non-RCT with dramatic effects to the top evidence tiers. For these types of observations, therapeutic efficacy may be such that randomization to a control arm may not be ethical[30]. The key is to balance the risk of authorizing a therapy that may later be disproven versus that of delaying adoption of a life-saving therapy by requiring a RCT that would likely take years to perform[30]. Indeed, there are quantifiable threshold values above which it is highly likely that effectiveness seen in non-randomized trials will consistently translate to improved survival.

In summary, contemporary levels-of-evidence hierarchies have already been broadened to acknowledge the important role played by non-RCTs (Fig. 1). Furthermore, powerful digital and

molecular technologies exist today that were inconceivable when the earliest levels of evidence were formulated, over 40 years ago[1]. Newer types of evidence are being exploited, including real-world data and the use of genomic sequencing and mechanism-based reasoning to select cancer patients for matched gene- and immune-targeted treatments. The COVID-19 pandemic has revealed that we can exploit novel types of evidence, including those generated by observational studies (Table 1) and by digital technologies, including downloadable apps. The latter can produce clinically relevant information self-reported by millions of individuals within a few weeks[20,21]. In all, the COVID-19 pandemic has shown that we must balance scientific rigor, reflected by classic levels of evidence, with the need for urgency. The lessons learned may expedite the discovery of important treatments for other deadly diseases.

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Ethics declarations

Competing interests

R.K. discloses the following: stock and other equity interests, IDbyDNA, CureMatch, Inc. and Soluventis; consulting or advisory role, Gaido, LOXO, X-Biotech, Actuate Therapeutics, Roche, NeoMed and Soluventis; speaker's fee, Roche; research funding, Incyte, Genentech, Merck Serono, Pfizer, Sequenom, Foundation Medicine, Guardant Health, Grifols, Konica Minolta and OmniSeq (all institutional); board member and cofounder, CureMatch, Inc; board member, CureMetrix.

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Exhibit B

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NEWS FEATURE | 06 October 2020

Face masks: what the data say

The science supports that face coverings are saving lives during the coronavirus pandemic, and yet the debate trundles on. How much evidence is enough?

Lynne Peeples

Illustration by Bex Glendining

When her Danish colleagues first suggested distributing protective cloth face masks to people in Guinea-Bissau to stem the spread of the coronavirus, Christine Benn wasn't so sure.

"I said, 'Yeah, that might be good, but there's limited data on whether face masks are actually effective,'" says Benn, a global-health researcher at the University of Southern Denmark in Copenhagen, who for decades has co-led public-health campaigns in the West African country, one of the world's poorest.

That was in March. But by July, Benn and her team had worked out how to possibly provide some needed data on masks, and hopefully help people in Guinea-Bissau. They distributed thousands of locally produced cloth face coverings to people as part of a randomized controlled trial that might be the world's largest test of masks' effectiveness against the spread of COVID-19.

Face masks are the ubiquitous symbol of a pandemic that has sickened 35 million people and killed more than 1 million. In hospitals and other health-care facilities, the use of medical-grade masks clearly cuts down transmission of the SARS-CoV-2 virus. But for the variety of masks in use by the public, the data are messy, disparate and often hastily assembled. Add to that a divisive political discourse that included a US president disparaging their use, just days before being diagnosed with COVID-19 himself. "People looking at the evidence are understanding it differently," says Baruch Fischhoff, a psychologist at Carnegie Mellon University in Pittsburgh, Pennsylvania, who specializes in public policy. "It's legitimately confusing."

RELATED

To be clear, the science supports using masks, with recent studies suggesting that they could save lives in different ways: research shows that they cut down the chances of both transmitting and catching the coronavirus, and

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some studies hint that masks might reduce the severity of infection if people do contract the disease.

But being more definitive about how well they work or when to use them gets complicated. There are many types of mask, worn in a variety of environments. There are questions about people's willingness to wear them, or wear them properly. Even the question of what kinds of study would provide definitive proof that they work is hard to answer.

"How good does the evidence need to be?" asks Fischhoff. "It's a vital question."

Beyond gold standards

At the beginning of the pandemic, medical experts lacked good evidence on how SARS-CoV-2 spreads, and they didn't know enough to make strong public-health recommendations about masks.

The standard mask for use in health-care settings is the N95 respirator, which is designed to protect the wearer by filtering out 95% of airborne particles that measure 0.3 micrometres (μm) and larger. As the pandemic ramped up, these respirators quickly fell into short supply. That raised the now contentious question: should members of the public bother wearing basic surgical masks or cloth masks? If so, under what conditions? "Those are the things we normally [sort out] in clinical trials," says Kate Grabowski, an infectious-disease epidemiologist at Johns Hopkins School of Medicine in Baltimore, Maryland. "But we just didn't have time for that."

So, scientists have relied on observational and laboratory studies. There is also indirect evidence from other infectious diseases. "If you look at any one paper — it's not a slam dunk. But, taken all together, I'm convinced that they are working," says Grabowski.

RELATED

Confidence in masks grew in June with news about two hair stylists in Missouri who tested positive for COVID-19[1].

Mounting evidence suggests coronavirus is airborne — but health advice has not caught up

Both wore a double-layered cotton face covering or surgical mask while working. And although they passed on the infection to members of their households, their clients seem to have been spared (more than half reportedly declined free tests). Other hints of effectiveness emerged from mass gatherings. At Black Lives Matter protests in US cities, most attendees wore masks. The events did not seem to trigger spikes in infections[2], yet the virus ran rampant in late June at a Georgia summer camp, where children who attended were not required to wear face coverings[3]. Caveats abound: the protests were outdoors, which poses a lower risk of COVID-19 spread, whereas the campers shared cabins at night, for example. And because many non-protesters stayed in their homes during the gatherings, that might have reduced virus transmission in the community. Nevertheless, the anecdotal evidence "builds up the picture", says Theo Vos, a health-policy researcher at the University of Washington in Seattle.

More-rigorous analyses added direct evidence. A preprint study[4] posted in early August (and not yet peer reviewed), found that weekly increases in per-capita mortality were four times lower in places where masks were the norm or recommended by the government, compared with other regions. Researchers looked at 200 countries, including Mongolia, which adopted mask use in January and, as of May, had recorded no deaths related to COVID-19. Another study[5] looked at the effects of US state-government mandates for mask use in April and May. Researchers estimated that those reduced the growth of COVID-19 cases by up to 2 percentage points per day. They cautiously suggest that mandates might have averted as many as 450,000 cases, after controlling for other mitigation measures, such as physical distancing.

"You don't have to do much math to say this is obviously a good idea," says Jeremy Howard, a research scientist at the University of San Francisco in California, who is part

of a team that reviewed the evidence for wearing face masks in a preprint article that has been widely circulated[6].

But such studies do rely on assumptions that mask mandates are being enforced and that people are wearing them correctly. Furthermore, mask use often coincides with other changes, such as limits on gatherings. As restrictions lift, further observational studies might begin to separate the impact of masks from those of other interventions, suggests Grabowski. “It will become easier to see what is doing what,” she says.

Although scientists can’t control many confounding variables in human populations, they can in animal studies. Researchers led by microbiologist Kwok-Yung Yuen at the University of Hong Kong housed infected and healthy hamsters in adjoining cages, with surgical-mask partitions separating some of the animals. Without a barrier, about two-thirds of the uninfected animals caught SARS-CoV-2, according to the paper[7] published in May. But only about 25% of the animals protected by mask material got infected, and those that did were less sick than their mask-free neighbours (as measured by clinical scores and tissue changes).

The findings provide justification for the emerging consensus that mask use protects the wearer as well as other people. The work also points to another potentially game-changing idea: “Masking may not only protect you from infection but also from severe illness,” says Monica Gandhi, an infectious-disease physician at the University of California, San Francisco.

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COVID has killed more than one million people. How many more will die?

Gandhi co-authored a paper[8] published in late July suggesting that masking reduces the dose of virus a wearer might receive, resulting in infections that are milder or even asymptomatic. A larger viral dose results in a more aggressive inflammatory response, she suggests.

She and her colleagues are currently analysing hospitalization rates for COVID-19 before and after mask mandates in 1,000 US counties, to determine whether the severity of disease decreased after public masking guidelines were brought in.

The idea that exposure to more virus results in a worse infection makes "absolute sense", says Paul Digard, a virologist at the University of Edinburgh, UK, who was not involved in the research. "It's another argument for masks."

Gandhi suggests another possible benefit: if more people get mild cases, that might help to enhance immunity at the population level without increasing the burden of severe illness and death. "As we're awaiting a vaccine, could driving up rates of asymptomatic infection do good for population-level immunity?" she asks.

Back to ballistics

The masks debate is closely linked to another divisive question: how does the virus travel through the air and spread infection?

The moment a person breathes or talks, sneezes or coughs, a fine spray of liquid particles takes flight. Some are large — visible, even — and referred to as droplets; others are microscopic, and categorized as aerosols. Viruses including SARS-CoV-2 hitch rides on these particles; their size dictates their behaviour.

Droplets can shoot through the air and land on a nearby person's eyes, nose or mouth to cause infection. But gravity quickly pulls them down. Aerosols, by contrast, can float in the air for minutes to hours, spreading through an unventilated room like cigarette smoke.


Visualization of the droplet spread when an N95 mask equipped with an exhalation port is used to impede the emerging jet.

Time-lapse images show how cough droplets spread from a person wearing an N95 mask that has a valve to expel exhaled air. Credit: S. Verma *et al.*/Phys. Fluids

What does this imply for the ability of masks to impede COVID-19 transmission? The virus itself is only about 0.1 μm in diameter. But because viruses don't leave the body on their own, a mask doesn't need to block particles that small to be effective. More relevant are the pathogen-transporting droplets and aerosols, which range from about 0.2 μm to hundreds of micrometres across. (An average human hair has a diameter of about 80 μm.) The majority are 1–10 μm in diameter and can linger in the air a long time, says Jose-Luis Jimenez, an environmental chemist at the University of Colorado Boulder. "That is where the action is."

Scientists are still unsure which size of particle is most important in COVID-19 transmission. Some can't even agree on the cut-off that should define aerosols. For the same reasons, scientists still don't know the major form of transmission for influenza, which has been studied for much longer.

Many believe that asymptomatic transmission is driving much of the COVID-19 pandemic, which would suggest that viruses aren't typically riding out on coughs or sneezes. By this reasoning, aerosols could prove to be the most important transmission vehicle. So, it is worth looking at which masks can stop aerosols.

All in the fabric

Even well-fitting N95 respirators fall slightly short of their 95% rating in real-world use, actually filtering out around 90% of incoming aerosols down to 0.3 μm. And, according to unpublished research, N95 masks that don't have exhalation valves — which expel unfiltered exhaled air — block a similar proportion of outgoing aerosols. Much less is known about surgical and cloth masks, says Kevin Fennelly, a pulmonologist at the US National Heart, Lung, and Blood Institute in Bethesda, Maryland.

In a review[9] of observational studies, an international research team estimates that surgical and comparable cloth masks are 67% effective in protecting the wearer.

In unpublished work, Linsey Marr, an environmental engineer at Virginia Tech in Blacksburg, and her colleagues found that even a cotton T-shirt can block half of inhaled aerosols and almost 80% of exhaled aerosols measuring 2 μm across. Once you get to aerosols of 4–5 μm, almost any fabric can block more than 80% in both directions, she says.

Multiple layers of fabric, she adds, are more effective, and the tighter the weave, the better. Another study[10] found that masks with layers of different materials — such as cotton and silk — could catch aerosols more efficiently than those made from a single material.

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Fast coronavirus tests: what they can and can't do

Benn worked with Danish engineers at her university to test their two-layered cloth mask design using the same criteria as for medical-grade ventilators. They found that their mask blocked only 11–19% of aerosols down to the 0.3 μm mark, according to Benn. But because most transmission is probably occurring through particles of at least 1 μm, according to Marr and Jimenez, the actual difference in effectiveness between N95 and other masks might not be huge.

Eric Westman, a clinical researcher at Duke University School of Medicine in Durham, North Carolina, co-authored an August study[11] that demonstrated a method for testing mask effectiveness. His team used lasers and smartphone cameras to compare how well 14 different cloth and surgical face coverings stopped droplets while a person spoke. "I was reassured that a lot of the masks we use did work," he says, referring to the performance of cloth and surgical masks. But thin polyester-and-spandex neck gaiters — stretchable scarves that can be pulled up over the mouth and nose — seemed to

actually reduce the size of droplets being released. “That could be worse than wearingD nothing at all,” Westman says.

Some scientists advise not making too much of the finding, which was based on justD one person talking. Marr and her team were among the scientists who responded withD experiments of their own, finding that neck gaiters blocked most large droplets. MarrD says she is writing up her results for publication.

“There’s a lot of information out there, but it’s confusing to put all the lines of evidenceD together,” says Angela Rasmussen, a virologist at Columbia University’s MailmanD School of Public Health in New York City. “When it comes down to it, we still don’t knowD a lot.”

Minding human minds

Questions about masks go beyond biology, epidemiology and physics. HumanD behaviour is core to how well masks work in the real world. “I don’t want someone whoD is infected in a crowded area being confident while wearing one of these clothD coverings,” says Michael Osterholm, director of the Center for Infectious DiseaseD Research and Policy at the University of Minnesota in Minneapolis.

Baseball players, one batting & one catching, and umpire standing behind, wearingD masks during the 1918 influenza pandemic

US baseball players wore masks while playing during the 1918 influenza epidemic. Credit: Underwood And Underwood/LIFE Images Collection/Getty

Perhaps fortunately, some evidence[12] suggests that donning a face mask might drive the wearer and those around them to adhere better to other measures, such as social distancing. The masks remind them of shared responsibility, perhaps. But that requires that people wear them.

Across the United States, mask use has held steady around 50% since late July. This is a substantial increase from the 20% usage seen in March and April, according to data from the Institute for Health Metrics and Evaluation at the University of Washington in Seattle (see go.nature.com/30n6kxv). The institute's models also predicted that, as of 23 September, increasing US mask use to 95% – a level observed in Singapore and some other countries – could save nearly 100,000 lives in the period up to 1 January 2021.

"There's a lot more we would like to know," says Vos, who contributed to the analysis. "But given that it is such a simple, low-cost intervention with potentially such a large impact, who would not want to use it?"

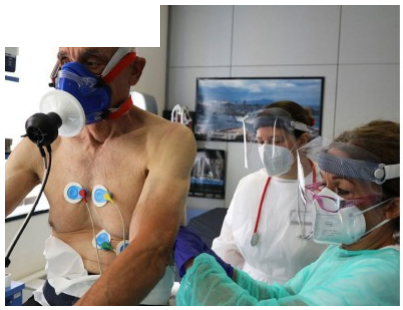
Further confusing the public are controversial studies and mixed messages. One study[13] in April found masks to be ineffective, but was retracted in July. Another, published in June[14], supported the use of masks before dozens of scientists wrote a letter attacking its methods (see go.nature.com/3jpvxpt). The authors are pushing back against calls for a retraction. Meanwhile, the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC) initially refrained from recommending widespread mask usage, in part because of some hesitancy about depleting supplies for health-care workers. In April, the CDC recommended that masks be worn when physical distancing isn't an option; the WHO followed suit in June.

There's been a lack of consistency among political leaders, too. US President Donald Trump voiced support for masks, but rarely wore one. He even ridiculed political rival Joe Biden for consistently using a mask – just days before Trump himself tested positive for the coronavirus, on 2 October. Other world leaders, including the president and prime minister of Slovakia, Zuzana Čaputová and Igor Matovič, sported masks early in the pandemic, reportedly to set an example for their country.

Denmark was one of the last nations to mandate face masks – requiring their use on public transport from 22 August. It has maintained generally good control of the virus through early stay-at-home orders, testing and contact tracing. It is also at the

forefront of COVID-19 face-mask research, in the form of two large, randomlyD controlled trials. A research group in Denmark enrolled some 6,000 participants,D asking half to use surgical face masks when going to a workplace. Although the study isD completed, Thomas Benfield, a clinical researcher at the University of Copenhagen andD one of the principal investigators on the trial, says that his team is not ready to shareD any results.

RELATED



The lasting misery of coronavirus long-haulers

Benn's team, working independently of Benfield's group, is in the process of enrolling around 40,000 people in Guinea-Bissau, randomly selecting half of the households to receive bilayer cloth masks — two for each family member aged ten or over. The team will then follow everyone over several months to compare rates of mask use with rates of COVID-like illness. She notes that each household will receive advice on how to protect themselves from COVID-19 — except that those in the control group will not get information on the use of masks. The team expects to complete enrolment in November.

Several scientists say that they are excited to see the results. But others worry that such experiments are wasteful and potentially exploit a vulnerable population. "If this was a gentler pathogen, it would be great," says Eric Topol, director of the Scripps Research Translational Institute in La Jolla, California. "You can't do randomized trials for everything — and you shouldn't." As clinical researchers are sometimes fond of saying, parachutes have never been tested in a randomized controlled trial, either.

But Benn defends her work, explaining that people in the control group will still benefit from information about COVID-19, and they will get masks at the end of the study. Given the challenge of manufacturing and distributing the masks, "under no circumstances", she says, could her team have handed out enough for everyone at the study's outset. In fact, they had to scale back their original plans to enrol 70,000

people. She is hopeful that the trial will provide some benefits for everyone involved. "But no one in the community should be worse off than if we hadn't done this trial," she says. The resulting data, she adds, should inform the global scientific debate.

For now, Osterholm, in Minnesota, wears a mask. Yet he laments the "lack of scientific rigour" that has so far been brought to the topic. "We criticize people all the time in the science world for making statements without any data," he says. "We're doing a lot of the same thing here."

Nevertheless, most scientists are confident that they can say something prescriptive about wearing masks. It's not the only solution, says Gandhi, "but I think it is a profoundly important pillar of pandemic control". As Digard puts it: "Masks work, but they are not infallible. And, therefore, keep your distance."

Nature **586**, 186-189 (2020)

doi: https://doi.org/10.1038/d41586-020-02801-8

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Exhibit C

Centers for Disease Control and Prevention

ISSN: 1080-6059

Volume 26, Number 5—May 2020

Policy Review

Nonpharmaceutical Measures for Pandemic Influenza in Nonhealthcare Settings—Personal Protective and Environmental Measures

On This Page

Figures

Tables

Downloads

Article

Appendix

Article & Appendix

RIS [TXT - 2 KB]

Article Metrics

Metric Details

Related Articles

Jingyi Xiao[1], Eunice Y. C. Shiu[1], Huizhi Gao, Jessica Y. Wong, Min W. Fong, Sukhyun Ryu, and Benjamin J. Cowling

Author affiliations: University of Hong Kong, Hong Kong, China

Cite This Article

Abstract

There were 3 influenza pandemics in the 20th century, and there has been 1 so far in the 21st century. Local, national, and international health authorities regularly update their plans for mitigating the next influenza pandemic in light of the latest available evidence on the effectiveness of various control measures in reducing transmission. Here, we review the evidence base on the effectiveness of nonpharmaceutical personal protective measures and environmental hygiene measures in nonhealthcare settings and discuss their potential inclusion in pandemic plans. Although mechanistic studies support the potential effect of hand hygiene or face masks, evidence from 14 randomized controlled trials of these measures did not support a substantial effect on transmission of laboratory-confirmed influenza. We similarly found limited evidence on the effectiveness of improved hygiene and environmental cleaning. We identified several major knowledge gaps requiring further research, most fundamentally an improved characterization of the modes of person-to-person transmission.

Influenza pandemics occur at irregular intervals when new strains of influenza A virus spread in humans (*1*). Influenza pandemics cause considerable health and social impact that exceeds that of typical seasonal (interpandemic) influenza epidemics. One of the characteristics of influenza pandemics is the high incidence of infections in all age groups because of the lack of population immunity. Although influenza vaccines are the cornerstone of seasonal influenza control, specific vaccines for a novel pandemic strain are not expected to be available for the first 5–6 months of the next pandemic. Antiviral drugs will be available in some locations to treat more severe infections but are unlikely to be available in the quantities that might be required to control transmission in the general community. Thus, efforts to control the next pandemic will rely largely on nonpharmaceutical interventions.

Most influenza virus infections cause mild and self-limiting disease; only a small fraction of case-patients require hospitalization. Therefore, influenza virus infections spread mainly in the community. Influenza virus is believed to be transmitted predominantly by respiratory droplets, but the size distribution of particles responsible for transmission remains unclear, and in particular, there is a lack of consensus on the role of fine particle aerosols in transmission (*2*,*3*). In healthcare settings, droplet precautions are recommended in addition to standard precautions for healthcare personnel when interacting with influenza patients and for all visitors during influenza seasons (*4*). Outside healthcare settings, hand hygiene is recommended in most national pandemic plans (*5*), and medical face masks were a common sight during the influenza pandemic in 2009. Hand hygiene has been proven to prevent many infectious diseases and might be considered a major component in influenza pandemic plans, whether or not it has proven effectiveness against influenza virus transmission, specifically because of its potential to reduce other infections and thereby reduce pressure on healthcare services.

In this article, we review the evidence base for personal protective measures and environmental hygiene measures, and specifically the evidence for the effectiveness of these measures in reducing transmission of laboratory-confirmed influenza in the community. We also discuss the implications of the evidence base for inclusion of these measures in pandemic plans.

Methods and Results

We conducted systematic reviews to evaluate the effectiveness of personal protective measures on influenza virus transmission, including hand hygiene, respiratory etiquette, and face masks, and a systematic review of surface and object cleaning as an environmental measure (Table 1). We searched 4 databases (Medline, PubMed, EMBASE, and CENTRAL) for literature in all languages. We aimed to identify randomized controlled trials (RCTs) of each measure for laboratory-confirmed influenza outcomes for each of the measures because RCTs provide the highest quality of evidence. For respiratory etiquette and surface and object cleaning, because of a lack of RCTs for laboratory-confirmed influenza, we also searched for RCTs reporting effects of these interventions on influenza-like illness (ILI) and respiratory illness outcomes and then for observational studies on laboratory-confirmed influenza, ILI, and respiratory illness outcomes. For each review, 2 authors (E.Y.C.S. and J.X.) screened titles and abstracts and reviewed full texts independently.

We performed meta-analysis for hand hygiene and face mask interventions and estimated the effect of these measures on laboratory-confirmed influenza prevention by risk ratios (RRs). We used a fixed-effects model to estimate the overall effect in a pooled analysis or subgroup analysis. No overall effect would be generated if there was considerable heterogeneity on the basis of I^2 statistic $\geq$75% (*6*). We performed quality assessment of evidence on hand hygiene and face mask interventions by using the GRADE (Grading of Recommendations Assessment, Development and Evaluation) approach (*7*). We provide additional details of the search strategies, selection of articles, summaries of the selected articles, and quality assessment (Appendix).

Personal Protective Measures

Hand Hygiene

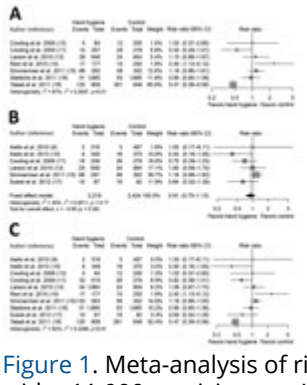

Figure 1. Meta-analysis of risk ratios for the effect of hand hygiene with or without face mask use on laboratory-confirmed influenza from 10 randomized controlled trials with >11,000 participants. A) Hand hygiene alone;...

We identified a recent systematic review by Wong et al. on RCTs designed to assess the efficacy of hand hygiene interventions against transmission of laboratory-confirmed influenza (*8*). We used this review as a starting point and then searched for additional literature published after 2013; we found 3 additional eligible articles published during the search period of January 1, 2013–August 13, 2018. In total, we identified 12 articles (*9*–*20*), of which 3 articles were from the updated search and 9 articles from Wong et al. (*8*). Two articles relied on the same underlying dataset (*16*,*19*); therefore, we counted these 2 articles as 1 study, which resulted in 11 RCTs. We further selected 10 studies with >10,000 participants for inclusion in the meta-analysis (Figure 1). We excluded 1 study from the meta-analysis because it provided estimates of infection risks only at the household level, not the individual level (*20*). We did not generate an overall pooled effect of hand hygiene only or of hand hygiene with or without face mask because of high heterogeneity in individual estimates (I^2 87 and 82%, respectively). The effect of hand hygiene combined with face masks on laboratory-confirmed influenza was not statistically significant (RR 0.91, 95% CI 0.73–1.13; I^2 = 35%, p = 0.39). Some studies reported being underpowered because of limited sample size, and low adherence to hand hygiene interventions was observed in some studies.

We further analyzed the effect of hand hygiene by setting because transmission routes might vary in different settings. We found 6 studies in household settings examining the effect of hand hygiene with or without face masks, but the overall pooled effect was not statistically significant (RR 1.05, 95% CI 0.86–1.27; I^2 = 57%, p = 0.65) (Appendix Figure 4) (*11*–*15*,*17*). The findings of 2 studies in school settings were different (Appendix Figure 5). A study conducted in the United States (*16*) showed no major effect of hand hygiene, whereas a study in Egypt (*18*) reported that hand hygiene reduced the risk for influenza by >50%. A pooled analysis of 2 studies in university residential halls reported a marginally significant protective effect of a combination of hand hygiene plus face masks worn by all residents (RR 0.48, 95% CI 0.21–1.08; I^2 = 0%, p = 0.08) (Appendix Figure 6) (*9*,*10*).

In support of hand hygiene as an effective measure, experimental studies have reported that influenza virus could survive on human hands for a short time and could transmit between hands and contaminated surfaces (*2*,*21*). Some field studies reported that influenza A(H1N1)pdm09 and influenza A(H3N2) virus RNA and viable influenza virus could be detected on the hands of persons with laboratory-confirmed influenza (*22*,*23*), supporting the potential of direct and indirect contact transmission to play a role in the spread of influenza. Other experimental studies also demonstrated that hand hygiene could reduce or remove infectious influenza virus from human hands (*24*,*25*). However, results from our meta-analysis on RCTs did not provide evidence to support a protective effect of hand hygiene against transmission of laboratory-confirmed influenza. One study did report a major effect, but in this trial of hand hygiene in schools in Egypt, running water had to be installed and soap and hand-drying material had to be introduced into the intervention schools as part of the project (*18*). Therefore, the impact of hand hygiene might also be a reflection of the introduction of soap and running water into primary schools in a lower-income setting. If one considers all of the evidence from RCTs together, it is useful to note that some studies might have underestimated the true effect of hand hygiene because of the complexity of implementing these intervention studies. For instance, the control group would not typically have zero knowledge or use of hand hygiene, and the intervention group might not adhere to optimal hand hygiene practices (*11*,*13*,*15*).

Hand hygiene is also effective in preventing other infectious diseases, including diarrheal diseases and some respiratory diseases (*8*,*26*). The need for hand hygiene in disease prevention is well recognized among most communities. Hand hygiene has been accepted as a personal protective measure in >50% of national preparedness plans for pandemic influenza (*5*). Hand hygiene practice is commonly performed with soap and water, alcohol-based hand rub, or other waterless hand disinfectants, all of which are easily accessible, available, affordable, and well accepted in most communities. However, resource limitations in some areas are a concern when clean running water or alcohol-based hand rub are not available. There are few adverse effects of hand hygiene except for skin irritation caused by some hand hygiene products (*27*). However,

because of certain social or religious practices, alcohol-based hand sanitizers might not be permitted in some locations (*28*). Compliance with proper hand hygiene practice tends to be low because habitual behaviors are difficult to change (*29*). Therefore, hand hygiene promotion programs are needed to advocate and encourage proper and effective hand hygiene.

Respiratory Etiquette

Respiratory etiquette is defined as covering the nose and mouth with a tissue or a mask (but not a hand) when coughing or sneezing, followed by proper disposal of used tissues, and proper hand hygiene after contact with respiratory secretions (*30*). Other descriptions of this measure have included turning the head and covering the mouth when coughing and coughing or sneezing into a sleeve or elbow, rather than a hand. The rationale for not coughing into hands is to prevent subsequent contamination of other surfaces or objects (*31*). We conducted a search on November 6, 2018, and identified literature that was available in the databases during 1946–November 5, 2018. We did not identify any published research on the effectiveness of respiratory etiquette in reducing the risk for laboratory-confirmed influenza or ILI. One observational study reported a similar incidence rate of self-reported respiratory illness (defined by ≥1 symptoms: cough, congestion, sore throat, sneezing, or breathing problems) among US pilgrims with or without practicing respiratory etiquette during the Hajj (*32*). The authors did not specify the type of respiratory etiquette used by participants in the study. A laboratory-based study reported that common respiratory etiquette, including covering the mouth by hands, tissue, or sleeve/arm, was fairly ineffective in blocking the release and dispersion of droplets into the surrounding environment on the basis of measurement of emitted droplets with a laser diffraction system (*31*).

Respiratory etiquette is often listed as a preventive measure for respiratory infections. However, there is a lack of scientific evidence to support this measure. Whether respiratory etiquette is an effective nonpharmaceutical intervention in preventing influenza virus transmission remains questionable, and worthy of further research.

Face Masks

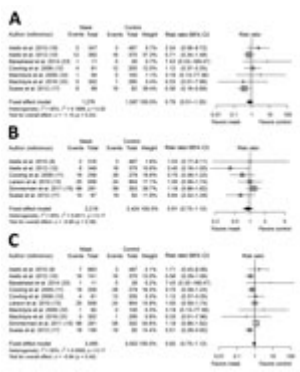

Figure 2. Meta-analysis of risk ratios for the effect of face mask use with or without enhanced hand hygiene on laboratory-confirmed influenza from 10 randomized controlled trials with >6,500 participants. A) Face mask...

In our systematic review, we identified 10 RCTs that reported estimates of the effectiveness of face masks in reducing laboratory-confirmed influenza virus infections in the community from literature published during 1946–July 27, 2018. In pooled analysis, we found no significant reduction in influenza transmission with the use of face masks (RR 0.78, 95% CI 0.51–1.20; I^2 = 30%, p = 0.25) (Figure 2). One study evaluated the use of masks among pilgrims from Australia during the Hajj pilgrimage and reported no major difference in the risk for laboratory-confirmed influenza virus infection in the control or mask group (*33*). Two studies in university settings assessed the effectiveness of face masks for primary protection by monitoring the incidence of laboratory-confirmed influenza among student hall residents for 5 months (*9*,*10*). The overall reduction in ILI or laboratory-confirmed influenza cases in the face mask group was not significant in either studies (*9*,*10*). Study designs in the 7 household studies were slightly different: 1 study provided face masks and P2 respirators for household contacts only (*34*), another study evaluated face mask use as a source control for infected persons only (*35*), and the remaining studies provided masks for the infected persons as well as their close contacts (*11*–*13*,*15*,*17*). None of the household studies reported a significant reduction in secondary laboratory-confirmed influenza virus infections in the face mask group (*11*–*13*,*15*,*17*,*34*,*35*). Most studies were underpowered because of limited sample size, and some studies also reported suboptimal adherence in the face mask group.

Disposable medical masks (also known as surgical masks) are loose-fitting devices that were designed to be worn by medical personnel to protect accidental contamination of patient wounds, and to protect the wearer against splashes or sprays of bodily fluids (*36*). There is limited evidence for their effectiveness in preventing influenza virus transmission either when worn by the infected person for source control or when worn by uninfected persons to reduce exposure. Our systematic review found no significant effect of face masks on transmission of laboratory-confirmed influenza.

We did not consider the use of respirators in the community. Respirators are tight-fitting masks that can protect the wearer from fine particles (*37*) and should provide better protection against influenza virus exposures when properly worn because of higher filtration efficiency. However, respirators, such as N95 and P2 masks, work best when they are fit-tested, and these masks will be in limited supply during the next pandemic. These specialist devices should be reserved for use in healthcare settings or in special subpopulations such as immunocompromised persons in the community, first responders, and those performing other critical community functions, as supplies permit.

In lower-income settings, it is more likely that reusable cloth masks will be used rather than disposable medical masks because of cost and availability (*38*). There are still few uncertainties in the practice of face mask use, such as who should wear the mask and how long it should be used for. In theory, transmission should be reduced the most if both infected members and other contacts wear masks, but compliance in uninfected close contacts could be a problem (*12*,*34*). Proper use of face masks is essential because improper use might increase the risk for transmission (*39*). Thus, education on the proper use and disposal of used face masks, including hand hygiene, is also needed.

Environmental Measures

Surface and Object Cleaning

For the search period from 1946 through October 14, 2018, we identified 2 RCTs and 1 observational study about surface and object cleaning measures for inclusion in our systematic review (*40*–*42*). One RCT conducted in day care nurseries found that biweekly cleaning and disinfection of toys and linen reduced the detection of multiple viruses, including adenovirus, rhinovirus, and respiratory syncytial virus in the environment, but this intervention was not significant in reducing detection of influenza virus, and it had no major protective effect on acute respiratory illness (*41*). Another RCT found that hand hygiene with hand sanitizer together with surface disinfection reduced absenteeism related to gastrointestinal illness in elementary schools, but there was no major reduction in absenteeism related to respiratory illness (*42*). A cross-sectional study found that passive contact with bleach was associated with a major increase in self-reported influenza (*40*).

Given that influenza virus can survive on some surfaces for prolonged periods (*43*), and that cleaning or disinfection procedures can effectively reduce or inactivate influenza virus from surfaces and objects in experimental studies (*44*), there is a theoretical basis to believe that environmental cleaning could reduce influenza transmission. As an illustration of this proposal, a modeling study estimated that cleaning of extensively touched surfaces could reduce influenza A infection by 2% (*45*). However, most studies of influenza virus in the environment are based on detection of virus RNA by PCR, and few studies reported detection of viable virus.

Although we found no evidence that surface and object cleaning could reduce influenza transmission, this measure does have an established impact on prevention of other infectious diseases (*42*). It should be feasible to implement this measure in most settings, subject to the availability of water and cleaning products. Although irritation caused by cleaning products is limited, safety remains a concern because some cleaning products can be toxic or cause allergies (*40*).

Top

Discussion

In this review, we did not find evidence to support a protective effect of personal protective measures or environmental measures in reducing influenza transmission. Although these measures have mechanistic support based on our knowledge of how influenza is transmitted from person to person, randomized trials of hand hygiene and face masks have not demonstrated protection against laboratory-confirmed influenza, with 1 exception (*18*). We identified only 2 RCTs on environmental cleaning and no RCTs on cough etiquette.

Hand hygiene is a widely used intervention and has been shown to effectively reduce the transmission of gastrointestinal infections and respiratory infections (*26*). However, in our systematic review, updating the findings of Wong et al. (*8*), we did not find evidence of a major effect of hand hygiene on laboratory-confirmed influenza virus transmission (Figure 1). Nevertheless, hand hygiene might be included in influenza pandemic plans as part of general hygiene and infection prevention.

We did not find evidence that surgical-type face masks are effective in reducing laboratory-confirmed influenza transmission, either when worn by infected persons (source control) or by persons in the general community to reduce their susceptibility (Figure 2). However, as with hand hygiene, face masks might be able to reduce the transmission of other infections and therefore have value in an influenza pandemic when healthcare resources are stretched.

therefore have value in an influenza pandemic when healthcare resources are stretched.

It is essential to note that the mechanisms of person-to-person transmission in the community have not been fully determined. Controversy remains over the role of transmission through fine-particle aerosols (*3*,*46*). Transmission by indirect contact requires transfer of viable virus from respiratory mucosa onto hands and other surfaces, survival on those surfaces, and successful inoculation into the respiratory mucosa of another person. All of these components of the transmission route have not been studied extensively. The impact of environmental factors, such as temperature and humidity, on influenza transmission is also uncertain (*47*). These uncertainties over basic transmission modes and mechanisms hinder the optimization of control measures.

In this review, we focused on 3 personal protective measures and 1 environmental measure. Other potential environmental measures include humidification in dry environments (*48*), increasing ventilation (*49*), and use of upper-room UV light (*50*), but there is limited evidence to support these measures. Further investigations on the effectiveness of respiratory etiquette and surface cleaning through conducting RCTs would be helpful to provide evidence with higher quality; evaluation of the effectiveness of these measures targeting specific population groups, such as immunocompromised persons, would also be beneficial (Table 2). Future cost-effectiveness evaluations could provide more support for the potential use of these measures. Further research on transmission modes and alternative interventions to reduce influenza transmission would be valuable in improving pandemic preparedness. Finally, although our review focused on nonpharmaceutical measures to be taken during influenza pandemics, the findings could also apply to severe seasonal influenza epidemics. Evidence from RCTs of hand hygiene or face masks did not support a substantial effect on transmission of laboratory-confirmed influenza, and limited evidence was available on other environmental measures.

Top

Ms. Xiao is a postgraduate student at the School of Public Health, University of Hong Kong, Hong Kong, China. Her primary research interests are influenza epidemiology and the dynamics of person-to-person transmission.

Top

Acknowledgments

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Top

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Top

Figures

Tables

Top

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[1]These first authors contributed equally to this article.

Table of Contents – Volume 26, Number 5—May 2020

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Top

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Exhibit D

— Other forms of evidence are available to judge effectiveness of this and other interventions

by Rossi A. Hassad, PhD, MPH
August 3, 2020

As we continue to endure the pain and burden of the COVID-19 pandemic with close to 16 million cases and more than 640,000 deaths worldwide, some reflection and acknowledgement is warranted, lest we forget and repeat our failures. Indeed, the unpredictability and uncertainty associated with this pandemic demands critical and innovative thinking, especially as we move into phase III studies of COVID-19 vaccine candidates to confirm safety, and evaluate their efficacy and effectiveness, recognizing the complexity of vaccine development including the fast-track process, referred to as "Operation Warp Speed." Other critical concerns include the extent to which SARS-CoV-2, the virus that causes COVID-19 is airborne, and the role of younger children in spreading the virus, amidst an expanding and terrifying pandemic, which is already eclipsing the historical images of the 1918 Spanish flu.

But foremost upon reflection, is the mask-wearing debacle, in which international and governmental authorities, for months, while there was explosive community spread of COVID-19 and an exponential death toll, emphatically communicated to the public, that mask-wearing in the community setting conferred no protection against COVID-19. They said the evidence just wasn't there, while noting that face masks should be reserved for healthcare workers. But as the world panicked, evidence emerged, and the headline now reads "CDC calls on Americans to wear masks to prevent COVID-19 spread." As well, in a complete reversal of position, the WHO (World Health Organization) now advises that "masks can be used either for protection of healthy persons (worn to protect oneself when in contact with an infected individual) or for source control (worn by an infected individual to prevent onward transmission)."

But what constitutes evidence in this context? There has been an almost exclusive focus on evidence from experimental studies, specifically the randomized controlled trial (RCT), which is characterized as the "gold standard" of research, as it allows for the determination of causality. However, the reason such evidence is still lacking, should be obvious – the RCT

unethical in the context of a deadly pandemic. At the minimum, an RCT would require manipulation of the intervention, by way of the researcher randomly assigning some members of the community to wear a face mask and others not to, and ensuring that both community groups are similar, based on key background characteristics, in other words, controlling for potential confounding factors.

An RCT may be theoretically perfect, but it is certainly not realistic in the context of mask-wearing and the COVID-19 pandemic. What is more relevant, meaningful, and available, is evidence from the observational research spectrum, primarily natural experiments. A natural experiment is an observational study where an intervention such as mask-wearing was implemented by forces outside the researcher's control, such as a governmental mandate, and the outcome (level of COVID-19 infection) can be used to explore a specific research question, for example: Does mask-wearing in the community setting reduce the level of COVID-19 infection? The results can be evaluated for causal inference, using a common epidemiological model known as the Bradford-Hill criteria.

Appreciating and using evidence other than from "true" experiments (i.e., an RCT) can be difficult especially in the health professions where the research culture is driven primarily by the hypothetico-deductive reasoning model; a closed or circular system of logic which can limit discovery and innovation. There is an urgent need for greater intellectual flexibility and curiosity.

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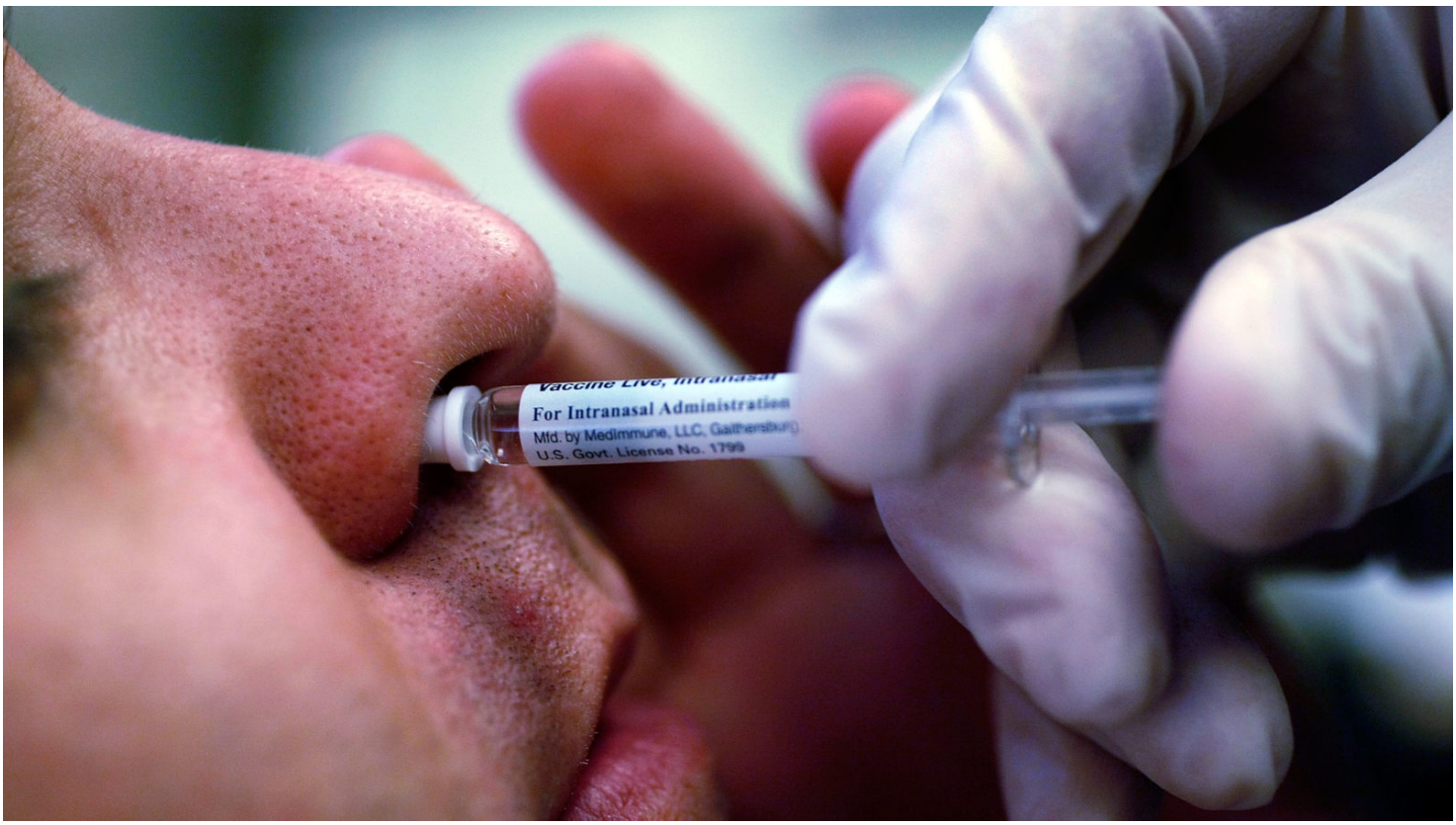
Last Updated August 03, 2020

Exhibit E

Why the ‘gold standard’ of medical research is no longer enough

By Tom FriedenAug. 2, 2017



The effectiveness of the nasal spray flu vaccine illustrates of the limitations of randomized controlled trials. *Joe Raedle/Getty Images*

Randomized controlled trials have long been held up as the “gold standard” of clinical research. There’s no doubt that well-designed trials are effective tools for testing a new drug, device, or other intervention. Yet much of modern medical care — perhaps most of it — is not based on randomized controlled trials and likely never will be. In this “dark matter” of clinical medicine, past practices and anecdotes all too often rule. We need to look beyond trials to improve medical care in these areas.

In a randomized controlled trial (RCT), participants are randomly assigned to receive either the treatment under investigation or, as a control, a placebo or the current standard treatment. The randomization process helps ensure that the

various groups in the study are virtually identical in age, gender, socioeconomic status, and other variables. This minimizes the potential for bias and the influence of confounding factors.

Despite their strengths, RCTs have substantial limitations. They can be very expensive to run. They can take many years to complete, and even then may not last long enough to assess the long-term effect of an intervention such as vaccine immunity, or to detect rare or long-term adverse effects. Findings from RCTs may not be valid beyond the study population — a trial that included a high-risk population in order to maximize the possibility of detecting an effect, for example, may not be relevant to a low-risk population. RCTs may not be practical for population-wide interventions and often aren't relevant for urgent health issues such as infectious disease outbreaks, for which public health decisions must be made quickly.

As I write this week in the New England Journal of Medicine, several other study types can generate data that are at least as effective as RCTs, or may be even more effective, at generating evidence for action, especially related to population-wide interventions.

The effectiveness of the nasal spray flu vaccine (also called the live attenuated vaccine) is a dramatic illustration of the limitations of RCTs. Trials suggested that the nasal spray vaccine was superior to flu shots, at least for some populations. In subsequent years, however, observational studies, including case-control studies, documented that, for reasons which are still unclear, the nasal spray *wasn't* effective against the flu. That led the Advisory Committee on Immunization Practices to recommend, and the CDC to accept the recommendation, that the nasal spray flu vaccine not be used in the 2016-2017 flu season.

For some public health issues, it isn't ethical to conduct an RCT. Take sudden infant death syndrome (SIDS). Early case-control studies suggested, but didn't prove, that babies who sleep on their stomachs are more likely to die of SIDS than babies who sleep on their backs. It wouldn't have been ethical to randomize some babies to stomach sleeping. A public program to implement putting children to sleep on their backs proved that this measure reduced the incidence of SIDS.

It would be difficult, if not impossible, to do an RCT of community-wide tobacco control measures. But analyses of the results of implementing tobacco control policies, such as taxes, smoke-free laws, and advertising bans, have generated robust evidence of effectiveness that could not have been accomplished through an RCT-style study.

For the several thousand rare diseases, RCTs are unlikely to be conducted due to the small number of people who have them and other logistical constraints. Detailed case studies, registries that collect information about specific conditions and diseases, and other study types can enhance understanding of a particular disease and its treatment to improve the health of affected patients.

The emerging use of "big data," including information from electronic health records and expanded patient registries, presents new opportunities to conduct large-scale studies with many of the benefits of RCTs but without the expense. One such study used data from the Veterans Health Administration and Medicare to examine outcomes of treatment for type 2 diabetes. This study was many times larger, with much longer follow-up and lower cost, than previous RCTs comparing the effectiveness of different diabetes drugs. It clearly showed that one class of drug, the thiazolidinediones, was much more effective than another class, the sulfonylureas, in reducing hospitalization and death.

Clinical and public health decisions are almost always made with imperfect data. There is no single, best approach to obtain better information about health interventions. Evidence grading systems, policy makers, and researchers must embrace other study types in addition to RCTs. Essential steps in interpreting findings and identifying data for action include promoting transparency in study methods, ensuring standardized data collection for key outcomes, and using new approaches to improve data synthesis.

Despite the global evidence base, around the world there are often claims that "there is no evidence tobacco harms health here" or that "soda isn't proven to drive obesity in this country." In part, such claims can be made because some formal systems of analyzing evidence give undue weight to RCTs and inappropriately discount other types of rigorously developed evidence.

A valid ideal is “evidence-based practice,” which means implementing in clinical care and public policy interventions that are proven to work. But it’s also important, and perhaps more so, to develop “practice-based evidence,” — that is, to implement programs and rigorously document whether or not they work. That would both save lives and expand the evidence base of effective interventions.

There will always be an argument for more research and for better data. But waiting for more data is often an implicit decision not to act, or to act on the basis of past practice rather than on the best available evidence. Glorifying RCTs above other approaches, even when these other approaches may be either superior or the only practical way to get an answer, relegates patients to receiving treatments that aren’t based on the best available evidence.

An approach that uses all appropriate evidence types and builds on the existing evidence base using proven best practices is the one most likely to result in clinical and public health action that will save lives.

Tom Frieden, M.D., served as director of the Centers for Disease Control and Prevention from 2009 to 2017.

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Exhibit F

Randomized Controlled Trial Int Forum Allergy Rhinol. 2020 Dec;10(12):1325-1328.
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Interim analysis of an open-label randomized controlled trial evaluating nasal irrigations in non-hospitalized patients with coronavirus disease 2019

Kyle S Kimura [1], Michael H Freeman [1], Bronson C Wessinger [2], Veerain Gupta [2], Quanhu Sheng [3], Li Ching Huang [3], Kate Von Wahlde [1], Suman R Das [1 4], Naweed I Chowdhury [1], Justin H Turner [1]

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Keywords: COVID-19; coronavirus; irrigation; nasal; saline.

Figures

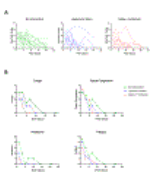

Figure 1. Days until symptom resolution in...

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RESEARCH NOTE | Free Access

Interim analysis of an open-label randomized controlled trial evaluating nasal irrigations in non-hospitalized patients with coronavirus disease 2019

Kyle S. Kimura MD, Michael H. Freeman MD, Bronson C Wessinger BE, Veerain Gupta BS, Quanhu Sheng PhD, Li Ching Huang PhD, Kate Von Wahlde MJ, Suman R. Das PhD ... See all authors

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Response to the coronavirus disease 2019 (COVID-19) pandemic has primarily focused on pharmacologic and medical interventions, including antivirals,[1] convalescent sera,[2] and vaccinations,[3] with each potentially critical in the fight against COVID-19, particularly among high-risk and hospitalized populations. Non-hospitalized patients with mild to moderate disease comprise an estimated 81% of those affected with COVID-19,[4] and there are currently no widely available interventions with proven ability to hasten symptom resolution or reduce viral shedding. We started an open-label randomized controlled trial (RCT) to evaluate the effect of nasal irrigation with hypertonic saline (HTS) or saline with surfactant on upper respiratory symptoms and viral load. Viral shedding is highest in the nasal cavity and nasopharynx,[5] and prior RCTs of saline irrigations for the common cold, including non–severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) coronaviruses, have shown that saline rinses can reduce symptom burden and decrease viral shedding.[6]

Patients and methods

We identified patients diagnosed with a positive qualitative real-time polymerase chain reaction (qRT-PCR) SARS-CoV-2 diagnostic test obtained from Vanderbilt University Medical Center and affiliated testing centers. Patients were enrolled in the study within 24 hours of testing and were given swabs, viral preservation media, and a symptom diary incorporating a modified version of the validated Wisconsin Upper Respiratory Symptom-21 Survey (WURSS-21)

(Supplementary Fig. 1).[7] Patients were randomized to 1 of 3 treatment arms: (1) twice-daily irrigation with 250 mL HTS; (2) twice-daily irrigation with HTS with 1% surfactant (HTSS); and (3) a nonintervention (NI) group (Supplementary Fig. 2). Participants performed scheduled mid-turbinate swabs and recorded daily temperatures and symptom scores over the 21-day study duration. A prior cross-sectional study found equivalent sensitivity and high correlation between patient-performed mid-turbinate swabs and nasopharyngeal swabs performed by healthcare workers.[8] Comprehensive details of study methodology and power analysis are provided in the Methods section in the Supplementary Appendix.

Results

We performed an interim analysis on the first 45 patients with completed symptom questionnaires, which included 17 patients in the NI group, and 14 each in the HTS and HTSS groups (Supplementary Table 1). The groups were similar with respect to age, sex, comorbidities, and other demographic and/or clinical factors. The median number of symptomatic days before diagnosis ranged from 2.0 to 2.5 and did not differ between the groups. Study completion was also similar with 3 patients lost to follow-up in each treatment arm.

The global symptom score for the question “How sick do you feel today,” continually declined during the study duration for all treatment groups (Fig. 1A), with a trend toward earlier time to symptom resolution in the intervention groups (median 14 days for NI, 10 days for HTS and HTSS; $p = 0.16$). There was a significant difference in median days to symptom resolution for nasal congestion (NI 14 days; HTS 5 days; HTSS 7 days; $p = 0.04$) and headache (NI, 12 days; HTS, 3 days; HTSS, 5 days; $p = 0.02$) (Fig. 1B, Table 1). Additionally, there was a trend toward differences between groups for cough ($p = 0.19$) and fatigue ($p = 0.17$). Comparison of viral load between groups is awaiting batch analysis and is pending completion of study enrollment.

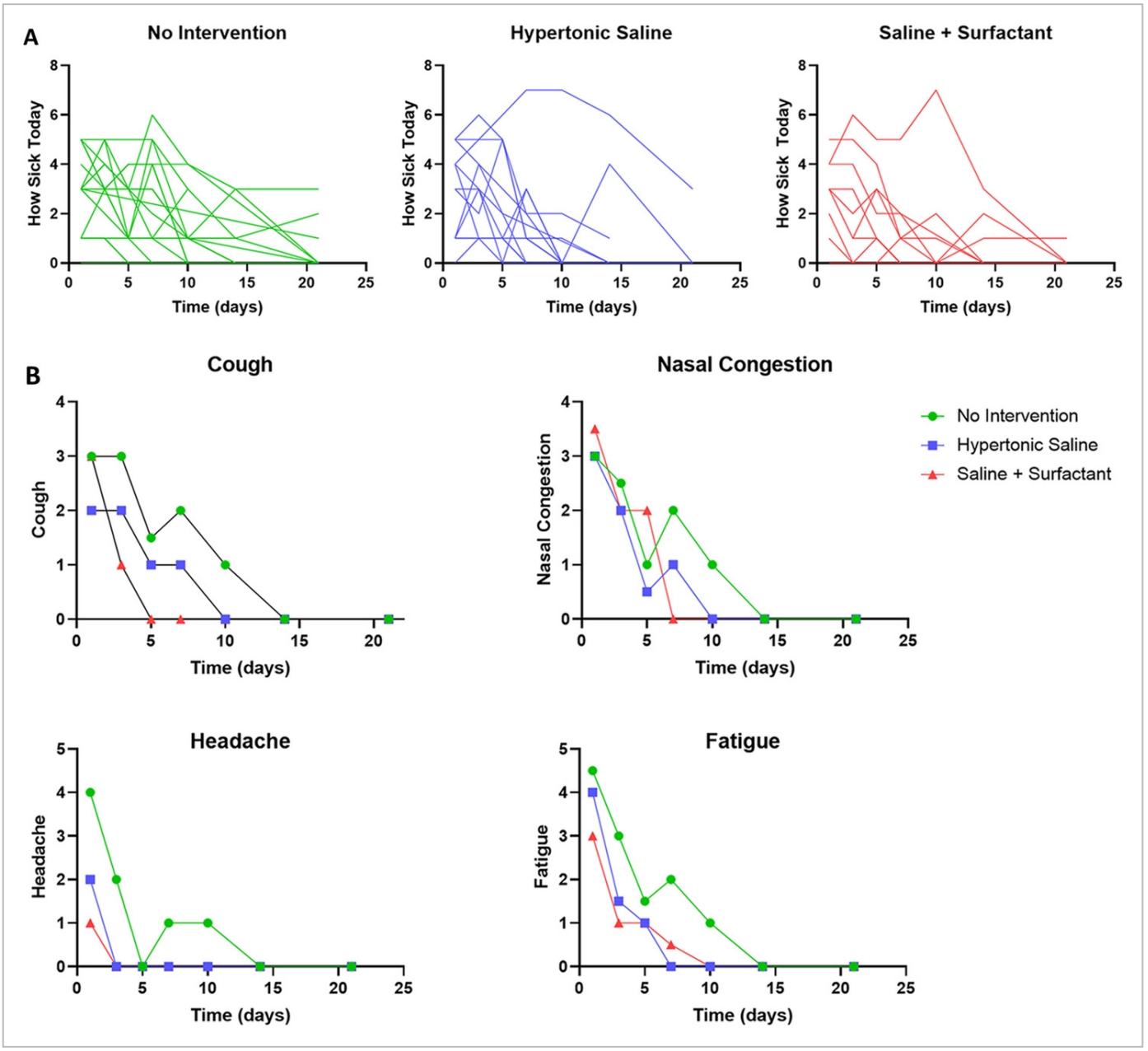

FIGURE 1

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. Days until symptom resolution in COVID-19 patients. (A) Daily reported symptom scores for individual participants during the 21-day study duration for the question "How sick do you feel today," (B) Daily median symptom scores for participants during the 21-day study duration for cough, nasal congestion, headache, and fatigue.

TABLE 1. Days until symptom resolution obtained from modified WURSS-21 questionnaire*

	Days until symptom resolution		
	No intervention	Hypertonic saline	Saline + surfactant

Symptom	(n = 17)	(n = 14)	(n = 14)	*p*
Symptom	No intervention (n = 17)	Hypertonic saline (n = 14)	Saline + surfactant (n = 14)	*p*
Cough	14 (10–21)	7 (5–14)	6 (5–21)	0.19
Nasal congestion	14 (7–21)	5 (3–11)	7 (4.5–21)	**0.04**
Headache	12 (5–21)	3 (3–9.3)	5 (3–7)	**0.02**
Fatigue	12 (5–21)	7 (5–10)	14 (7–21)	0.17
Muscle/joint pain	8.5 (5–21)	7 (4–8.5)	10 (5–21)	0.40
Altered smell/taste	10 (6–17.5)	12 (5.5–21)	12 (3–21)	0.94
Think clearly	8.5 (3–13)	10 (4–15.5)	10 (6.5–17.5)	0.65
Sleep well	7 (5–14)	10 (5–21)	8.5 (6.5–21)	0.75
Breath easily	7 (3–21)	10 (3–12.8)	8.5 (4–18.3)	0.99
Walk/climb stairs	7 (3–21)	10 (7–21)	6 (3–12.8)	0.49

*Data presented as medians with interquartile range. Bold indicates $p < 0.05$ by Kruskal-Wallis test.

WURSS-21 = Wisconsin Upper Respiratory Symptom-21 Survey.

Discussion

Nasal saline irrigation is a commonly accepted and inexpensive therapy with proven efficacy as a treatment for viral upper respiratory infections and has been proposed as a potentially beneficial treatment for COVID-19.[9] Here, we present initial findings from the first RCT evaluating nasal irrigations in non-hospitalized patients with COVID-19. The effect of nasal irrigation on symptom resolution was substantial, with nasal congestion and headache resolving a median of 7 to 9 days earlier in the intervention groups. Our analysis suggests that nasal irrigations may shorten symptom duration and may have potential as a widely available and inexpensive intervention to reduce disease burden among those affected. The additive effects of surfactant remain unclear, because the impact of HTS and HTSS on symptom resolution was fairly equivalent, and it has been reported that surfactant nasal irrigations are associated with some tolerability issues in a subset of patients.[10] However, the addition of

surfactant may have beneficial effects on viral shedding and/or maturation given their reported ability to rapidly induce membrane dissolution and lysis of many viruses and other microorganisms.

A note of caution is indicated when considering use of nasal saline irrigations in patients with confirmed COVID-19 because irrigation could potentially disperse viral particles or contaminate surfaces in the immediate vicinity. The SARS-CoV-2 virus can remain on plastic and metal surfaces for extended periods of time,[11] and other types of viruses can be detected in nasal lavage fluid.[12] Given these concerns, the current study only enrolled patients who could self-isolate and perform irrigation in a bathroom separate from other household contacts. Similar precautions would need to be taken by any COVID-19 patient considering this intervention.

Although the current study provides evidence to suggest that topical saline irrigation can reduce symptom burden in patients with COVID-19, we are not yet able to determine whether irrigations affect viral load and/or shedding. We hypothesize that both HTS and HTSS will reduce viral shedding as has been reported for some cold viruses,[6] and will present these findings after batch qRT-PCR analysis of nasal swabs from all study participants. In the interim, we would advocate the use of hypertonic nasal saline irrigations in non-hospitalized COVID-19 patients as a safe and inexpensive intervention to reduce symptom burden.

Supporting Information

Filename	Description
alr22703-sup-0001-SuppMat.docx 36.3 KB	**Figure 1**. COVID-19 daily symptom survey (modified from Wisconsin Upper Respiratory Symptom Survey – 21[2]). **Figure 2**. Consort flow diagram for screening, enrollment, and randomization of study participants. **Table 1**. Clinical and demographic characteristics of study participants.
alr22703-sup-0002-figureS1.tif 1.6 MB	Supplementary Material
alr22703-sup-0003-figureS2.tif 556.4 KB	Supplementary Material

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Rapid initiation of nasal saline irrigation to reduce morbidity and mortality in COVID+ outpatients: a randomized clinical trial compared to a national dataset

Amy L. Baxter, Kyle R. Schwartz, Ryan W. Johnson, Ann-Marie Kuchinski, Kevin M. Swartout, Arni S. R. Srinivasa Rao, Robert W. Gibson, Houlton M. Boomer, Erica Cherian, Taylor Giller, Matthew Lyon, Richard Schwartz

doi: https://doi.org/10.1101/2021.08.16.21262044

Abstract

Importance SARS-CoV-2 enters the nasopharynx to replicate; mechanical debridement with nasal irrigation soon after diagnosis could reduce morbidity and mortality.

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Setting Single-lab community testing facility associated with the emergency department (ED) in Augusta, GA.

Participants A consecutive sample of outpatients 55 years and older were contacted from daily COVID-19+ lab reports between September 24 and December 21 of 2020. Patients without supplemental oxygen use or cognitive barriers agreeing to same-day irrigation initiation were remotely consented. Among 826 screened, 321 were unable to be reached, 132 were ineligible, 294 refused participation, and 79 participants were enrolled.

Interventions Participants were randomly assigned adding 2.5 mL povidone-iodine 10% or 2.5 mL sodium bicarbonate to 240ml of isotonic nasal irrigation twice daily for 14 days.

Main Outcomes and Measures The primary outcome was hospitalization or death from COVID-19 within 28 days of enrollment by daily self-report confirmed with phone calls and hospital records, compared to the CDC Surveillance Dataset covering the same time. Secondary outcomes compared symptom resolution by irrigant additive.

Results Seventy-nine participants were enrolled (mean [SD] age, 64 [8] years; 36 [46%] women; 71% Non-Hispanic White). Analyzed by intention-to-treat, by day 28, COVID-19 symptoms resulted in 1/42 hospitalizations in those irrigating with alkalinization, 0/37 in the povidone-iodine group, (1.27%) and no deaths. Of nearly three million CDC cases, 9.14% were known to be hospitalized, with an additional 1.5% mortality in those without hospitalization data. The total risk of hospitalization or death (10.6%) was 8.4 times that of enrolled patients (SE=2.74; P=.006). 62 completed daily surveys (78%), averaging 1.8 irrigations/day. Eleven had irrigation complaints, and four discontinued. There were no significant differences by additive.

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Trial Registration ClinicalTrial.gov Identifier: NCT04559035

Author Approval All authors have filled out ICMJE and approved submission.

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Competing Interest Statement

The authors have declared no competing interest.

Clinical Trial

NCT04559035

Funding Statement

Materials were provided by Neilmed Inc. and Rhinosystems Inc. The investigator-initiated study was supported by funding from the Bernard and Anne Gray Donor Advised Fund Community Foundation for Greater Atlanta, Neilmed Inc., and Rhinosystems.

Author Declarations

I confirm all relevant ethical guidelines have been followed, and any necessary IRB and/or ethics committee approvals have been obtained.

Yes

The details of the IRB/oversight body that provided approval or exemption for the research described are given below:

Institutional Review Board Office Augusta University 1120 15th St., CJ-2103 Augusta GA 30912-7621 Email: IRB@augusta.edu Phone: 706-721-3110 http://www.augusta.edu/research/irboffice/

All necessary patient/participant consent has been obtained and the appropriate institutional forms have been archived.

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I understand that all clinical trials and any other prospective interventional studies must be registered with an ICMJE-approved registry, such as ClinicalTrials.gov. I confirm that any such study reported in the manuscript has been registered and the trial registration ID is provided (note: if posting a prospective study registered retrospectively, please provide a statement in the trial ID field explaining why the study was not registered in advance).

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I have followed all appropriate research reporting guidelines and uploaded the relevant EQUATOR Network research reporting checklist(s) and other pertinent material as supplementary files, if applicable.

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Meaning In older outpatients testing positive for SARS-CoV-2 who initiated nasal irrigation rapidly after diagnosis, risk of hospitalization or death was eight times lower than national rates reported by the CDC.

Background and Objectives

Mechanical binding of the SARS-CoV-2 spike protein-receptor to the ACE2 receptor is a target for pharmacologic and immunologic COVID-19 therapeutics. Genetic and clinical factors support an additional mechanical therapeutic option – nasal irrigation – may be uniquely effective. Sungnak et al. localized the necessary co-expression of ACE2 and TMPRSS2 primarily in the ciliated nasal epithelia.(**1**) The finding that viral "spike" protein mutations and N-Methylation of the nasal ACE2 receptor increased infectiousness further support a mechanical relationship between the viral particle and receptor.(**2**)

Ignaz Semmelweis pioneered handwashing to reduce infection in 1847. In emergency medicine and surgery, debriding infectious material with copious high-powered irrigation is standard practice. Nasal irrigation under pressure, or "nasal lavage", has been demonstrated to reduce the duration and severity of both *Coronaviridae* and illnesses like flu with shorter incubation periods.(**3**) Irrigation should work best in clinical scenarios with long incubation and local non-hematogenous spread, where reducing viral load impacts severity. Otolaryngology reviews supported the concept and safety of nasal antimicrobials and irrigation.(**4**-**6**) If clinically effective, irrigation could be an inexpensive option rapidly available worldwide.

Given research supporting the virucidal activity of povidone-iodine against MERS and SARS-CoV-2(**7**-**9**) and the possible impact of alkalinization to reduce SARS-CoV-1 viral cell fusion and entry,(**10**) patients were randomized to add alkalinization or povidone-iodine to pressurized nasal lavage. We hypothesized rapid initiation of nasal irrigation after testing positive would reduce the severity of COVID-19. Our primary outcome was COVID-19 hospitalization or death, with secondary outcomes of symptom duration and severity.(**11**, **12**)

Methods

Trial Design

The Rapid Initiation of Nasal Saline Irrigation (RINSI) study was an unblinded randomized clinical trial of alkalinized vs povidone-iodine nasal irrigation in outpatients aged 55 years and older recently PCR positive for SARS-CoV-2, nested in a prospective case:cohort using laboratory-confirmed cases in the CDC COVID-19 Case Surveillance dataset of patients 50 and older.(**13**) The trial protocol and statistical analysis plan appear in Supplement 1. The study was approved by the institutional review board at Augusta University in Augusta, Georgia. All participants provided remote informed consent in English.

Study Setting and Recruitment

This trial was conducted in Augusta, Georgia. Patients testing positive for COVID-19 by nasal swab or saliva PCR processed at a single lab at the Augusta University were recruited from September 24, 2020 to December 21, 2020. The 28-day follow-up was completed January 18, 2021. Signs at the testing site informed patients of the irrigation trial and eligibility criteria, and a recruitment flyer was given with testing. The daily laboratory-generated list of COVID-19 tests was screened for patient age, first positive test in the system, and location within 25 miles of Augusta University. Prospective participants were called consecutively between the hours of 9:00am through the early afternoon five to six days a week. On days where sufficient staffing for deliveries was a concern, the list was randomized for calling order. Participants interested in participation were assessed over the phone for inclusion criteria, and remote informed consent was completed per IRB policy.

Study materials were delivered to their residence by a member of the research team using COVID-19 precautions (masks, maintaining 6 ft. or more physical distance, door drop off) later that day. Materials consisted of a nasal irrigation device with 28+ accompanying saline pods/packets, two gallon jugs of distilled water, a physical copy of the consent form, an instructional sheet, and the randomly allocated additive (baking soda or povidone-iodine) with a 2.5ml scoop. The detailed instruction sheet contained directions to mix the irrigant materials, as well as links to a YouTube video demonstrating how to conduct irrigation with the relevant device. A previous study found nasal irrigation units under pressure (squeezing or pump) were superior to gravitational units.(**14**) In order to avoid bias toward any particular product, participants were assigned on alternate days to one of two pressure-based nasal irrigation systems (NAVAGE, Rhinosystems Inc.) or Neilmed Sinus Rinse (Neilmed Inc.). Patients were instructed to initiate irrigation on the same day of contact and

enrollment. An investigator called the patient or their designated contact at day 2, 7, 14, and 28 to verify ED visits, hospitalization, or answer any questions.

Eligibility Criteria

Eligible patients had to be able to read the informed consent in English, agree to nasal lavage for 14 days with a 14-day follow-up, provide a back-up contact for clinical follow-up, and be available to receive materials and initiate irrigation that day. Exclusion criteria included current supplemental oxygen therapy, unwillingness to try nasal irrigation or current use of nasal irrigation, nasal surgery within the past year or chronic sinusitis, prior COVID-19 infection or positive test, symptoms longer than 7 days prior to testing, inability to complete surveys by computer or smartphone, and an allergy to iodine or shellfish. Hospital employees were initially excluded, given the unknown impact of greater risk due to exposure or lower risk due to T-cell immunity, but this exclusion criteria was ultimately removed.

Randomization

Patients were randomized to rinse with 240cc saline including 0.5 mL 10% povidone-iodine (0.1% final concentration) or 0.5 mL sodium bicarbonate twice daily for 14 days. Randomization was stratified by sex in 10 blocks of 10 random numbers by the first author using Random.org. With odd numbers signifying alkaline and even povidone-iodine, numbered opaque envelopes were prepared in separate sequences for male or female participants to be opened after consent, indicating the appropriate additive to be given to the patient.

Interventions and Masking

To avoid withholding effective treatment and to better assess adherence to nasal irrigation, all enrolled participants were given a pressurized nasal saline irrigation unit. Masking of study treatments (alkalinization or 0.1% povidone-iodine) was not undertaken. Study personnel reviewing hospital records to verify admission or death were masked to intervention.

Main Outcomes and Measures

The primary outcome was hospitalization for COVID-19 symptoms within 28 days of enrollment, by self-report and phone calls verified by the testing site hospital's electronic medical records. Secondary outcomes in enrolled patients compared symptom resolution and home exposure, adherence to nasal irrigation, and any impact of antimicrobial or alkalization addition to the irrigant. Prompts to complete study materials were sent to participants via email from Qualtrics twice daily for the duration of the study period. To verify irrigation, patients uploaded pictures of used irrigation materials daily into the Qualtrics system.

In addition to demographic data, patients were asked preexisting medical history as found on the CDC person of interest form, including Chronic Lung disease (Emphysema, COPD), Asthma, Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus, Cardiovascular Disease, Hypertension, Chronic Renal Disease, weight and height to calculate obesity defined as BMI>30, Immunocompromised condition, and symptoms. Symptoms included the number of days since first subjectively sick, loss of smell, loss of taste, fatigue, presence or absence of fever >100.4˚F, chills, muscle aches, runny nose, cough (new onset or worsening of chronic cough), shortness of breath, nausea or vomiting, headache, abdominal pain, and diarrhea.

Hospitalization and mortality data were compared to the National CDC Case Surveillance Public Use Dataset.(**13**) This dataset has 12 elements for all COVID-19 cases shared with CDC, including first positive specimen, first report to CDC, first day of illness, a summary “case earliest date”, laboratory-confirmed or suspected, symptom onset, demographic data, and a binary measure for pre-existing clinical conditions. Hospitalization, ICU, and mortality data have four options: yes, no, unknown (marked on form), or missing (nothing recorded). Following CDC research recommendations, we identified laboratory confirmed cases by “case earliest date” to match the testing dates in our sample. Between September 23, 2020 and December 21, 2020, 45% of laboratory-confirmed cases in the dataset included known hospitalization status. While a December Georgia Department of Public Health analysis indicated a minimum admission rate in age 50+ of 16.4% (K. Krohnert, email communication December 2020), this information was not published. For the same period, the COVID-19 Case Surveillance Public Use Data with Geography for Georgia-specific information had hospitalization data for only 5%, with a hospitalization rate of 50%, so the 12-item National CDC Case Surveillance dataset was selected as the most comprehensive and conservative comparison group.

Statistical Analysis

At the time we initiated the study, admission rates in older and African American communities were 40%,(**15**) and the population in our study environment was 50% Black. We assumed a baseline admission rate of 25%, with a large effect size of irrigation alone (Cohen’s d=7) and a smaller impact of the additive to the irrigant (Cohen’s d=3). To reduce hospitalizations by 60%, 100 patients per group would be required based on irrigant composition, and 79 based on irrigation versus control. After three months, the patient outcome data was evaluated and the admission and mortality rate in the patients enrolled was zero. Given the percentage of patients admitted in Georgia (16.4%), and the public health burden of COVID-19 the decision was made to stop enrollment and compare outcomes at the end of the observation period.

For patient characteristics between irrigant groups and devices used, t-tests were used unless non-normal distribution was noted, in which case the Mann Whitney U test was used. Baseline measures of duration of symptoms and ongoing reported symptoms by day were compared across the conditions. To test the effect of treatment group in symptom persistence, either t-tests for normally distributed data or the Mann-Whitney U test for non-normally distributed data was used.

The results are compared to laboratory-confirmed case data from the CDC.(**13**) Chi-square was used to evaluate differences in demographic proportions of sex, race, and age by 10-year tronche. We used an exact binomial test with Clopper-Pearson confidence intervals to compare observed hospital admission rates among participants compared with national rates of severe disease (admission or death) published by CDC. The exact binomial test is well-suited to assess the probability of observing the proportion of patients in this study. That proportion comparison is supplemented by an odds comparison across the study and CDC data (MedCalc Software Ltd. Odds ratio calculator. https://www.medcalc.org/calc/odds_ratio.php (Version 20.009)).

To avoid overestimating by reporting bias, we calculated the admission rate retaining cases where hospitalization status was missing or unreported in the denominator. Since only half of CDC cases included hospitalization status, our number should underestimate true national admissions. We included deaths only when hospitalization was unknown or missing as a rate over the total confirmed case denominator. Thus, our rate underestimates deaths. We did not include deaths in cases where hospitalizations are reported in our analysis to avoid counting outcomes with increased severity twice.

Results

During the study period, 826 unique patients aged 55 and older were eligible to be contacted within 24 hours of a positive PCR; 79 were able to be enrolled and receive irrigation materials on the day of contact.(Figure 1). After enrollment, 11 patients complained of discomfort or spotty epistaxis, with four discontinuing irrigation.

There was one COVID-19 related admission out of 79 patients assigned to nasal irrigation, 0/37 assigned to povidone-iodine and 1/42 patients in the alkalinization group (1.3%). One patient in the alkalinization group had a COVID-19 related ED visit but was not admitted. In addition to COVID-19 healthcare utilization, one patient reported an ED visit for a minor trauma, and one patient had a syncopal episode requiring admission for evaluation in the follow up period after resolution of COVID symptoms. These events were verified in the EHR database, and there were no additional ED visits or hospitalizations found in consented patients.

During the same enrollment period, in patients 50+ years the CDC reported 2937299 laboratory-confirmed cases with 268607 known hospitalizations, or 9.14% admission rate. There were 44,358 deaths where hospitalization was not reported, or 1.5%. Hospitalization or death occurred in for a total of 10.6% (OR:.125, 95%CI .017 to 0.9.) The CDC comparison rate of hospitalization and mortality was therefore 8.39 times higher than the hospital admission rate of patients enrolled in this nasal irrigation study (z[78]= -2.522; SE=2.74; P=.006). These reported data were not adjusted for underreporting of hospitalizations and disease cases.(**16**) The CDC group had a lower reported proportion of minority patients (**Table 1**), but 36.6% did not specify race. For the 785,285 CDC cases for whom both hospitalization and death were reported, 8.22% of patients expired. There were no deaths in our cohort.

Table 1:

View inline

Of the 79 enrolled, 57 patients completed the initial symptom and history questionnaire; patients reported a median of 3.3 days (IQR 2,5) of symptoms prior to enrollment. Twelve patients received their materials but didn't record their first irrigation until the following day.

An online daily symptom and irrigation data collection survey was completed by 62 patients (median of 12 of 14 days, IQR 1,13.75). Of 631 daily online surveys, patients reported irrigating once per day (7.29%), twice daily (88.43%), or none (4.25%), averaging 1.79 irrigations per day. (**Table 2**) Patients were asked to take pictures of used irrigation materials to corroborate irrigation, but the number of used packets over time became difficult to assess for confirmation. Eleven patients had concerns with irrigation, four discontinued use.(**Table 3**)

Table 2:

View inline

All participants with completed intake surveys (n=53)

Table 3

View inline

Outcomes by Irrigant and Unit

Presenting symptoms present in over 50% of patients included fever, muscle aches, congestion, and headache. In other studies evaluating COVID symptoms, fatigue, headaches, anosmia and congestion persisted.(**17**) There were no statistical differences in symptomatic outcomes by irrigation unit used, (Figure 2) but symptom resolution of all or only one mild symptom among headache, fatigue, anosmia and congestion in 14 days was more likely in the povidone-iodine group (21/27) than the alkalinization group (17/35, p=0.0192).

Ten participants (12.7% by intention to treat) had household contacts who tested positive at least one day after enrollment, compared to 18.8% in a published meta-analysis.(**11**) There was no difference in risk of household spread by additive or irrigation unit. (**Table 3**)

Discussion

To the extent that our results generalize, pressurized nasal irrigation offers a safe and over the counter measure with potentially vital public health impact. Nationally, the reduction from 10.6% to 1.3% as of the month this writing (August 2021) would have corresponded in absolute terms to almost 780,000 fewer patients 55 and older requiring admission. Improved patient outcomes would be accompanied by corresponding reductions in pressure on ICU capacity as well as stress and risk to healthcare providers.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pathogen is a single stranded positive sense RNA virus, with a similar spike protein-receptor binding mechanism as SARS-CoV and MERS-CoV. Clinically COVID-19 differs notably from previous *Coronaviridae*: children are less impacted; obesity, diabetes, African American race and hypertension are independent risk factors; the relatively pathognomonic symptom of anosmia is present in up to 80% of patients,(**18**, **19**) and the duration from infection to mild symptoms to severe symptoms in the alpha variant was prolonged.

The clinical differences in presentation support the current understanding that SARS-CoV-2 primarily enters and replicates in the nasopharynx, with olfactory neuroepithelium ACE2 expressed at 700 times the expression in lungs.(**19**, **20**) Conditions increasing nasal ACE2 expression or sinus size (obesity, hypertension, pollution, older male sex) correlate with increased severity, further supporting targeting viral fusion in the nasopharynx.(**21**, **22**) In contrast, populations lacking fully developed sinus area (children), with a high baseline practice of nasal irrigation (Laos, Vietnam), or higher mask compliance have decreased severity.(**23**, **24**)

The size of the nasal cavity (and thus available ciliated epithelia) correlate with age and male sex. (**23**, **25**) At a protein level, obesity and diabetes both increased expression of nasal ACE2 receptors, as did pollution and age.(**26**, **27**) Together, the nasal cavity size, ACE2 expression and variolation explanation could account for lower pediatric severity and spread.(**28**) The degree of methylation of the ACE2 receptors (and thus stiffness and ease of viral attachment) is related to both race and epigenetic stress.(**29**, **30**) Thus, increased virulence correlating with increased stability of the spike proteins in variants supports the mechanical hypothesis.

Given the local cell to cell rather than hematogenous spread and delay in activation of lung TMPRRS2,(31) the potential exists that mechanically debriding viral particles lodged in the ACE2 receptor, but not yet fused, can reduce severity. Furthermore, the variation in severity with methylation implies that not all particles are securely attached. The size variations in the entire nasal cavity, rather than just anterior nares, support the concept that full nasal cavity irrigation may be superior to nasal spray. Finally, the number of asymptomatic cases and the correlation of illness severity with viral load implied that even after PCR positivity, a window exists wherein lowering the viral load through irrigation could be clinically advantageous.

While nasal irrigation reduced symptoms of other *Coronaviridae*, flu,(3) and bacterial carriage in otolaryngology(32, 33), pathology from local spread and aspiration and the continued production of viral load locally suggest a potentially greater impact on COVID-19. Association of viral load with severity (24, 34, 35) suggests a different kind of cumulative pathology related to immune response, as well as the potential for reducing severity after the fact by debridement. Multiple studies have demonstrated immediate viral load reductions in vitro and in vivo with direct oral or nasal application of antivirals,(7-9) or the theoretical benefit of lavaging and gargling. (36-38) However, a small study of gargles and sprays of effective povidone-iodine did not show a significant reduction in viral load,(39) nor were clinical differences seen in a small randomized trial using twice daily nasal irrigation without an effective virucidal.(17)

Multiple commentaries have supported the concept and safety(37) of debridement.(4, 38, 40, 41) A host of investigators coming to the above-detailed conclusions initiated multiple prospective studies varying additives (Neem oil, ozone, surfactant, lactobacillis, virucidals), concentration, and chronicity for COVID prevention or treatment. Several of these ongoing studies in prevention have been limited by pandemic enrollment difficulties, or by statistical challenges from a lower-than-expected incidence in healthcare workers who may have T cell protection initially unanticipated during power analyses.

To our knowledge this is the largest prospective clinical trial using both twice daily large volume irrigation with a virucidal arm, and with documented adherence to irrigation. Moreover, the older and higher risk population in this study (with concurrent larger sinus area) may be most suitable to reducing morbidity and mortality.

Limitations

Our results support that irrigation, whether accompanied by alkalinization or an effective virucidal, reduces the likelihood of hospitalization. There are a number of limitations to our study design and

execution.

The primary concern without a matched control group is the generalizability of our sample. The CDC database did not differ significantly by sex or age, but too many patients were missing race/ethnicity to meaningfully evaluate. The greatest risk of bias comes from preferentially reporting cases with hospitalization or death. Data suggests cases and hospitalizations are underreported rather than over,(**16**) however, and the CDC admission rate of 9.14% is lower than rates in other prospective studies with older populations. In a study of monoclonal antibodies delivered to outpatients testing positive, Chen et al found a 15% admission rate in patients 65+ or with BMI > 35.(**42**) Our sample came from a socioeconomically challenged catchment area, with average age 64 and BMI >30. In a similar health system to ours, Price-Haywood et al found a 39.7% admission rate; a Cochran database of minority patients' admission rates in similar time periods and demographic location to our enrollment period consistently found admission rates as high as 60%. (**15**, **43**)

While the goal was to initiate irrigation as quickly as possible after a positive test, healthcare infrastructure and testing turnaround time may limit the potential for fast intervention. The requirement to participate on 24-hour notice could have biased our sample toward healthier, technologically connected, higher socioeconomic status patients. The bias from feeling too well or too sick to participate could have been an issue, but approximately equal numbers declined participation due to "brain fog" as declined because they felt well and deemed any intervention unnecessary.

While irrigation could be an effective mechanical protection against variants in vaccinated people, adoption of a new hygiene intervention – or any intervention – is a barrier. Of the 537 patients contacted, 28 did not want to perform nasal irrigation. Of those who initiated irrigation, most continued twice daily use, but eleven had concerns about irrigating that were communicated to our staff. While only four discontinued irrigation, without the discussion and coaching adherence in the general population could be lower.

Finally, our data suggested that povidone-iodine might be superior to alkalinization. While most studies find low concentrations to be safe over months of application in younger patients,(**6**) studies using tenfold higher concentrations for gargling did find transient increases that resolved after the study.(**39**) For prolonged use, thyroid function testing in older individuals may be warranted.

Conclusion

As an intervention, pressurized nasal irrigation showed promise to reduce the severity of COVID-19 infection when initiated within 24 hours of a positive test. As large unvaccinated populations pressure evolution of variants, an effective mechanical outpatient intervention to reduce viral entry and hospitalizations can save lives and reduce the stress on hospital staff. Further research into the frequency and adjuvants of irrigation will be important not just for this pandemic, but for future viruses to come.

Data Availability

Data is available from the University of Augusta Department of Emergency Medicine Research Office, and online from the CDC COVID-19 Case Surveillance Public Use Data

https://data.cdc.gov/Case-Surveillance/COVID-19-Case-Surveillance-Public-Use-Data/vbim-akqf/data

Footnotes

- The initial analysis evaluated hospitalization risk only for cases where the status was known. To reduce the risk of reporting bias, statistical analysis was revised to include all laboratory-confirmed cases in the denominator, and only deaths where hospitalization data was unreported. Figure 1 was revised to include only unique cases testing positive. Tables 1-2 were revised, and Table 3 was added to compare irrigation and device data. Authors with ICMJE forms were added.

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Potential Role of Xylitol Plus Grapefruit Seed Extract Nasal Spray Solution in COVID-19: Case Series

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Abstract

The SARS-CoV-2 virus has created an unprecedented impact on healthcare globally. Being a novel virus, several treatments have been explored against COVID-19. During the early stages of the disease, treatment is mainly supportive. While several studies have suggested different treatment modalities, there is still no definitive treatment against COVID-19. Re-purposing already established medications, with excellent safety profiles, is a possible approach for treating the disease in its early stage. Having a mode of transmission as a droplet mode, several studies have supported how the nose can contain the primary route of entry of SARS-CoV-2. Hence, we postulated that re-purposing a commercially available nasal spray containing xylitol and grapefruit seed extract (GSE), namely Xlear Nasal Spray® (Xlear, Inc., American Fork, USA) could be used as an adjunct treatment of COVID-19. With a well-established safety profile, the components of this nasal spray have been studied and have been shown to have potential efficacy against viral pathogens, including coronavirus, and may potentially regulate pathways important in the initial entry of infection, replication, and systemic response to SARS-CoV-2. We present a series of three mild-moderate risks, symptomatic, COVID-19 patients, treated with the

intranasal combination, as an adjuvant to their ongoing treatment, with rapid clinical improvement and shorten time to negativization on repeat intranasal swab test via PCR. No safety issues were noted during the course of treatment. Xlear nasal spray, containing xylitol plus GSE, given its established safety profile and compelling clinical results described here, could be a potential adjunct treatment option in mild-moderate COVID-19 cases.

Keywords: grapefruit seed extract, xylitol, covid-19, sars-cov-2, intranasal, therapeutics

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel virus and the causative agent of the Coronavirus disease 2019 (COVID-19). Currently, the management and therapeutic options for COVID-19 are limited, including self-quarantine and supportive care, usually indicated for mild cases. In contrast, a moderate disease in high-risk patients and patients with severe conditions generally require hospitalization [1]. COVID-19 has caused a significant impact on the healthcare systems of various countries across the globe. As of October 3, 2020, COVID-19 has infected over 34,790,000 and caused over 1,031,000 deaths worldwide. In the United States alone, over 7,379,000 COVID-19 cases and over 200,000 deaths have been reported [2]. This situation has led to an urgent need for therapeutic options prompting increased interest in re-purposing the existing medications that might play a role in the treatment of COVID-19. Interestingly, it has been documented that both angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2), which are present not only in the bronchial epithelium and alveolar type II epithelium cells but also in the nasal epithelium, are associated with the virus entry into the cell. Pharmacological agents such as nasal sprays might be optimal therapeutic candidates for providing better outcomes in COVID-19 patients if used in the early stages of the disease [1-3]. In their systematic review, Gengler et al. have reported that viral shedding appears to be largely from the nasal cavity further suggesting that the nasal cavity could be a major source of COVID-19 transmission. Moreover, the nasal shedding could place various healthcare workers, such as the ones participating in rhinologic procedures, at a higher risk of COVID-19 [4].

Prior studies suggest that candidate agents with potential activity against SARS-CoV-2, which can be administered intranasally, might play a pivotal role in the treatment against COVID-19. In this vein, we have identified two candidate agents with potential activity against SARS-CoV-2 which can be administered intranasally, namely, xylitol and grapefruit seed extract (GSE). Moreover, the antiviral effects of xylitol as well as its antimicrobial properties are evaluated and have been documented [5]. Properties of GSE were evaluated in vitro with several viruses such as the avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and it showed activity against enveloped viruses such as the AIV and NVD but resistance with non-enveloped viruses namely IBDV [6]. In a study conducted by Bansal et al., they concluded that out of three samples of iota-carrageenan, all three were effectively able to inhibit the SARS-CoV-2 [5]. The third sample containing xylitol in combination with addition to iota-carrageenan was able to demonstrate an antiviral effect against SARS-CoV-2 at various concentrations which were tested in the study suggesting its potential role in COVID-19 [5]. Preliminary studies conducted by our group at two different laboratories have tested the individual components xylitol and GSE of commercially available intranasal spray (Xlear Nasal Spray®; Xlear, Inc., American Fork, USA). The results of the aforementioned preliminary studies seem to point toward the conclusion that the components exert a significant virucidal effect.

Accordingly, taking into consideration the current COVID-19 pandemic situation and the antimicrobial and virucidal effects of xylitol and GSE, we hereby present a case series of three COVID-19 positive patients who were prescribed the commercially available Xlear nasal spray, which contains xylitol and

GSE, for a duration of seven days. The present cases highlight the potential efficacy of intranasal xylitol and GSE, as a therapeutic aid for the management and treatment of COVID-19.

Case presentation

Case 1

A 16-year-old Hispanic female, with a past medical history of iron deficiency anemia, hemoglobin levels unknown, and was treated previously with ferrous sulfate, which was diagnosed three years ago, tested positive for COVID-19 on July 7, 2020. The patient is a non-smoker, with no past surgical history and not taking any maintenance medications. The patient complained of sore throat, dry mouth, nasal congestion, runny nose, productive cough with yellow sputum, anosmia, and ageusia, in addition to reporting waking up at night due to the coughing episodes, two days before consultation. Afebrile, no abdominal pain, diarrhea, no shortness of breath, weakness, or lethargy were reported. The patient also reported taking self-medication for two days with warm water and tea, which did not help alleviate the symptoms. A consultation with a primary care physician was pursued followed by a COVID-19 reverse transcriptase-polymerase chain reaction (RT-PCR) test via nasopharyngeal swab performed on the patient. Two days later, the patient tested positive, subsequently enrolled in this case series, and was given the experimental treatment. The patient was instructed to spray Xlear nasal spray twice per nostril four times a day every six hours for seven days, which was an adjunct to her self-medication. The patient continued to self-medicate with warm water and tea, and supportive treatment. On day 1, the patient complained of a stuffy nose, anosmia, ageusia, tiredness, cough, stuffiness, and congestion. Oxygenation 98% on room air, pulse rate 78 beats per minute, afebrile, with mild symptoms on Symptoms Assessment Score (SAS). Patients rated generalized pain as three on the Visual Analogue Score (VAS) and the Numerical Rating Scale (NRS). On day 3, an improvement was noted in her symptoms, particularly anosmia which she reported being able to smell strong substances. On average, the documented resolution of anosmia is two weeks [7]. An improvement of cough was also noted. The patient's labs were drawn on day 4 with normal levels of c-reactive protein (CRP) and d-dimer (Appendices). On day 7, the patient noted an improvement in the overall symptoms with a reduced degree of tiredness, absence of cough, congestion, and stuffiness. Although mild ageusia is still present, it was markedly improved compared to day 1. The patient remained afebrile throughout the duration of the trial. Improvement of symptoms was noted during the trial duration. On day 7, the patient was retested for COVID-19 RT-PCR via nasopharyngeal swab with non-reactive results. Repeat testing of COVID-19 RT-PCR was also done on day 8 which also showed non-reactive results (Appendices). A follow-up was done on day 14 and the patient reported no symptoms with a return to baseline health.

Case 2

A 60-year-old Hispanic male was tested positive for COVID-19 on July 7, 2020. The patient had a past medical history of leukemia, which was diagnosed in 2012. Currently in remission, post-chemotherapy and radiation therapy, a heavy smoker, and occasional alcoholic beverage user. The patient reported using maintenance medications: zolpidem 10 mg once a day and bupropion HCL 100 mg two times a day. Two days before consulting the primary care physician, the patient started to experience sore throat, dry mouth, sneezing, nasal congestion, runny nose associated with anosmia and ageusia, and a low-grade fever at 101 Fahrenheit (F). No abdominal pain, diarrhea, shortness of breath weakness, or increased tiredness were noted. Furthermore, self-medication with warm water and tea did not help alleviate the symptoms that were reported by the patient. Upon consultation with the primary care physician, the patient was tested for COVID-19 RT-PCR via the nasopharyngeal swab. Following the positive COVID-19 test, the patient was subsequently enrolled in the case series experimental group. The patient was instructed to use the Xlear nasal spray four times a day every six hours for seven days,

which was an adjuvant to his self-medication. The patient self-medicated with acetaminophen for fever, multivitamins, tea, and warm water. On day 1, the patient complained of a stuffy nose, sneezing, congestion, sandy and watery eyes, with oxygen saturation at 97% room air and pulse rate of 86 beats per minute. The patient also complained of anosmia, fever, 101 F. Rated overall symptoms as mild on SAS and generalized pain as three VAS and NRS. On day 2, the patient was noted to have tiredness and productive cough, with awakenings at night time due to coughing episodes. The patient also noted increasing tiredness. The patient also complained of ageusia. However, the patient was now afebrile with stable oxygenation and pulse rate. On day 3, the patient noted improvement of symptoms with only sandy eyes, anosmia, and ageusia. The patient also noted that he was now able to start smelling strong substances. On day 4, the patient's CRP and d-dimer were tested and were unremarkable (Appendices). On day 7, the patient reported symptoms of tiredness, ageusia, and anosmia with a 70-80% improvement of the ability to smell. The patient remained afebrile starting on day 2 with noted improvement during the entire seven days. Subsequently, on the same day, the patient was retested for COVID-19 with RT-PCR via nasopharyngeal swab with non-reactive results. The patient continued to smoke 10 tobacco sticks per day throughout the duration of the trial. A repeat test for COVID-19 RT-PCR was done and yielded a negative result (Appendices). A follow-up was done on day 14 and the patient reported no symptoms with the return to baseline health.

Case 3

A 38-year-old Hispanic male tested positive for COVID-19 on September 26, 2020. The patient had a past surgical history of arthroscopy 20 years ago and was currently taking n-acetyl glucosamine, vitamin C, and vitamin D daily. The patient has grade 1 obesity (body mass index 30), a non-smoker, non-alcoholic beverage drinker, and does not use illicit drugs. Two days before the consultation in urgent care, the patient started to experience cold-like symptoms, sinus pressure, night sweats, and unquantified fever. Consultation at urgent care was then pursued. On the chest X-ray, diminished lung volumes with hypoventilatory changes at the lung bases with basilar atelectasis were noted. The patient was prescribed azithromycin 250 mg for five days along with albuterol nebulization (Appendices). Five days after the consultation, the patient continued to feel worse despite taking the prescribed medications. The patient then opted to have himself tested for COVID-19 after learning that his uncle, whom he interacted with a few days prior, tested positive for COVID-19. The patient subsequently tested positive. The patient was then prescribed with Xlear nasal spray and was instructed to spray twice per nostril four times a day every six hours for seven days, as an adjuvant to his ongoing previously mentioned treatment. On day 1, the patient complained of a runny and stuffy nose, tiredness, productive cough, nasal congestion, diarrhea, with oxygen saturation at 94%, and afebrile. The patient also complained of headache, rated two on the VAS and NRS scale, and rated overall symptoms as mild. On day 3, the patient noticed an improvement of symptoms with only tiredness, nasal congestion, and cough. The patient demonstrated stable vital signs and oxygenation and reported that he has regained his sense of smell and his sense of taste. On day 4, the patient's CRP and d-dimer were tested and were unremarkable (Appendices). On day 7, the patient reported symptoms of tiredness, and cough, although overall symptoms significantly improved as compared to previous days. The patient remained afebrile throughout the trial with noted improvement in all seven days. The patient was also retested for COVID-19 with RT-PCR via nasopharyngeal swab and showed non-reactive result (Appendices). Follow-up was done on day 14 and the patient reported no symptoms with a return to baseline health. No repeat chest X-ray was done post-treatment.

Discussion

These reported cases are the first to shed some light regarding the potential efficacy of utilizing intranasal xylitol plus GSE as an adjunct treatment against COVID-19 and reduction to the time of negativization on nasal RT-PCR. While it is difficult to have definitive proof of efficacy in a form of a case series, we believe that the present series provides a rationale for initiating larger randomized placebo-controlled clinical trials evaluating the utilization of xylitol plus GSE in the form of an intranasal spray in COVID-19 patients.

The patients we reported also had risk factors that could increase the risk of morbidity and mortality: patient 1 had iron deficiency anemia, corrected with iron therapy; patient 2 had a significant risk factor given his smoking status and history of cancer, chemotherapy, and radiation; and patient 3, although relatively healthy, was mildly obese with history of arthroscopy. The above-mentioned patients have a higher than average risk of COVID-19 morbidity and mortality [8]. Neither of the patients progressed to severe disease and all patients showed improvement in the symptoms with the intranasal use of xylitol plus GSE, with a reduced number of days to testing positive to negative via COVID-19 RT-PCR nasal swab test.

Two sprays per nostril every six hours were administered to these three patients. A standard dose per nasal spray contains up to 140 µL per spray. Based on the nasal cycle, each dose of 140 µL per spray delivered properly into the nasal cavity with an estimated nasal airway surface liquid volume in the range of 50-375 µL should remain in the cavity an average of four to six hours [9-14]. A study conducted by Hou et al. stated that there is a strong association between the high levels of ACE2 and SARS-CoV-2 infectivity [15]. Studies show a higher ACE2 level in the nasopharyngeal tract compared to the lower respiratory tract [15,16]. In a genomic map of COVID-19 by the University of North Carolina at Chapel Hill, it was found that there is a gradient with greater expression of ACE2 receptors and SARS-CoV-2 infectivity in the nose compared to the peripheral lung tissue. These case series support our rationale that therapeutic strategies should aim at reducing viral load in the nose early in the disease using nasal sprays or lavages [17]. The underlying role of xylitol and GSE on influencing the ACE2 levels in the nasopharyngeal tract remains to be elucidated.

Lastly, the time to negativization is important to note. The average time to negativization was found to be approximately an average of 14 days [18]. These cases have shown that by using xylitol plus GSE in the form of an intranasal spray (Xlear nasal spray), as an adjunct to the ongoing treatment, the time to negativization was reduced to seven days, a 50% reduction compared to the usually known average. It should be noted that in this case series, a combination of intranasal xylitol plus GSE nasal spray was mainly used as an adjunct therapy to ongoing treatments for COVID-19.

Conclusions

In summary, the three patients reported in this article, with minimal to moderate risk for morbidity and mortality from COVID-19, demonstrated an improvement in the symptoms and a reduction in the clinical course post use of xylitol plus GSE in the form of a nasal spray, commercially available as Xlear nasal spray, as an adjunct to their ongoing treatment. This combination could play a potential role in improving the outcome in mild to moderate COVID-19 patients. While relatively safe for general use, larger randomized, placebo-controlled clinical trial studies are mandated which could shed further light on this topic.

Appendices

Figure 1

Open in a separate window

Chest X-ray image of patient 3

Table 1

Laboratory results of patients.

CRP: C-reactive protein, RT-PCR: reverse transcriptase-polymerase chain reaction.

Day	Laboratory Test	Normal Values	Patient 1	Patient 2	Patient 3
Day 4	CRP	0.0-9.9 mg/L	0.4	0.4	0.4
Day 4	D-Dimer	<0.50 mg/L	0.19	0.19	0.19
Day 7	COVID-19 RT-PCR	Non-reactive	Non-reactive	Non-reactive	Non-reactive

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Gustavo Ferrer- research grants from Xlear and work on research and development with Xlear

Notes

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Human Ethics

Consent was obtained by all participants in this study

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Exhibit I

- Research
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Rhinovirus prevalence as indicator for efficacy of measures against SARS-CoV-2

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Abstract

Background

Non-pharmaceutical measures to control the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) should be carefully tuned as they can impose a heavy social and economic burden. To quantify and possibly tune the efficacy of these anti-SARS-CoV-2 measures, we have devised indicators based on the abundant historic and current prevalence data from other respiratory viruses.

Methods

We obtained incidence data of 17 respiratory viruses from hospitalized patients and outpatients collected by 37 clinics and laboratories between 2010-2020 in Germany. With a probabilistic model for Bayes inference we quantified prevalence changes of the different viruses between months in the pre-pandemic period 2010-2019 and the corresponding months in 2020, the year of the pandemic with noninvasive measures of various degrees of stringency.

Results

We discovered remarkable reductions δ in rhinovirus (RV) prevalence by about 25% (95% highest density interval (HDI) [−0.35,−0.15]) in the months after the measures against SARS-CoV-2 were introduced in Germany. In the months after the measures began to ease, RV prevalence increased to low pre-pandemic levels, e.g. in August 2020 δ=−0.14 (95% HDI [−0.28,0.12]).

Conclusions

RV prevalence is negatively correlated with the stringency of anti-SARS-CoV-2 measures with only a short time delay. This result suggests that RV prevalence could possibly be an indicator for the efficiency for these measures. As RV is ubiquitous at higher prevalence than SARS-CoV-2 or other emerging respiratory viruses, it could reflect

the efficacy of noninvasive measures better than such emerging viruses themselves with their unevenly spreading clusters.

Peer Review reports

Background

The Coronavirus Disease 2019 (COVID-19) pandemic is caused by the severe acute respiratory syndrome coronavirus (SARS-CoV-2) [1]. SARS-CoV-2 is transmitted person-to-person predominantly via respiratory droplets and aerosols produced by breathing, coughing or sneezing. These particles are deposited directly on mucosal surfaces, or on fomites [2–4]. Recent evidence suggests that SARS-CoV-2 is quite resilient and may remain infectious in aerosols for hours and on surfaces for days [5]. With no effective treatment or vaccine available, the containment of SARS-CoV-2 depends on measures such as physical distancing, restrictions on mobility, increased personal hygiene, and use of face masks [6–9]. These measures appear to be effective [10] but they also exert substantial social and economic burden [11]. Therefore, governments should tune these measures to limit the spread of SARS-CoV-2, while also allowing a maximum degree of normalcy. To this end, we need reliable indicators for the efficacy of the measures against SARS-CoV-2.

Rigorous measures against SARS-CoV-2 will most likely slow down the spread of the virus, as we have seen in the first COVID-19 wave in different countries around the world. Thus, for the weeks and months after the measures are introduced we expect a reduction in the relative frequency (prevalence) of individuals who test positive for SARS-CoV-2. As the measures against SARS-CoV-2 begin to be relaxed to a point where they are no longer effective in containing the virus, we anticipate resurgence in the prevalence of SARS-CoV-2. This implies that we may use the information about changes in SARS-CoV-2 prevalence over time to assess the efficacy of the measures.

With SARS-CoV-2 being a novel virus, the use of SARS-CoV-2 prevalence as indicator for efficacy of the measures comes with a number of caveats. For instance, due to lack of comparable epidemiological data for this virus from the previous years we have limited understanding of its seasonal variation in transmission, which is likely to exert a strong influence on the dynamics of the pandemic, as we know from other respiratory viruses. An example is provided by the influenza virus: in Europe we observe high influenza prevalence between December and April [12]. For the remaining months of the year, however, the prevalence of influenza is negligible. The lack of historic prevalence data for SARS-CoV-2 means that we cannot disentangle effects of measures from potential seasonal effects. Thus, by ignoring the seasonal variation in transmission, we may under- or overestimate SARS-CoV-2 prevalence, and as a result misjudge the efficacy of measures. Moreover, accurate assessment of the SARS-CoV-2 prevalence depends on robust infrastructure (e.g. testing laboratories, experts, kits, digital platforms for sharing of SARS-CoV-2 data) for rapid detection and reporting of SARS-CoV-2 infections, including widespread screening for asymptomatic SARS-CoV-2 infections [13]. As of yet, such infrastructure is lacking across many parts of the globe, partially blinding us.

In the study presented here, we describe an indirect yet more robust approach for quantifying the efficacy of the measures against SARS-CoV-2. Our core assumption is that efficient measures against SARS-CoV-2 will also suppress the spread of other respiratory viruses that have similar features as SARS-CoV-2, such as transmission routes, viability in different environments, etc. To test this hypothesis, we obtained epidemiological data on the incidences of 17 different respiratory viruses spanning the years 2010-2020. Using a probabilistic model, we inferred the monthly prevalence of these viruses in the pre-pandemic period 2010-2019, and then compared this with the prevalence of the same virus in the months of 2020, i.e., the period following the introduction of the measures against SARS-CoV-2. Thus, we were able to determine to which degree the prevalence of the different respiratory viruses is affected by the measures against SARS-CoV-2, while properly accounting for seasonal effects. Strong deviations in viral prevalence are interpreted and discussed here in the context of the features of the different viruses. A key finding of this study is that rhinovirus prevalence is a suitable indicator for the effectiveness of public health measures against SARS-CoV-2.

Methods

Virus prevalence data

Incidence data on 17 different respiratory viruses in hospitalized patients was obtained from the Respiratory Viruses Network (RespVir) [14]. The data was collected from 37 clinics and laboratories across Germany in the period from 2010 to 2020 (up to and including October 2020) (Supplementary Section 1). From RespVir we also obtained incidence data on SARS-CoV-2 collected from 14 laboratories across Germany in the period from 24.01.2020 to 27.10.2020 (Supplementary Section 1). From this data we computed frequencies (counts) of positive tests for each virus that originate from a specific laboratory in a given month and year, including the total number of tests made.

Statistical modeling of monthly prevalence of 17 respiratory viruses

We used data from the years 2010 to 2020 to model the monthly prevalence of different respiratory viruses. For laboratory $l \in \{1,\ldots,37\}$, month $m \in \{1,\ldots,12\}$ and year $y \in \{2010,\ldots,2020\}$, we observed Y^v_{lmy} positive cases of the virus v among N^v_{lmy} tested patients. We set the design variable X^v_{my} 0 for months in the pre-pandemic period (years 2010 to 2019) and 1 for months in 2020. That is, the X^v_{my} are indicator variables for whether we are in the pandemic year or in a prepandemic year.

We aimed at inferring from these data the mean prevalence of a given virus in each month of the year in the pre-pandemic and pandemic period. Additionally, we aimed at quantifying the effect of the anti-SARS-CoV-2 measures on the prevalence in the different months of the year 2020. For this purpose we designed a likelihood model M for Bayesian inference. M describes the positive case count as a binomial model:

$$p\left(Y^v_{lmy} \middle| M\right) = \text{Binomial}\left(\pi^v_{lmy}, N^v_{lmy}\right), \tag{1}$$

where π is the probability of positive tests, defined as the inverse-logit function of $\hat{\pi}$:

$$\pi^v_{lmy} = \text{logit}^{-1}\left(\hat{\pi}^v_{lmy}\right), \tag{2}$$

where $\text{logit}^{-1}(x) = 1/(1+\exp(-x))$. The raw prevalence varied substantially between individual laboratories. To account for this source of variation, the model treats the corresponding coefficients $\hat{\pi}$ as random samples drawn from a normal distribution:

$$\hat{\pi}^v_{lmy} \sim \text{Normal}\left(\alpha^v_{my}, \tau^v\right), \tag{3}$$

where the inverse-logit of α^v_{my} is the mean prevalence of virus v in month m of year y, and τ^v is the standard deviation of virus v that accounts for the variance in prevalence between the individual laboratories.

Empirically, we know that the prevalence of most respiratory viruses follows specific seasonal patterns. Hence, the viral prevalence in a given month of the year is not completely independent across the different years in the period from 2010 to 2020. Hierarchical models, such as M, enable sharing of information between parameters across the different years by partial pooling [15]. For a specific virus and month, the corresponding coefficients α are treated as random samples drawn from a population of parameters:

$$\alpha^v_{my} \sim \text{Normal}\left(\mu^v_m + \beta^v_m X^v_{my}, \sigma^v_m\right) \tag{4}$$

With a Bayesian approach we can then infer shared parameters for month *m* and virus *v*, such as the mean pre-pandemic prevalence $\text{logit}^{-1}(\mu_m^v)$, the mean pandemic prevalence $\text{logit}^{-1}(\mu_m^v + \beta_m^v)$, where coefficient β_m^v is the effect of the anti-SARS-CoV-2 measures; and the standard deviation σ_m^v, while simultaneously accounting for the within-year variability. The weakly informative priors assigned to β,μ and σ and τ are defined by:

$$\beta_m^v \sim \text{Normal}(\mu = 0, \sigma = 10) \tag{5}$$

$$\mu_m^v \sim \text{Normal}(\mu = 0, \sigma = 10) \tag{6}$$

$$\sigma_m^v \sim \text{Cauchy}^+(\mu = 0, \gamma = 1) \tag{7}$$

$$\tau^v \sim \text{Cauchy}^+(\mu = 0, \gamma = 1) \tag{8}$$

For virus *v* and month *m*, we estimate the change in mean prevalence (δ) between the pandemic and the pre-pandemic period as:

$$\delta_m^v = \text{logit}^{-1}(\mu_m^v + \beta_m^v) - \text{logit}^{-1}(\mu_m^v) \tag{9}$$

For months where δ<0 and the 95% Highest Density Intervals (HDIs) of δ lie mostly or completely below 0 (0 = null effect), we have strong evidence of reduced viral prevalence in the year 2020 compared to that in the years 2010 to 2019. On the other hand, for months where δ>0 and the 95% HDI of δ lie mostly or completely above 0, we have strong evidence of increased viral prevalence. Distributions with the 95% HDIs more or less centered around 0 indicate that there is no evidence for a clear change in the monthly viral prevalence in the year 2020. Note that unclear evidence is not equivalent to no change, because for a month with $\delta \approx 0$ we may also have a wide 95% HDI, including possibilities for positive or negative change.

Notably, the inference of each coefficient β relies on only one small data set (one value per contributing lab) for each month of year 2020. Therefore, the β coefficients will be highly uncertain and have wide 95% HDIs. Consequently, the coefficients δ will also be uncertain.

M was implemented in Stan [16]. Inference of the parameters of *M* was executed with rstan using the No-U-Turn sampler by running a Markov chain Monte Carlo (MCMC) simulation with six chains of 10,000 iterations each, including 3,000 warm-ups (R-package rstan, version 2.19.2). To test the validity of our model, we performed posterior predictive checks. We used the potential scale reduction factor (PSRF), the effective number of samples (N_{eff}) and information provided by rstan on divergences during the MCMC sampling to check for a successful convergence. For each parameter we report its posterior median and 95% HDI.

Statistical modeling of monthly SARS-CoV-2 prevalence

For laboratory $l \in \{1,\ldots,14\}$ and month $m \in \{1,\ldots,10\}$ in 2020, we observe Y_{lm} positive cases of SARS-CoV-2 among N_{lm} tested patients. From this data we infer the overall monthly prevalence of positive cases. To this end we designed a likelihood model M_{SC2} for Bayesian inference. With M_{SC2} we describe the count of positive SARS-CoV-2 cases as a binomial model:

$$p(Y_{lm}|M_{SC2}) = \text{Binomial}(\pi_{lm}, N_{lm}), \tag{10}$$

where π is the probability of positive tests, defined as the inverse-logit of α:

$$\pi_{lm} = \text{logit}^{-1}(\alpha_{lm})$$

(11)

The SARS-CoV-2 prevalence varied substantially between individual laboratories. To account for this source of variation, the model treats the corresponding coefficients α as random samples drawn from a population of parameters:

$$\alpha_{lm} \sim \text{Normal}(\mu_m, \sigma),$$
(12)

where μ_m is the mean SARS-CoV-2 prevalence for a specific month m, and σ is the standard deviation. The weakly informative priors assigned to μ_m and σ are defined by:

$$\mu_m \sim \text{Normal}(\mu = 0, \sigma = 10)$$
(13)

$$\sigma \sim \text{Cauchy}^{+}\,(\mu = 0, \gamma = 1)$$
(14)

M_{SC2} was implemented in Stan and executed with MCMC simulation settings identical to those introduced for model M. For each parameter we report its posterior median and 95% HDI.

With M_{SC2} we can also infer the mean SARS-CoV-2 prevalence in different weeks of the year based on the RespVir data on SARS-CoV-2 frequencies in different lab- oratories and weeks of year 2020 (Supplementary FigureS2). For this analysis we have to substitute the month-specific index $m \in \{1,\ldots,10\}$ with the week-specific index $m \in \{4,\ldots,43\}$ in M_{SC2}.

Results

SARS-CoV-2 prevalence in 2020

We evaluated the monthly SARS-CoV-2 prevalence in 2020 (Fig. 1A). For January 2020 our data does not contain positive cases for SARS-CoV-2. Hence, the mean SARS-CoV-2 prevalence was 0 [0,0.009] (in the following we give the 95% HDIs behind numbers in square brackets to quantify uncertainty). In February 2020 a few of our laboratories found SARS-CoV-2 (SupplementaryFigure S1), however, the overall mean SARS-CoV-2 prevalence remained low (0.005 [0.002,0.013]). SARS-CoV-2 prevalence reached its peak at 0.067 [0.035,0.126] in March 2020. Around mid-March 2020, the initial set of measures against SARS-CoV-2 was introduced in Germany [17]. This was followed by a drop in mean SARS-CoV-2 prevalence to 0.032 [0.016,0.066] in April 2020 and to 0.009 [0.004,0.018] in May 2020. Between June 2020 and September 2020, the SARS-CoV-2 prevalence remained approximately flat. In June, July, August and September 2020, the mean SARS-CoV-2 prevalence was 0.004 [0.002,0.009],0.007 [0.003,0.016],0.005 [0.002,0.012] and 0.006 [0.003,0.014], respectively. In October 2020 new SARS-CoV-2 cases surged again [18], increasing the mean SARS-CoV-2 prevalence to 0.016 [0.007,0.035] (Fig. 1A).

Fig. 1

Monthly prevalence of SARS-CoV-2 and RV. **a** Blue circles and bars: mean SARS-CoV-2 prevalence between January 2020 and October 2020 with the corresponding 95% HDIs. Orange circles and bars: mean pandemic RV prevalence between January 2020 and October 2020 with the corresponding 95% HDIs. Colored rectangles along the x-axis at dates of specific measures, relaxations or other important events in 2020 [17]. 27.01: first confirmed case of SARS-CoV-2 in Germany; 09.03: large events are canceled; 16.03: schools, child care, shops, churches, bars, etc. are closed; 23.03: contact ban; 20.04: shops (partially) reopen; 27.04: mandatory use of face masks; 30.04: museums, temples, zoos and playgrounds reopen; 04.05: schools (partially) reopen; 16.05: restaurants reopen; 15.06: European Union and Schengen countries reopen borders; 17.06: more than 1,000 meat-factory workers test positive for SARS-CoV-2. **b** Blue rectangles: 95% HDIs of the mean pre-pandemic RV prevalence in each month of the year. Orange circles and bars refer to RV as in panel (**a**)

Full size image ›

Among 17 respiratory viruses, rhinovirus is most strongly affected by anti-SARS-CoV-2 measures

As a result of the measures against SARS-CoV-2, we also expect a reduction in the spread of other respiratory viruses that have similar features as SARS-CoV-2. To test this hypothesis, we compared the monthly prevalence of 17 different respiratory viruses between the years 2010-2019 (pre-pandemic period) and 2020 (pandemic period) in Germany (Supplementary Figure S3). For each month of the year we report the change in mean prevalence (δ) of each respiratory virus between the year 2020 and the period 2010-2019 (Supplementary Figure S4). For almost all respiratory viruses, δ values are predominantly negative, i.e. these viruses have lower prevalence in 2020 (Fig. 2A).

Fig. 2

Change in mean monthly prevalence (δ) of different respiratory viruses between the pandemic (2020) and pre-pandemic (2010-2019) period. **a** Colored circles: median coefficients δ for different months of the year (x-axis) and different respiratory viruses (y-axis). Random vertical jitter was added to avoid overplotting. **b** Change in mean RV prevalence. Black circles and bars: median coefficients δ with the corresponding 95% HDIs. Dashed lines at δ=0

Full size image ›

For rhinovirus (RV) we observed exceptionally strong suppression of its prevalence during that period (Fig. 2A/B). Other viruses, such as human respiratory syncytial virus (HRSV), human parainfluenza virus 3 (HPIV-3), human adenovirus (HAdV) and enterovirus (EV), decreased moderately from April 2020 to October 2020, compared to the respective months from 2010 to 2019 (Fig. 2A, Supplementary Figure S3). For several other respiratory viruses (human bocavirus (HBoV) to influenza A virus subtype H3N2 (FLUA(H3N2)) in Fig. 2A) there was a small to negligible trend to lower δ, and only 2 of the 17 viruses, human coronavirus HKU1 (HCoV-HKU1) and influenza A virus subtype H1N1 (FLUA(H1N1)), had a positive δ trend.

Virus seasonality is one determinant of the degree to which the prevalence of different respiratory viruses reflects the measures against SARS-CoV-2. RV and several of the other viruses (HAdV, HPIV-3) whose prevalence are moderately suppressed by the measures, are continuously present in the general population at a prevalence of a few percent (Supplementary Figure S3). Only this high baseline prevalence allowed us to detect moderate or strong reduction in their prevalence in the period from April 2020 to October 2020. HRSV prevalence, on the other hand, varies strongly between different seasons, i.e. during winter and early spring we observe high HRSV prevalence in Europe, and low prevalence during the rest of the year [12]. Thus, we were able to detect moderate reduction in HRSV prevalence from April 2020 to June 2020 when HRSV prevalence was still sufficiently high, but not for July to October 2020 when HRSV has a low prevalence anyway.

The remaining respiratory viruses either have transmission patterns that vary drastically between different seasons, or have low prevalence throughout the year (Sup-plementary Figure S5). In either case, these viruses were barely present in Germany in the period from April 2020 to October 2020, so that changes in their prevalence in that period due to anti-SARS-CoV-2 measures could not be detected reliably.

Rhinovirus prevalence

In the above analysis, rhinovirus (RV) had particularly strong changes in prevalence between April and October 2020, which made it most promising as an indicator of efficacy of anti-SARS-CoV-2 measures. Hence, we focus on RV in the following.

First, in January 2020 and February 2020, the months before the anti-SARS-CoV-2 measures were introduced in Germany, the mean RV prevalence (orange circles and bars in Fig. 1B) is consistent with the pre-pandemic mean RV prevalence (blue rectangles in Fig. 1B). The mean RV prevalence in March 2020 is slightly lower yet still has large overlap with the mean pre-pandemic RV prevalence (Fig. 1B).

Second, between April 2020 and June 2020, the mean RV prevalence falls completely outside the 95% HDI of the pre-pandemic mean RV prevalence for the respective months (Fig. 1B). The drastic reduction in mean RV prevalence in April 2020 (δ=−0.2 [−0.26,−0.14]), May 2020 (δ=−0.28 [−0.36,−0.2]) and June 2020 (δ = −0.29 [−0.37,−0.22]) may be attributed to the measures against SARS-CoV-2. In July 2020 (δ = −0.23 [−0.34,−0.06]), August 2020 (δ=−0.14 [−0.28,0.12]) and September 2020 (δ=−0.29 [−0.46,−0.01]) we observed a moderate resurgence in mean RV prevalence to low pre-pandemic levels. In October 2020 (δ=−0.32 [−0.44,−0.14]) the mean RV prevalence once again falls completely outside the 95% HDI of the pre-pandemic mean RV prevalence for the respective month (Fig. 1B).

Third, while from 2010 to 2019 the RV prevalence exhibited a clear seasonal upward trend from February to June (upward shift of blue rectangles in Fig. 1B), the data for 2020 shows an unbroken downward trend from February to June (orange circles in Fig. 1A/B), contrary to the seasonal trend. It seems that these dynamics in 2020 could have started even before the implementation of anti-SARS-CoV-2 measures in mid-March, possibly because many individuals, alerted by the intensive news coverage, have changed their behavior.

Discussion

Transmission properties of RV and SARS-CoV-2 explain their dynamics during the pandemic

To contain the spread of SARS-CoV-2 in Germany, a package of diverse measures was introduced around mid-March 2020 [17]. With SARS-CoV-2 presumed to spread by both airborne and contact-based pathways, the measures were aimed at reducing the number of person-to-person contacts by e.g. physical distancing and closure of shops and schools, and by more rigorous personal hygiene with e.g. more frequent hand washing, use of face masks, and disinfecting surfaces. Soon after the measures were enacted, we observed a reduction in SARS-CoV-2 prevalence (Fig. 1A). As a byproduct of the measures against SARS-CoV-2, the transmission of other respiratory viruses, such as RV, appears to have been diminished as well (Fig. 2B, Supplementary Figure S4). Between mid-April 2020 and mid-June 2020, gradual relaxation of the measures took place, including partial re-opening of shops and schools, lifting of travel restrictions and reopening of playgrounds and churches [17]. Soon thereafter, namely between July 2020 and September 2020, the RV prevalence increased to low pre-pandemic levels, while the SARS-CoV-2 prevalence remained approximately flat. In October 2020 the number of new SARS-CoV-2 cases in Germany surged, and so did our estimated SARS-CoV-2 prevalence. In contrast to this, the RV prevalence decreased in October 2020, though with a still large uncertainty (Fig. 1B). To better understand why SARS-CoV-2 and RV exhibit similar dynamics between April 2020 and June 2020, and divergent dynamics between July 2020 and October 2020, we examine some of their features more closely.

RV is the most common respiratory pathogen of humans and a major causative agent of the common cold [19, 20]. RV is likely transmitted via respiratory aerosols produced by coughing or sneezing, and by contact with surfaces contaminated with nasal secretions [20]. Owing to its stability, RV may remain infectious on surfaces for days and in aerosols for hours [21, 22]. While the assessment of SARS-CoV-2 transmission and resilience in different environments is still an area of active research, recent studies have reported similar transmission routes and degree of resilience for different human coronaviruses (HCoVs) including for SARS-CoV-2 [5, 22]. If we assume that transmission routes of RV and SARS-CoV-2 have a large overlap, then we can expect that the rigorous anti-SARS-

CoV-2 measures will affect the spread of both viruses to a similar degree. This is a plausible explanation for the consistent decrease in RV and SARS-CoV-2 prevalence between April 2020 and June 2020.

However, there are also differences between RV and SARS-CoV-2 that have to be considered, namely differences in (1) seasonal patterns, and (2) the degree of dissemination in the human population.

First, the introduction of anti-SARS-CoV-2 measures have certainly lowered the prevalence of RV and SARS-CoV-2 but even these lower levels are still modulated by seasonal patterns. We know empirically that there is a seasonal upwards trend in RV prevalence from February to September (Fig. 1B). Conversely, there is a downward trend in the prevalence of different HCoVs during summer and autumn [23] (Supplementary Figure S3). If we assume that SARS-CoV-2 follows a seasonal trend that is similar to that of other HCoVs, then the combined effect of the season and of anti-SARS-CoV-2 measures is probably responsible for the flat SARS-CoV-2 prevalence between July and September 2020. In the same vein, the divergence of RV and SARS-CoV-2 prevalence courses in summer 2020 could also be due to seasonal changes that modulate the low level RV and SARS-CoV-2 prevalence still suppressed by anti-SARS-CoV-2 measures. As the activity of HCoVs peaks in the coming months of fall and winter and the activity of RV declines (Fig. 1B), we expect to see a rebound of SARS-CoV-2 prevalence to a level similar to the RV prevalence and again a crossing of the two prevalence curves as observed between February and March 2020. Already in October 2020 we see evidence in support of such dynamics between RV and SARS-CoV-2 (Fig. 1A). As a result of the combined effect of the new anti-SARS-CoV-2 measures introduced in Germany in November 2020 [17], and the seasonal suppression of RV being close to its inflection point, we expect again a roughly parallel decay of RV and SARS-CoV-2 prevalence if the measures are effective.

Second, we know that RV is widespread in the human population and environment [19]. Hence, rapid resurgence in RV prevalence might be possible in response to the relaxations. With SARS-CoV-2 being less widely disseminated within the European population, it is also possible that it takes longer for SARS-CoV-2 to reemerge in response to the relaxations.

In summary, generally low levels of RV prevalence between January and October 2020 are consistent with the effectiveness of anti-SARS-CoV-2 measures in Germany, though we emphasize that seasonality of viral prevalence cannot be neglected. These results are corroborated by reports from the United Kingdom [24] and Australia [25, 26] where after lock-downs low RV prevalence values were observed that then bounced back after the easing of restrictions [27].

RV prevalence is the most suitable indicator of efficacy for the anti-SARS-CoV-2 measures among all studied viruses

Our study reveals reduced prevalence of the respiratory viruses HRSV, HPIV-3, HAdV and EV between April 2020 and October 2020. HRSV, HAdV and EV are transmitted similarly to RV and SARS-CoV-2, namely via respiratory droplets and aerosols, and direct or indirect contact [21, 28, 29], while limited experimental data for HPIV-3 hints at fomites as its main route of transmission [21]. There are several arguments in favor of using the RV prevalence over the prevalence of the other respiratory viruses as indicator of efficacy for the measures against SARS-CoV-2.

First, RV is constantly circulating in the population and is therefore subject to significantly lower seasonal fluctuations than other respiratory viruses, such as HRSV (Supplementary Figure S3). Second, the prevalence of RV is typically higher throughout the year than the prevalence of HRSV, HPIV-3, HAdV and EV (SupplementaryFigure S3). Hence, larger decreases in prevalence, which are also easier to detect by our approach, are possible for RV as a result of the measures (Fig. 1B). Third, we see that the prevalence of HRSV, HPIV-3, HAdV and EV increases with a longer time delay in response to the relaxations of the anti-SARS-CoV-2 measures in comparison to the RV prevalence (Supplementary Figure S3). These features favor RV prevalence as a quickly responding indicator of anti-SARS-CoV-2 measure efficacy.

In other geographical regions where respiratory viruses exhibit different seasonal patterns of transmission, another respiratory virus might be a more appropriate indicator of the anti-SARS-CoV-2 measure efficacy. For instance, several studies of seasonal influenza virus in Japan [30], South Korea [31], Singapore [32], Australia, Chile, South

Africa and the United States [33] have reported suppressed influenza prevalence in the period after the implementation of non-pharmaceutical measures against SARS-CoV-2. Owing to the low seasonal prevalence of influenza in Germany in the period between April 2020 and October 2020, our study shows only a negligible reduction in influenza prevalence (SupplementaryFigure S3).

Conclusions

Using one virus, such as RV, to monitor measures against another virus, such as SARS-CoV-2, is seemingly paradoxical. However, a mixture of factors such as the high transmissibility of SARS-CoV-2, its initially complex dissemination pattern of exponential growth in many clusters, and the limited testing capacities at the begin of the pandemic, had left us partially blinded regarding the efficacy of anti-viral measures in the first months. In such a situation, the prevalence of RV, a ubiquitous respiratory virus with a long historic record, moderate seasonality and transmission routes similar to SARS-CoV-2, is likely a better indicator of the efficacy of anti-SARS-CoV-2 measures than the prevalence of the latter virus itself. This logic also applies to RV in relation to other respiratory viruses that are candidates for causing future epidemics or pandemics.

Availability of data and materials

Data and source code are available upon reasonable request from Rolf Kaiser and Ortwin Adams (data) and Simo Kitanovski (source code).

Abbreviations

COVID-19:

Coronavirus Disease 2019

EV:

enterovirus

FLU:

influenza

HAdV:

human adenovirus

HBoV:

human bocavirus

HCoV:

human corona virus

HDI:

Highest Density Interval

HMPV:

human metapneumovirus

HPIV:

human parainfluenza virus

HRSV:

human respiratory syncytial virus

MCMC:

Markov chain Monte Carlo

N_{eff}*:*

effective number of samples

PSRF:

potential scale reduction factor

RespVir:

Respiratory Viruses Network

RV:

rhinovirus

SARS-CoV-2:

severe acute respiratory syndrome coronavirus 2.

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Respiratory Virus Network

Contributions

SK: concept of the current analysis, analysis of the data, and writing the manuscript; GHR: concept of the current analysis, interpretation of results and review of the manuscript; OA: coordination of the RespVir network, review of the manuscript and interpretation of results; BG: coordination of the RespVir network, review of the manuscript and interpretation of results; TL: review and preparation of the manuscript, interpretation of results; DH: concept of the current analysis, writing the manuscript and interpretation of results; RK: concept of the current analysis, coordination of the RespVir network and interpretation of results; All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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Supplementary Information

Additional file 1

This file includes Supplementary Section 1 and Supplementary Figure S1–S5.

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Exhibit J

New Results

A Nasal Spray Solution of Grapefruit Seed Extract plus Xylitol Displays Virucidal Activity Against SARS-Cov-2 *In Vitro*

Gustavo Ferrer, Arian Betancourt, Camille Celeste Go, Hector Vazquez, Jonna B. Westover, Valeria Cagno, Caroline Tapparel, Marcos A. Sanchez-Gonzalez

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ABSTARCT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the ongoing pandemic coronavirus disease 2019 (COVID-19) has triggered worldwide concerted efforts in an attempt to identify effective therapies. In the present study, we have identified two candidate agents with potential activity against SARS-CoV-2 which can be administered intranasally, namely, xylitol and grape seed fruit extract (GSE). A commercially available nasal spray (Xlear) combining xylitol and GSE has been available for years, but the antiviral effects of this solution have not been documented. This *in vitro* study examined the virucidal effect of Xlear against SARS-CoV-2. To this end, two independent sets of experiments were carried out to test the hypothesis that Xlear is an effective (Experiment I) and replicable (Experiment II) means to deactivate SARS-CoV-2. When tested against SARS-CoV-2, the test compound GSE 0.2% was the only compound effective at reducing >3 log10 CCID50 infectious virus from, 3.67 log10 CCID50/0.1 mL to an undetectable amount of infectious virus. The present results validated by two independent sets of experiments, performed by different

labs, on different viral strains, provide early evidence to encourage further pilot and clinical studies aimed at investigating the use of Xlear as a potential treatment for COVID-19

1 Introduction

The initial global outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the ongoing pandemic coronavirus disease 2019 (COVID-19), was initially identified in Wuhan, China in December 2019. As of July 2020, there were more than 13.3 million confirmed cases worldwide, with total deaths exceeding 573,000 (**Dong et al., 2020**). Worldwide concerted efforts have been made in an attempt to characterize the disease and identify effective therapies targeting SARS-CoV-2 including lines of studies focusing on the route of infection, the potential routes of administration of therapeutic agents as well as the potential efficacy of antiseptics (**Meister et al., 2020**). In this vein, a landmark study found that the coronavirus infects the nasal cavity via the angiotensin-converting enzyme 2 (ACE2) protein which appears to be the host-cell receptor for SARS-CoV-2 (**Hoffmann et al., 2020**). Since the nasal epithelium cells have the highest percentage of ACE2 expressing ciliate cells in the proximal airways, it is plausible to suggest that pharmacological agents such as sprays that are used via the intranasal route of administration might be optimal candidates for providing effective therapies against COVID-19 (**Jia et al., 2005**).

In a recent literature review conducted by Higgins et al. it is highlighted that intranasal drug delivery represents an important area of research for viral diseases and COVID-19 (**Higgins et al., 2020**). They concluded that the intranasal method of drug delivery has potential relevance for future clinical trials in the setting of disease prevention and treatment of SARS-CoV-2 in addition to other viral diseases (**Higgins et al., 2020**). Subsequently, **Siddiqi et.al (2020)**, in a diagram of COVID-19 disease progression, illustrated that the viral response phase is highest during the early infection of the disease process, of which patients manifest mild constitutional symptoms. Taken together the aforementioned studies support our rationale that therapeutic strategies should be aimed at reducing the viral load in the nose by targeting this mild-moderate phase of the disease process, and hence the use of a nasal spray might be an effective means to accomplish this therapeutic strategy.

In the present study, we have identified two candidate agents with potential activity against SARS-CoV-2 which can be administered intranasally, namely, xylitol and grape seed fruit extract (GSE). Xylitol, a sweetener with antimicrobial and anti-inflammatory properties, has been shown effective in decreasing the incidence of dental caries and improving chronic rhinitis as well as important microbiota and immunological modulatory effects (**Akgül et al., 2020**; **Haukioja et al., 2008**; **Weissman et al., 2011**; **Xu et al., 2016**). Xylitol has been reported to have multiple health benefits

as well as is generally safe and well-tolerated for most adults in doses up to 35 grams per day and up to 20 grams per day in children (**Salli et al., 2019**; **Storey et al., 2007**; **Ur-Rehman et al., 2015**). A derivative of grapefruit seeds, GSE, is associated with abundant health benefits due to the presence of antioxidants and proanthocyanidin complexes (**Chacón et al., 2009**). Also, GSE has been documented to have inhibitory effects against the avian influenza virus, Newcastle disease virus, infections bursal disease virus, as well as other pathogenic enteric viruses (**Komura et al., 2019**; **Su and D'Souza, 2011**). A commercially available nasal spray combining xylitol and GSE, marketed as Xlear (American Fork, UT, USA), has been widely used in the United States for several decades, but the antiviral effects of this solution have not been documented. Accordingly, the aim of the present *in vitro* study was to examine the virucidal effect of Xlear against SARS-CoV-2. To this end, two independent sets of experiments were carried out to test the hypothesis that Xlear is an effective (Experiment I) and replicable (Experiment II) means to deactivate SARS-CoV-2 the causative microorganism of COVID-19.

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2 MATERIALS AND METHODS

2.1 Experiment I: Xlear Virucidal Activity Efficacy

2.1.1 Procedure

SARS-CoV-2, USA-WA1/2020 strain, virus stock was prepared before testing by growing 2 passages in Vero 76 cells. Culture media for prepared stock (test media) was MEM with 2% fetal bovine serum (FBS) and 50 µg/mL gentamicin. Human rhinovirus 16, strain 11757 purchased from ATCC (Gaithersburg, Maryland, USA), was grown in 3 passages of HeLa cells in MEM with 2% fetal bovine serum (FBS), 25 mM MgCl2, and 50 µg/mL gentamicin. Test media is the growth media with 5% FBS.

2.1.2 Virucidal Assay

Test compounds including commercially available Xlear containing purified water, 11% Pure Xylitol (Shandon Lujian, Shandong, China), 0.6%NaCL (Saline), and 0.015% GSE (Chemie Research & Manufacturing Co., Casselberry, FL, USA) were obtained from the manufacturer in liquid form and stored at room temperature. The test compound 11% xylitol in saline was diluted 1:2 with water before testing. Each solution was mixed directly with virus stock so that the final concentration was 90% of each test compound and 10% virus stock. A single concentration was tested in triplicate. Test media without virus was added to duplicate tubes of the compounds to serve as toxicity and neutralization controls. Ethanol (90%) was tested in parallel as a positive control and water only as a virus control. The test solutions were incubated at room temperature (22 ± 2˚C) for 15 minutes with SARS-CoV-2 or Rhinovirus-16. The solutions were then neutralized by a 1/10 dilution in the test

media of each specific virus. The virucidal assays were performed in triplicate, then after neutralization, the triplicate samples were pooled, serially diluted, and assayed for infectious virus.

2.1.3 Virus Quantification

The surviving virus from each sample was quantified by standard end-point dilution assay. Briefly, the neutralized samples were pooled and serially diluted using eight log dilutions in test medium. Then 100 µL of each dilution was plated into quadruplicate wells of 96-well plates containing 80-90% confluent Vero 76 (SARS-CoV-2) or HeLa cells (Rhino-16). The toxicity controls were added to an additional 4 wells of Vero 76 or HeLa cells and 2 of those wells at each dilution were infected with virus to serve as neutralization controls, ensuring that the residual sample in the titer assay plate did not inhibit growth and detection of the surviving virus. Plates were incubated at 37 ± 2˚C with 5% CO_2 for 5 days and at 33 ± 2˚C with 5% CO2 for 4 days for the SARS-CoV-2 assay and the Rhinovirus-16 assay, respectively. Each well was then scored for the presence or absence of an infectious virus. The titers were measured using a standard endpoint dilution 50% cell culture infectious dose (CCID50) assay calculated using the Reed-Muench (1948) equation and the log reduction value (LRV) of each compound compared to the negative (water) control was calculated.

2.2 Experiment II: Xlear Virucidal Activity Replication

2.2.1 Procedure

SARS-CoV2/Switzerland/GE9586/2020 virus stock was amplified and titrated in Vero E6 cells by plaque assay cultured in DMEM HG with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

DOSE-RESPONSE ASSAY

Xlear nasal spray was serially diluted in DMEM HG and incubated with SARS-CoV2 (MOI 0.003 corresponding to 200 pfu/well) for 1 hour at 37˚C and subsequently added on Vero E6 cells for 1 hour at 37˚C. The inoculum was then removed, cells were washed and overlaid with DMEM HG with 5% FBS and Avicel 0.8%. 48hpi cells were fixed with PFA 4% and stained with crystal violet. Plaques were counted and percent of infection calculated in comparison with untreated wells. The experiments were performed twice independently, and each was performed in duplicate.

VIRUCIDAL ASSAY

Xlear spray was mixed in different concentrations with SARS-CoV2 stock (10^5pfu). The compound was mixed directly with the virus solution with a final concentration of respectively 90%, 80%, 60%, or 20%. PBS was used as control. The solution and virus were incubated at 37 ˚C for 1 hour. The solution was then neutralized by a 1/10 dilution in test media. A 60% condition was repeated in two

independent experiments while the other dilutions were performed in a single experiment in duplicate.

The infectious virus from each sample was quantified by standard end-point dilution assay. 100 µL of each dilution were plated into quadruplicate wells of 96-well plates containing 80-90% confluent Vero 76 cells. Plates were incubated at 37˚C with 5% CO2 for three days. Each well was then scored for the presence or absence of the virus. The end-point titers (TCID50) values were calculated using the Reed-Muench (1948) equation.

2.2.2 Toxicity assay

Vero-E6 (13000 cells per well) were seeded in 96-well plate. Xlear was serially diluted in DMEM supplemented with 5% FBS and added on cells for 1h, followed by a washout, addition of DMEM supplemented with 5% FBS for additional 48h hours. MTT reagent (Sigma Aldrich) was added on cells for 3h at 37˚C according to manufacturer instructions, subsequently cells were lysed with pure DMSO and absorbance read at 570 nm. Percentages of viability were calculated by comparing the absorbance in treated wells and untreated.

3 RESULTS

3.1 Experiment I

Virus titers and LRV of Rhinovirus-16 and SARS-CoV-2 when incubated with a single concentration of the Xlear solutions are shown in **Table 1**. After a 15-minute contact time, the Xlear nasal spray was not effective at reducing the infectious Rhino-16 virus. When tested against SARS-CoV-2, the test compound GSE 0.2% was the only compound effective at reducing >3 log10 CCID50 infectious virus from, 3.67 log10 CCID50/0.1 mL to an undetectable amount of infectious virus (**Table 1**). The Xlear nasal spray and the GSE 0.2% had some toxicity in the top rows (1/10 dilution of the test sample) which may have contributed to the virucidal effect of the GSE. The 11% xylitol and 11% erythritol had no cytotoxicity. The positive control and neutralization control performed as expected.

Table 1.

Virucidal efficacy of Xlear compounds against Rhinovirus-16 and SARS-CoV-2 after a 15-minute incubation with virus at 22 ± 2°C.

3.2 Experiment II

SARS-Cov2 is inhibited in the dose-response assay (**Figure 1**) by different concentrations of Xlear spray. However, the dilution 1:2 in medium evidenced damage to the cells with almost complete loss of the cells, while with the dilution 1:6 a partial damage to the cell was evidenced, while no

morphologic changes in cells were visible from dilution 1:12 onwards. These results were further confirmed with toxicity assays (**Figure 1b**).

Figure 1.

a) SARS-CoV-2 dose-response inhibition. Xlear was incubated at different dilutions with SARS-CoV2 (200 pfu) for 1h at 37 C. At the end of the incubation, mixtures were serially diluted and added for 1h at 37°C on Vero-E6 cells. Mixtures were then removed, and cells overlaid with medium containing 0.8% avicel. Cells were fixed 48hpi and plaques were counted. Results are mean and SEM of 2 independent experiments performed in duplicate. b) Xlear toxicity evaluation. Different dilutions of the nasal spray were incubated for 1h (followed by addition of medium for 47h) or for 48h on cells in DMEM 5% FBS. At the end of the incubation MTT reagent was added on cells and percentages of viability were evaluated by comparing treated and untreated wells.

In the virucidal assays (**Figure 2**), Xlear showed virucidal activity at the different concentrations tested. Complete inhibition of viral infectivity was observed for the 90%, 80%, 60% condition, and a reduction of 2.17 log of viral titer in the 20% condition. In this assay, the mixture of virus and Xlear was neutralized by a 1/10 dilution before addition on cells, therefore diluting the compound below the toxic doses determined in the toxicity assay (**Figure 1b**).

Figure 2.

SARS CoV-2 virucidal assay. Xlear was incubated with SARS-CoV2 ($5*10^5$ pfu) for 1h at 37 C. At the end of the incubation, mixtures were serially diluted and added on Vero-E6 cells. Cells were fixed 48hpi and scored for presence or absence of cytopathic effect and TCID50/ml was determined. Results are mean and SD of two independent experiments.

4 DISCUSSION

The present study sought to evaluate the *in vitro* virucidal effects of a solution combining xylitol and GSE in a nasal spray formulation known as Xlear. The novel results of this study support our hypothesis that Xlear displays virucidal activity against SARS-CoV-2. The present results validated by two independent sets of experiments, performed by different labs, on different viral strains, provide early evidence to encourage further pilot and clinical studies aimed at investigating the use of Xlear as a potential treatment for COVID-19.

Xlear is a solution of xylitol and GSE, in line with previous reports, the latter displayed antiviral activity. Komura et al. demonstrated the efficacy of GSE as an antimicrobial agent on avian pathogens including avian influenza virus, Newcastle disease virus, infectious bursal disease virus, *Salmonella Infantis*, and *Escherichia coli* (**Komura et al., 2019**). Also, GSE has shown similar antiviral activities against human enteric pathogens including Hepatitis A virus in a dose-dependent manner (**Su and D'Souza, 2011**). Interestingly, GSE antiviral activity seems to be particularly effective on enveloped viruses. Since SARS-CoV-2 is an enveloped virus the GSE characteristics to induced or target the viral envelope should not be overlooked as candidate therapies for COVID-19 emerge

(**Schoeman and Fielding, 2019**). On the other hand, xylitol did not show *in vitro* virucidal properties in the present study. However, it seems that the viral protective effects of xylitol are evident *in vivo* a suggested by studies demonstrating ameliorating effects against human respiratory syncytial virus and changes in the microbiota when consumed orally (**Uebanso et al., 2017**; **Xu et al., 2016**).

The precise mechanism of action of GSE is poorly understood. However, according to the present virucidal tests, the active component of the spray is the GSE, which is in line with previous reports demonstrating that the extract was is effective to inactivate different enveloped and non-enveloped viruses (**Su and D'Souza, 2011**).

Moreover, it seems that the mechanism of action of GSE targets the viral adsorption (or viral binding) to a greater extent than viral replication. It is worth mentioning that studies of the precise mechanism of action of GSE are beyond the scope of this work.

As with any research study, the present experimental design is not free from some limitations. The minimum time required for the Xlear solution to exert the virucidal effect was not investigated. Furthermore, to assess the relevance of the time-dependent effect of Xylitol effect *in vivo*, it will be important to verify if the addition of the spray-on cells previously infected at nontoxic doses would exert a reduction of the viral titer. Also, whether pre-treating the cells with the spray and subsequently adding the virus would decrease the rate of infection would be needed to assess the possible preventive use of the nasal spray.

CONCLUSIONS

This study demonstrates the strong virucidal effects against SARS-CoV-2 of the Xlear nasal spray compound with xylitol and GSE. Using a virucidal nasal spray could become a cutting-edge element in the prevention and treatment of COVID-19 disease. To further ascertain the impact of this nasal spray in SARS-CoV-2, we propose to perform further a randomized placebo-controlled study of intranasally delivered Xlear in patients with mild to moderate SARS-CoV-2 and randomized placebo-controlled preventive trial in healthcare workers.

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Exhibit K

Confirmatory Results

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In Vitro Analysis of the Anti-viral Potential of nasal spray constituents against SARS-CoV-2

Mark L Cannon, Jonna B. Westover, Reiner Bleher, Marcos A. Sanchez-Gonzalez, Gustavo A. Ferrer

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Abstract

Viral pandemics have taken a significant toll on humanity and the world now is contending with the SARS-CoV-2 epidemic. Readily available economical preventive measures should be immediately explored. Xylitol has been reported to reduce the severity of viral infections as well as the severity of pneumonia, and increase the survivability of animal subjects. Since pneumonia and acute respiratory distress syndrome are potentially fatal complications of COVID-19, the present study tested the *in vitro* effectiveness of xylitol against SARS-CoV-2. Virus titers and LRV of SARS-CoV-2, were incubated with a single concentration of nasal spray. Toxicity was observed in the top dilution (1/10). Virus was seen below that dilution so it did not affect calculations of virus titer or LRV. After a 25-minute contact time, the nasal spray (11% Pure Xylitol, 0.85%NaCL (Saline), and 0.20% grapefruit seed extract) reduced virus from 4.2 to 1.7 log10 CCID50 per 0.1 mL, a statistically significant reduction ($P<0.001$) of 2.5 log10 CCID50. STEM Images obtained at the BIoCryo Laboratory revealed virus contained on the cell wall but none intra-cellular, possibly due to D-xylose (xylitol) production of

glycoaminoglycans decoy targets. Xylitol and grapefruit seed extract are not exotic nor expensive rare high technology answers to viral epidemics. The potential in saving lives and the economies of the world by using X-GSE combination therapy should inspire large clinical trials, especially in those nations whereas the healthcare system would be dangerously compromised by the adoption of less effective and significantly more financially demanding therapies. Because there are no risk factors in using the X/GSE combination therapy, and the nasal spray is over the counter available without a prescription, and the spray allows for comfortable long term mask-wearing, adoption of this preventive anti-viral therapy should be encouraged.

Competing Interest Statement

The authors have declared no competing interest.

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Confirmatory Results

In Vitro Analysis of the Anti-viral Potential of nasal spray constituents against SARS-CoV-2

Mark L Cannon, Jonna B. Westover, Reiner Bleher, Marcos A. Sanchez-Gonzalez, Gustavo A. Ferrer

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Abstract

Viral pandemics have taken a significant toll on humanity and the world now is contending with the SARS-CoV-2 epidemic. Readily available economical preventive measures should be immediately explored. Xylitol has been reported to reduce the severity of viral infections as well as the severity of pneumonia, and increase the survivability of animal subjects. Since pneumonia and acute respiratory distress syndrome are potentially fatal complications of COVID-19, the present study tested the *in vitro* effectiveness of xylitol against SARS-CoV-2. Virus titers and LRV of SARS-CoV-2, were incubated with a single concentration of nasal spray. Toxicity was observed in the top dilution (1/10). Virus was seen below that dilution so it did not affect calculations of virus titer or LRV. After a 25-minute contact time, the nasal spray (11% Pure Xylitol, 0.85%NaCL (Saline), and 0.20% grapefruit seed extract) reduced virus from 4.2 to 1.7 log10 CCID50 per 0.1 mL, a statistically significant reduction ($P<0.001$) of 2.5 log10 CCID50. STEM Images obtained at the BIoCryo Laboratory revealed virus contained on the cell wall but none intra-cellular, possibly due to D-xylose (xylitol) production of

glycoaminoglycans decoy targets. Xylitol and grapefruit seed extract are not exotic nor expensive rare high technology answers to viral epidemics. The potential in saving lives and the economies of the world by using X-GSE combination therapy should inspire large clinical trials, especially in those nations whereas the healthcare system would be dangerously compromised by the adoption of less effective and significantly more financially demanding therapies. Because there are no risk factors in using the X/GSE combination therapy, and the nasal spray is over the counter available without a prescription, and the spray allows for comfortable long term mask-wearing, adoption of this preventive anti-viral therapy should be encouraged.

Introduction

Viral pandemics have taken a significant toll on humanity and the world now is contending with the SARS-CoV-2 epidemic. As of November, 28st, 2020, the estimate of cases and related fatalities for the world are reported as 61,877,685 and 1,447,246 respectively.[1] The societal cost of COVID-19 is very difficult to measure, but millions have lost their livelihoods and the Federal (USA) expenditures top 3 trillion dollars, more than the amount spent on all scientific research in the history of federal expenditures.[2] And yet there is little to show for this Herculean effort and expenses as of this date. For example, the total cost of World war II was 4 trillion dollars in today's dollars over 4 years with 481,000 fatalities for the United States of America. It appears that more Americans will die early in just one year due to the COVID-19 epidemic. Readily available and economical preventive measures should be immediately explored.

Newly published research has demonstrated the antiviral properties of polyols. Xylitol has been reported to reduce the severity of viral infections. The effect of dietary xylitol on hRSV infection was investigated in a mouse model with significant results reported.[4] The mice received xylitol for 14 days before virus exposure and for a further three days post-viral exposure. The mice receiving xylitol had significantly reduced viral lung titers than the controls receiving phosphate-buffered saline (PBS). Fewer CD3+ and CD3+CD8+ lymphocytes, whose numbers are indicative of inflammatory status, were recruited in the mice receiving xylitol. These results demonstrated improved hRSV infection outcomes and reduced inflammation-associated immune responses to hRSV infection with dietary xylitol. The same researchers previously reported positive effects of xylitol on mice with influenza A virus infection (H1N1) also with a decrease in recruitment of inflammatory lymphocytes.[5] It has been reported that a decrease in CD3+CD8+ lymphocytes is a predictor of mortality for COVID-19 patients. The antiinflammatory and antiviral properties of D-xylose/xylitol in respiratory conditions are subject to a patent application (number WO1999048361A1) filed in 1998 in the United States. 6 Subsequently, xylitol is a main active ingredient in nasal spray products, such as Xlear Sinus Care.

Xylitol has also been demonstrated to reduce the severity of pneumonia, and increase the survivability of animal subjects.[7, 8] Pneumonia and acute respiratory distress syndrome are potentially fatal complications of COVID-19. 9 Interestingly, xylitol has been used in clinical trials to decrease *Pseudomonas aeruginosa* in patients with cystic fibrosis.[10, 11] The idea of using xylitol in the ventilators dedicated to COVID-19 patients was not, however, put into practice due to the number of clinical trials with other funded treatments that later demonstrated limited success. [12] Further research into the effectiveness of xylitol against SARS-CoV-2 is therefore required.

Materials and Methods

Virucudal Assay

SARS-CoV-2, USA-WA1/2020 strain, virus stock was prepared before testing by growing in Vero 76 cells. Culture media for prepared stock (test media) was MEM with 2% fetal bovine serum and 50 µg/mL gentamicin. The compound was mixed directly with virus solution so that the final concentration was 90% of the compound preparation and 10% virus solution. A single concentration was tested in triplicate. Test media without virus was added to one tube of the prepared compound to serve as toxicity controls. Ethanol (70%) was tested in parallel as a positive control and water only as a virus control. Solution and virus were incubated at room temperature (22 ± 2˚C) for 25 minutes. The solution was then neutralized by a 1/10 dilution in test media.

Surviving virus from each sample was quantified by standard end-point dilution assay. Briefly, samples were serially diluted 1/10 in test medium. Then 100 µL of each dilution were plated into quadruplicate wells of 96-well plates containing 80-90% confluent Vero 76 cells. Plates were incubated at 37 ± 2˚C with 5% CO2 for 6 days. Each well was then scored for the presence or absence of virus. The end-point titers (CCID50) values were calculated using the Reed-Muench (1948) equation. Three independent replicates of each sample were tested, and the average and standard deviation were calculated. Results were compared with untreated controls by one-way ANOVA with Dunnett's multiple comparison tests using GraphPad Prism (version 8) software. Controls: Virus controls were tested in water and the reduction of virus in test wells compared to virus controls was calculated as the log reduction value (LRV). Toxicity controls were tested with media not containing virus to determine if the samples were toxic to cells. Neutralization controls were tested to ensure that virus inactivation did not continue after the specified contact time, and that residual sample in the titer assay plates did not inhibit growth and detection of surviving virus. This was done by adding toxicity samples to titer test plates then spiking each well with a low amount of virus that would produce an observable amount of CPE during the incubation period.

Electron Microscopy

Cell pellets were fixed in 3% glutaraldehyde, 2% formaldehyde in 0.1 M PIPES, pH 7.2 for 72 hours. After fixation, cells were enrobed in 10% gelatin, rinsed in 0.1 M PIPES for 3 x 10 minutes and postfixed in 1% osmium tetroxide for 1 hour. After two rinses for 10 minutes in DI water, cells were en bloc stained with 2% uranyl acetate in DI water and rinsed 2 x 5 minutes with DI water. Samples were dehydrated in an ascending series of ethanol (25%, 50%, 75%, 95% and 3 x 100%) for 15 minutes each and infiltrated at RT with EMBed 812 resin/ethanol mixture 1:1 for 30 minutes, 3:1 for 60 minutes, and with pure resin overnight. The next day, samples were transferred into fresh resin in silicon molds and polymerized at 65□C for 48 hours. Sections of ca. 80 nm thickness were generated with a diamond knife (Diatome, Hatfield, PA) using a Leica Ultracut-S ultramicrotome. The sections were placed on TEM grids and images were recorded with a Hitachi HD2300 STEM at 200 kV acceleration voltage.

(Chemicals were from EMS, Hatfield, PA)

Results

Virucidal Assay

Virus titers and LRV of SARS-CoV-2, when incubated with a single concentration of nasal spray, are shown in **Table 1**. Toxicity was observed in the top dilution (1/10). Virus was seen below that dilution so it did not affect calculations of virus titer or LRV. After a 25-minute contact time, the nasal spray reduced virus from 4.2 to 1.7 log10 CCID50 per 0.1 mL, a statistically significant reduction of 2.5 log10 CCID50. Neutralization controls demonstrated that residual sample did not inhibit virus growth and detection in the endpoint titer assays. Virus controls and positive controls performed as expected.

Table 1.

Virucidal efficacy of nasal spray against SARS-CoV-2 after a 25-minute incubation with virus at 22 ± 2°C.

Imaging

Scanning transmission electron microscope Images obtained at the BIoCryo Laboratory (Northwestern University) revealed virus contained on the cell wall but none intra-cellular, possibly due to D-xylose (xylitol) production of glycoaminoglycans decoy targets. A very recently published article correlated d-xylose and xylitol to the severity and morbidity related factors in COVID-19. 13

D-xylose is the initiating element for sulfated glycosaminoglycans (GAG) that attach to the core protein. D-xylose can be derived from xylitol by d-xylose reductase action replenishing this carbohydrate that is targeted by the SARS-CoV-2 virus. If the virus attaches to the d-xylose position on the GAG, such as heparin sulfate, the virus can then contact the ACE2 receptor. Additionally, xylitol serves as a decoy target for the virus, preventing it from successfully reaching the ACE2 receptor. Further research into the mechanism that xylitol initiates with cells to prevent viral penetration and replication is essential and should be prioritized.

Discussion

Xylitol has a long history of being safe and beneficial in preventing bacterial pathogen infections.[14] It is considered a prebiotic due to its positive effect on the microbiome, reducing pathogenic proliferation.[15] The use of xylitol in oral health to prevent dental caries and periodontal disease has been well documented as safe and effective.[16, 17] Studies have shown xylitol inhibits the formation of mixed species biofilms, which include *Porphyromonas gingivalis* in vitro.[16, 18] Long term clinical studies have demonstrated that children with dental problems grow up to be adults with heart disease. For example, Kids with dental abscesses were followed for 27 years and they developed pre-clinical signs of coronary heart disease. 19 In addition, general morbidity and mortality rates are very closely associated with advanced periodontal disease, and there are also well documented connections to inflammatory Alzheimer's disease and atherosclerosis.[20–25]

By inhibiting P gingivalis with xylitol and erythritol, the innate and adaptive immune response of the human host should be more robust.[26] Also, the possibility of salivary spread of oral pathogens should be reduced, preventing onset of the acute respiratory distress syndrome. Indeed, there have been three important recently published peer review articles, two in Medical Hypothesis and one in the British Dental Journal that reinforce how important oral health care is in regards to COVID-19 itself. Periodontal disease is another of the pre-disposing co-morbidities.[27] This is certainly not surprising as PD creates systemic inflammation, increase in proinflammatory cytokine levels. This would exacerbate the cytokine storm of COVID-19, and the oral pathogens in the saliva could cause an increase in the pneumonia risk.[28]

Xylitol and grapefruit seed extract are not exotic nor expensive rare high technology answers to viral epidemics. The potential in saving lives and the economies of the world by using X-GSE combination therapy should inspire large clinical trials, especially in those nations whereas the healthcare system would be dangerously compromised by the adoption of less effective and significantly more financially demanding therapies. Also, the potential mechanism by which xylitol may pose as a

decoy target for the SARS-CoV-2 virus was just recently published. [13] Because there are no risk factors in using the X/GSE combination therapy, and the nasal spray is over the counter available without prescription, and the spray allows for comfortable long term mask wearing, adoption of this preventive anti-viral therapy should be encouraged.

Conclusion

Combination therapy with GSE and xylitol may prevent spread of viral respiratory infections not just for SAR-CoV-2 but also for future H1N1 or other viral epidemics. GSE significantly reduces the viral load while xylitol prevents the virus attachment to the core protein on the cell wall.

Figure 1.

SARS-CoV-2 virus outside Vero 76 immortalized cells

Figure 2.

SARS-CoV-2 on cell wall. No viral inclusion bodies noted within cell cytoplasm.

Acknowledgement

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Back to top

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Exhibit L

STUDY REPORT

Antiviral Efficacy Against Virus Infections in Human-Derived Tracheal/Bronchial Epithelial Cells

USU Study Number:	10-20-21-3D XLEAR
Report date:	1 December 2021
Test compounds:	Sorbitol, Erythritol, Xylitol, GSE, Chlorpheniramine Maleate
Viruses:	Influenza A/CA/07/09 (H1N1) Respiratory Syncytial Virus (RSV) strain A2 SARS-CoV-2 strain USA/PHC658/2021 (B.1.617.2; delta)

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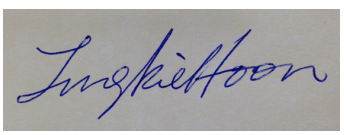

Kie Hoon Jung, Ph.D.

Introduction

The antiviral activity of 5 compounds provided by Xlear were evaluated against Influenza A/CA/07/09 (H1N1), Respiratory Syncytial Virus (RSV) strain A2, and SARS-CoV-2 strain USA/PHC658/2021 (B.1.617.2; delta) in a highly differentiated, three-dimensional (3-D), *in vitro* model of normal, human-derived tracheal/bronchial epithelial (TBE) cells. The compounds were tested at the concentrations indicated in Tables 1-3 in duplicate inserts of the 3D tissue models of the human airway (MatTek Life Sciences). Antiviral activity was measured by virus yield reduction assays on day 3 (H1N1), day 5 (RSV), or day 6 (SARS-C-V2) after infection.

Materials and Methods

Compounds: The compounds received as solids were dissolved in the MatTek culture medium (AIR-100-MM) and further diluted to the test dilutions. Sorbitol (45%) and GSE (43%) were received in solution and were further diluted to the test dilutions in the culture medium. Ribavirin (ICN Pharmaceuticals, Inc. Costa Mesa, CA) or Remdesivir (MedChemExpress, cat# HY-104077) were tested as the positive control.

Cell Culture: The EpiAirway™ Model consists of normal, human-derived tracheal/bronchial epithelial (TBE) cells which have been cultured to form a multi layered, highly differentiated model which closely resembles the epithelial tissue of the respiratory tract. The cell cultures were made to order by MatTek Life Sciences (https://www.mattek.com) (Ashland, MA) and arrived in kits with either 12- or 24-well inserts each. The TBE cells were grown on 6mm mesh disks in transwell inserts. During transportation the tissues were stabilized on a sheet of agarose, which was removed upon receipt. One insert was estimated to consist of approximately 1.2 x 10^6 cells. Kits of cell inserts (EpiAirway™ AIR-100, AIR-112) originated from a single, healthy, non-smoker donor #9831. Upon arrival, the cell transwell inserts were immediately transferred to individual wells of a 6-well plate according to manufacturer's instructions, and 1 mL of MatTek's proprietary culture medium (AIR-100-MM) was added to the basolateral side, whereas the apical side was exposed to a humidified 5% CO_2 environment. The TBE cells were cultured at 37°C for a minimum of one day before the start of the experiment. After the equilibration period, the mucin layer, secreted from the apical side of the cells, was removed by washing with 400 µL pre-warmed 30 mM HEPES buffered saline solution 3X. Culture medium was replenished to the basal side following the wash steps. The tissues were then allowed to rest in a 37°C and 5% CO_2 environment for a minimum of 1 hour prior to the assay.

Viruses: The virus stocks were diluted in AIR-100-MM and infected at MOI 0.01 (H1N1), MOI 0.01 (RSV) and MOI 0.02 (SARS-CoV-2) $CCID_{50}$ per cell, respectively.

Experimental design: Each compound treatment (140 µL) is applied to the apical side, and culture medium only is applied to the basal side (1 mL), for a 2 h incubation. Virus is then added (140 µL) to the apical side for a 2 h infection period. As a virus control, some of the cells were treated with placebo (cell culture medium only). Following the infection, the apical medium was removed, wells are washed once with media, and fresh test compound is added to the apical side. The basal side was replaced with fresh medium. The cells were

maintained at the air-liquid interface. On days 3 (H1N1), 5 (RSV), or 6 (SARS-CoV-2) post-infection, the medium was removed and discarded from the basal side. Virus released into the apical compartment of the TBE cells was harvested by the addition of 400 µL of culture medium that was pre-warmed at 37°C. The contents were incubated for 30 min, mixed well, collected, thoroughly vortexed and plated on MDCK (H1N1), MA-104 cells (RSV), or Vero E6 cells (SARS-CoV-2) for VYR titration. Triplicate wells were used for virus controls.

Determination of virus titers from each treated cell culture: MDCK (H1N1), MA-104 cells (RSV), or Vero E6 cells (SARS-CoV-2) cells were seeded in 96-well plates and grown overnight (37°C) to confluence. Samples containing virus were diluted in 10-fold increments in infection medium and 200 µL of each dilution transferred into respective wells of a 96-well microtiter plate. Four microwells were used for each dilution to determine 50% viral endpoints. After 3-7 days of incubation, each well was scored positive for virus if any cytopathic effect (CPE) was observed as compared with the uninfected control. The virus dose that was able to infect 50% of the cell cultures ($CCID_{50}$ per 0.2 mL) was calculated by the Reed-Muench method (1948). The VYR data and log reduction values (LRV) are summarized in Tables 1-3.

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Table 1. Antiviral efficacy against Influenza A/CA/07/09 (H1N1)

Test Compounds	Concentration (%)	[a]Log_{10} $CCID_{50}$ virus per 0.2 mL	[b]LRV
Sorbitol	5 5	6.30 5.30	1.47
Erythritol	5 5	3.50 4.67	3.17
Xylitol	5 5	6.00 6.00	1.27
GSE	0.2 0.2	1.50 1.50	5.77[d]
Chlorpheniramine Maleate	1 1	1.50 1.00	6.02[d]
Ribavirin	100 μg/ml 10 1	0.67 5.50 7.30	[c]EC_{90} 3.1 SI >32
Virus Control Influenza A/CA/07/09 (H1N1)	MOI 0.01	7.30 7.00 7.50	Avg. 7.27

Each well was scored positive for virus if any CPE was observed as compared with the uninfected control.
[a]Titer results from the virus yield reduction assay.
[b]LRV (log reduction value) is the average reduction of virus compared to the average virus control
[c]EC_{90} = 90% effective concentration (reduce virus yield by 1 log_{10}) as determined by regression analysis.
[d]Some cell cytotoxicity was observed and may have contributed to the antiviral effects.

Table 2. Antiviral efficacy against Respiratory Syncytial Virus (RSV) strain A2

Test Compounds	Concentration (%)	[a]Log_{10} $CCID_{50}$ virus per 0.2 mL	[b]LRV
Sorbitol	5 5	2.00 2.00	2.49
Erythritol	5 5	3.00 2.30	1.84
Xylitol	5 5	2.00 1.67	2.65
GSE	0.2 0.2	2.00 2.30	2.34[d]
Chlorpheniramine Maleate	1 1	1.50 1.50	2.99[d]
Ribavirin	100 μg/ml 10 1	1.30 3.30 4.67	[c]EC_{90} 4.6 SI >22
Virus Control RSV A2 ATCC VR-1540	MOI 0.01	4.50 4.67 4.30	Avg. 4.49

Each well was scored positive for virus if any CPE was observed as compared with the uninfected control.
[a]Titer results from the virus yield reduction assay.
[b]LRV (log reduction value) is the average reduction of virus compared to the average virus control
[c]EC_{90} = 90% effective concentration (reduce virus yield by 1 log_{10}) as determined by regression analysis.
[d]Some cell cytotoxicity was observed and may have contributed to the antiviral effects.

Table 3. Antiviral efficacy against SARS-CoV-2 strain USA/PHC658/2021 (B.1.617.2; delta).

Test Compounds	Concentration (%)	[a]Log_{10} $CCID_{50}$ virus per 0.2 mL	[b]LRV
Sorbitol	5 5	1.50 1.67	3.5
Erythritol	5 5	1.67 2.00	3.29
Xylitol	5 5	1.50 1.00	3.84
GSE	0.2 0.2	1.50 1.50	3.59[d]
Chlorpheniramine Maleate	1 1	2.30 2.50	2.69[d]
Remdesivir	5 μM 0.5 0.05	0.67 2.50 5.00	[c]EC_{90} 0.12 SI >42
Virus Control SARS-CoV-2 USA /PHC658 /2021 (B.1.617.2; delta)	MOI 0.02	5.30 5.30 4.67	Avg. 5.09

Each well was scored positive for virus if any CPE was observed as compared with the uninfected control.
[a]Titer results from the virus yield reduction assay.
[b]LRV (log reduction value) is the average reduction of virus compared to the average virus control
[c]EC_{90} = 90% effective concentration (reduce virus yield by 1 log_{10}) as determined by regression analysis.
[d]Some cell cytotoxicity was observed and may have contributed to the antiviral effects.

Exhibit M

FDA NEWS RELEASE

FDA Approves First Treatment for COVID-19

For Immediate Release:

October 22, 2020

Español (/news-events/press-announcements/la-fda-aprueba-el-primer-tratamiento-para-el-covid-19)

Today, the U.S. Food and Drug Administration approved (https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/214787Orig1s000lbl.pdf) the antiviral drug Veklury (remdesivir) for use in adult and pediatric patients 12 years of age and older and weighing at least 40 kilograms (about 88 pounds) for the treatment of COVID-19 requiring hospitalization. Veklury should only be administered in a hospital or in a healthcare setting capable of providing acute care comparable to inpatient hospital care. Veklury is the first treatment for COVID-19 to receive FDA approval.

This approval does not include the entire population that had been authorized to use Veklury under an Emergency Use Authorization (EUA) originally issued on May 1, 2020. In order to ensure continued access to the pediatric population previously covered under the EUA, the FDA revised the EUA for Veklury to authorize the drug's use for treatment of suspected or laboratory confirmed COVID-19 in hospitalized pediatric patients weighing 3.5 kg to less than 40 kg or hospitalized pediatric patients less than 12 years of age weighing at least 3.5 kg. Clinical trials assessing the safety and efficacy of Veklury in this pediatric patient population are ongoing.

"The FDA is committed to expediting the development and availability of COVID-19 treatments during this unprecedented public health emergency," said FDA Commissioner Stephen M. Hahn, M.D. "Today's approval is supported by data from multiple clinical trials that the agency has rigorously assessed and represents an important scientific milestone in the COVID-19 pandemic. As part of the FDA's Coronavirus Treatment Acceleration Program (/drugs/coronavirus-covid-19-drugs/coronavirus-treatment-acceleration-program-ctap), the agency will to continue to help move new medical products to patients as soon as possible, while at the same time determining whether they are effective and if their benefits outweigh their risks."

Under the Federal Food, Drug, and Cosmetic Act, approval of a new drug product requires substantial evidence of effectiveness and a demonstration of safety for the drug's intended use(s). In considering approval of a drug, the FDA conducts a benefit-risk assessment based on rigorous scientific standards to ensure that the product's benefits outweigh its risks for the

intended population. This is different from the standard used in the issuance of an EUA (https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#abouteuas).

The approval of Veklury was supported by the agency's analysis of data (https://www.accessdata.fda.gov/drugsatfda_docs/nda/2020/214787Orig1s000Sumr.pdf) from three randomized, controlled clinical trials that included patients hospitalized with mild-to-severe COVID-19.

One randomized, double-blind, placebo-controlled clinical trial (https://clinicaltrials.gov/ct2/show/NCT04280705) (ACTT-1), conducted by the National Institute of Allergy and Infectious Diseases, evaluated how long it took for subjects to recover from COVID-19 within 29 days of being treated. The trial looked at 1,062 hospitalized subjects with mild, moderate and severe COVID-19 who received Veklury (n=541) or placebo (n=521), plus standard of care. Recovery was defined as either being discharged from the hospital or being hospitalized but not requiring supplemental oxygen and no longer requiring ongoing medical care. The median time to recovery from COVID-19 was 10 days for the Veklury group compared to 15 days for the placebo group, a statistically significant difference. Overall, the odds of clinical improvement at Day 15 were also statistically significantly higher in the Veklury group when compared to the placebo group.

A second randomized, open-label multi-center clinical trial (https://clinicaltrials.gov/ct2/show/NCT04292730) of hospitalized adult subjects with moderate COVID-19 compared treatment with Veklury for five days (n=191) and treatment with Veklury for 10 days (n=193) with standard of care (n=200). Researchers evaluated the clinical status of subjects on Day 11. Overall, the odds of a subject's COVID-19 symptoms improving were statistically significantly higher in the five-day Veklury group at Day 11 when compared to those receiving only standard of care. The odds of improvement with the 10-day treatment group when compared to those receiving only standard of care were numerically favorable, but not statistically significantly different.

A third separate, randomized, open-label multi-center clinical trial (https://www.clinicaltrials.gov/ct2/show/NCT04292899) of hospitalized adult subjects with severe COVID-19 compared treatment with Veklury for five days (n= 200) and treatment with Veklury for 10 days (n= 197). Researchers evaluated the clinical status of subjects on Day 14. Overall, the odds of a subject's COVID-19 symptoms improving were similar for those in the five-day Veklury group as those in the 10-day Veklury group, and there were no statistically significant differences in recovery rates or mortality rates between the two groups.

Important information about using Veklury to treat COVID-19 for its approved use is available in the prescribing information which includes dosing instructions, potential side effects and drug interactions. Possible side effects include: increased levels of liver enzymes, which may be

a sign of liver injury; and allergic reactions, which may include changes in blood pressure and heart rate, low blood oxygen level, fever, shortness of breath, wheezing, swelling (e.g., lips, around eyes, under the skin), rash, nausea, sweating or shivering. Similar safety information about using Veklury to treat COVID-19 in certain hospitalized pediatric patients under the EUA is available in the fact sheets for health care providers (https://www.fda.gov/media/137566/download) and patients/caregivers (https://www.fda.gov/media/137565/download).

The FDA granted this application Fast Track (/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/fast-track) and Priority Review (/patients/fast-track-breakthrough-therapy-accelerated-approval-priority-review/priority-review) designations. The Agency also granted this application a Material Threat Medical Countermeasure Priority Review Voucher (/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/21st-century-cures-act-mcm-related-cures-provisions), which provides additional incentives for certain medical products intended to treat or prevent harm from specific chemical, biological, radiological and nuclear threats.

The FDA granted approval and reissued the revised EUA to Gilead Sciences Inc.

The FDA, an agency within the U.S. Department of Health and Human Services, protects the public health by assuring the safety, effectiveness, and security of human and veterinary drugs, vaccines and other biological products for human use, and medical devices. The agency also is responsible for the safety and security of our nation's food supply, cosmetics, dietary supplements, products that give off electronic radiation, and for regulating tobacco products.

Related Information

- Frequently Asked Questions for Veklury (remdesivir), including information on additional clinical trials (https://www.fda.gov/media/137574/download)
- Remdesivir EUA Letter of Authorization (https://www.fda.gov/media/137564/download)
- CDER Statement: FDA's Veklury (remdesivir) approval for the treatment of COVID-19—The Science of Safety and Effectiveness (/drugs/news-events-human-drugs/fdas-approval-veklury-remdesivir-treatment-covid-19-science-safety-and-effectiveness)
- Emergency Use Authorization: Therapeutics (https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#coviddrugs)
- Coronavirus Treatment Acceleration Program (CTAP) (/drugs/coronavirus-covid-19-drugs/coronavirus-treatment-acceleration-program-ctap)
- Coronavirus Disease (COVID-19) (/emergency-preparedness-and-response/mcm-issues/coronavirus-disease-2019-covid-19)

217
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Exhibit N

Ear Nose Throat J. 2021 Mar 26;1455613211002929. doi: 10.1177/01455613211002929.
Online ahead of print.

Effective Nasal Disinfection as an Overlooked Strategy in Our Fight against COVID-19

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Free article

Abstract

Although the recent advent of a vaccine and other therapeutic aids in our fight against COVID-19 has brought us a step closer to controlling the pandemic, our fight is far from over. Handwashing, masks, and social distancing practices are considered reasonable measures to control the spread of the disease have been well accepted by government officials and public health officials despite scarce and conflicting scientific evidence. Taking into consideration the aforementioned measures, there is an additional perhaps overlooked practice that warrants our attention-nasal disinfection and hygiene.

Keywords: COVID-19; anti-viral; disinfection; nasal sprays; virucidal.

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PERSONAL VIEW | VOLUME 21, ISSUE 9, E296-E301, SEPTEMBER 01, 2021

Importance of non-pharmaceutical interventions in lowering the viral inoculum to reduce susceptibility to infection by SARS-CoV-2 and potentially disease severity

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Summary

Adherence to non-pharmaceutical interventions to prevent the transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been highly variable across settings, particularly in the USA. In this Personal View, we review data supporting the importance of the viral inoculum (the dose of viral particles from an infected source over time) in increasing the probability of infection in respiratory, gastrointestinal, and sexually transmitted viral infections in humans. We also review the available evidence the relationship of the viral inoculum to disease severity. Non-pharmaceutical interventions might the susceptibility to SARS-CoV-2 infection by reducing the viral inoculum when there osu

an infectious source. Data from physical sciences research suggest that masks protect the wearer by filtering virus from external sources, and others by reducing expulsion of virus by the wearer. Social distancing, handwashing, and improved ventilation also reduce the exposure amount of viral particles from an infectious source. Maintaining and increasing non-pharmaceutical interventions can help to quell SARS-CoV-2 as we enter the second year of the pandemic. Finally, we argue that even as safe and effective vaccines are being rolled out, non-pharmaceutical interventions will continue to play an essential role in suppressing SARS-CoV-2 transmission until equitable and widespread vaccine administration has been completed.

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Introduction

Given the heterogeneity in both disease severity and incidence of COVID-19 worldwide, some experts have suggested that adherence to non-pharmaceutical interventions (eg, social distancing and mask-wearing) is an important and intervenable contributor to these observed differences.[1] Non-pharmaceutical interventions have been severely underused as part of the COVID-19 response in the USA, especially with later infection surges of COVID-19. An average of 49% of Americans reported wearing facial masks daily during the months of June to August, 2020, compared with 95% observed adherence in Hong Kong and 100% reported adherence in Vietnam in the same period[1] An emerging hypothesis is that the viral inoculum, and the interventions that might decrease it, could not only limit infections but also lead to less severe COVID-19 disease if these interventions fail to prevent infection.[2, 3, 4, 5, 6] In this Personal View, we review data supporting the importance of the viral inoculum for susceptibility to respiratory, gastrointestinal, and sexually transmitted viral infections, and the available evidence linking the inoculum to disease severity. We also argue that, even as safe and effective vaccines are being rolled out, non-pharmaceutical interventions will continue to play an essential and ongoing role in suppressing the transmission of SARS-CoV-2 and any further mutations.

Inoculum, host susceptibility, and outcomes for human pathogens

The importance of pathogen inoculum (ie, the number of organisms to which a host is exposed as a function of the concentration, duration, and viral load of the source's infectious material) on the resulting probability of infection has been well described in humans for several viral pathogens, such as influenza viruses,[7, 8, 9, 10] respiratory syncytial virus,[11, 12, 13] adenovirus,[14] enterovirus,[15] poliovirus,[16] rhinovirus,[17, 18] and rotavirus,[19] and also for several bacteria and parasites, particularly in the context of food safety.[20, 21] An important example of the relationship between source viral load and infectivity is HIV, for which a study among HIV serodiscordant couples in Rakai, Uganda showed that the transmission rate was monotonically related to a higher viral load set point of the partner with HIV, with no transmissions observed with a viral load of less than 1500 copies per mL.[22] For respiratory viruses, the relationship between infection and the viral inoculum has been documented through controlled human infection studies, which typically involve increasing the dose of challenge viruses in a stepwise way, by use of intranasal mucosal atomisation devices until mild or moderate illness occurs.[7, 8, 9, 10, 12] For instance, in an influenza A virus subtype H1N1 human challenge model, no infections occurred until participants received 10^5 or higher 50% tissue culture infectious dose ($TCID_{50}$), with a higher proportion of participants showing viral shedding with each ten-fold increase in $TCID_{50}$.[23] Respiratory viruses typically follow this pattern of higher inoculum leading to a higher probability of infection in human challenge experiments, including influenza viruses, rhinovirus, and respiratory syncytial virus,[7, 8, 9, 10, 12] although there are exceptions, given the complexity of the pathogen-host interaction.[24, 25, 26] In addition, the dose needed to infect 50% of the human population (human infectious dose, HID_{50}) varies between pathogens and their subtypes or strains, which has been documented for influenza viruses, rhinovirus, and others.[21]

The influence of the inoculum on disease severity is more challenging to study in humans, given that some viruses only cause mild clinical manifestations in immunocompetent human hosts. Viruses that cause severe disease symptoms are too dangerous to study experimentally in humans, and disease severity is more challenging to measure than is infection itself. However, in one controlled experiment with respiratory syncytial virus, five of the seven human participants who were successfully infected with undiluted, higher dose virus inoculum (10^5 plaque-forming units) administered intranasally, and had an infection confirmed by viral culture, developed symptomatic respiratory syncytial virus disease, whereas none of the 16 participants infected with the diluted, lower dose virus inoculum ($10^{2·7}$ plaque-forming units) developed symptomatic disease.[11] In a human challenge experiment with influenza A virus subtype H3N2, 16 (55%) of 29 volunteers inoculated intranasally with 10^6 or higher $TCID_{50}$ showed viral shedding, but those inoculated with the highest dose self-reported the highest symptom severity score.[9] Other previous experiments that studied the relationship between inoculum and symptom severity did not stratify results by confirmed infections, making the data more difficult to interpret.[10, 17, 23]

The association between dose and disease severity for some viruses is more easily shown in animal models. The relationship between inoculum and mortality in BALB/c mice has been shown for a mouse adapted-H5N1 strain of influenza A virus, although 100% mortality occurred at relatively low doses. For coronaviruses, there are few human data for dose and response, although there is some evidence of a dose-response relationship from mouse models of severe acute respiratory syndrome coronavirus (SARS-CoV), which is structurally similar to SARS-CoV-2,[27] as well as from murine hepatitis virus, which has been studied as a model of human coronavirus.[28] Intranasally administered, higher dose inocula of SARS-CoV in a BALB/c mouse model showed a dose-dependent association with higher mortality, weight loss, and higher viral titres in the respiratory tract.[29] In an murine hepatitis virus-1 mouse model, escalating doses of virus were associated with increasing mortality rates (p=0·01) in 20 mice with positive viral cultures.[30] Findings in animal models might not, however, translate the complexity of pathogen-host interactions in humans.

Viral inoculum, host susceptibility, and outcomes for SARS-CoV-2

Given the severity of illness associated with SARS-CoV-2 infection, human challenge studies are controversial.[31] However, in an animal model of SARS-CoV-2 infection, Syrian hamsters were successfully infected with two different doses of SARS-CoV-2, intranasally and intraocularly, and the higher dose was associated with greater weight loss and more severe lung abnormalities on chest imaging.[32] In another experiment, when a surgical mask partition was placed between the cages of infected and uninfected Syrian hamsters, only six (25%) of 24 hamsters protected by the surgical mask partition were infected, compared with ten (67%) of the 15 control animals without mask partitions (p=0·018).[33] Several mouse model studies of SARS-CoV-2 examined dose-response effects on disease severity,[34, 35, 36] although the relationship between dose and mortality was difficult to interpret due to the absence of confirmed infection at lower doses in one of these,[34] and there was difficulty in distinguishing between increased incubation period and increased severity in another.[35] However, in one mouse model of infection with an adapted SARS-CoV-2, a ten-fold increase in inoculation dose resulted in a 60% mortality, compared with a 20% mortality among BALB/c mice, all with pulmonary infections confirmed via viral culture (p=0·09).[36]

Some epidemiological data[1, 2, 3, 4, 5, 37] suggestive of the viral inoculum effect with SARS-CoV-2 are also worthy of notice. A natural experiment of sorts occurred in the Swiss Alps between March 25, 2020, and April 14, 2020, in two spatially separated homogenous cohorts of soldiers of similar age (median age 21) and without substantial comorbidities.[4] After a COVID-19 outbreak occurred in one of the cohorts, physical distancing and surgical mask-wearing was implemented in both cohorts. An outbreak of COVID-19 in the previously unaffected cohort occurred after the implementation of this policy, with 13 (15%)

asymptomatic soldiers later confirmed to have COVID-19 through mass testing (66 [43%] of 154 were not tested) and none of the 154 recruits developing symptoms. In the cohort impacted before mask-wearing and social distancing were implemented, 102 (47%) of the 215 soldiers who tested positive were symptomatic (132 [37%] of 354 were not tested).[4]

Another study that enrolled participants with PCR-confirmed SARS-CoV-2 infection and their close contacts during the spring 2020 outbreak of SARS-CoV-2 in Catalonia, Spain did a post-hoc analysis of transmission dynamics in a cluster randomised trial of post-exposure prophylaxis with hydroxychloroquine.[38] An outbreak field team visited cases and contacts in homes or nursing homes from March 17, 2020 to April 28, 2020, and measured SARS-CoV-2 viral loads from nasopharyngeal swabs at day 1 and 14. This study found a dose-response relationship between viral load of the index case and the probability of symptomatic disease among contacts, including when adjusting for symptom status of the index case. The viral load of the index case was proportionally related to transmissibility and inversely related to duration of the incubation period the infected contact went through, with higher index viral loads in the cases associated with shorter incubation periods among contacts.[38] The authors suggest that the viral load of cases is an important driver of transmission. Finally, epidemiological data show a higher basic reproduction number (R_0) of SARS-CoV-2 compared with Middle East respiratory syndrome coronavirus or severe acute respiratory syndrome virus[39] (although the relationship of R_0 with HID_{50} is unknown[40]), and the period of asymptomatic transmission with SARS-CoV-2 probably also plays an important role.

Non-pharmaceutical interventions to reduce viral inoculum

Masks

Surgical masks worn by infected individuals reduce transmission by blocking the release of virions into the air, as has been shown for coronaviruses or influenza viruses.[41] Evidence regarding the ability of cloth face coverings to reduce also the size of the inward viral inoculum was already available for other respiratory viruses,[42, 43, 44, 45, 46] and has been accumulating for droplets and aerosols that simulate SARS-CoV-2.[47, 48] Increasing evidence from physical sciences research on how cloth masks might protect the wearer (filtration for personal protection), as well as the long-standing evidence on how masks protect others (so-called source control) led to a change in guidance from the US Centers for Disease Control and Prevention, whose original public health recommendation for the public to wear face coverings, from April 3, 2020, provided the reason that masks protect others.[49] However, a scientific brief by the same agency, updated on November 20, 2020, revised the guidance to indicate that masks protect both the user and others, which also help to increase mask-wearing compliance in the USA.[50]

Rather than criticising the efficacy of cloth masks, investments should be made for the production of high-quality surgical or other types of face coverings to increase their availability outside of health-care settings.[51] Standardisation of recommendations for surgical masks (which use electrostatic-based filtration) and, if not available, high-quality cloth masks (at least two-ply and high-thread count),[52] will reduce confusion.[53] Availability and uniform provision of consistently produced and effective facial coverings could also reduce some mask-wearing hesitancy because a greater perceived efficacy might increase compliance in populations with less adherence to mask-wearing.[54]

In a study in Denmark in April 3, 2020 to June 2, 2020, individuals were randomly assigned to a group where surgical masks were recommended and provided, versus a standard-of-care group. The trial pointed towards a potential association between mask-wearing and reduced SARS-CoV-2 transmission during a low-incidence period, with the point estimate suggesting only a modest benefit.[55] However, several design limitations of the trial probably hindered its ability to show the benefits of mask-wearing for the prevention of COVID-19—underpowering; randomisation at the individual, rather than community, level; flawed outcome measures; and self-reported adherence to mask-wearing in a setting where mask-wearing was not the community norm[56, 57, 58] —which suggests that the accumulating epidemiological and physical sciences evidence for the efficacy of masks might be more compelling than this study showed.[59, 60, 61, 62] A modelling study done in the USA has found a correlation between universal mask-wearing and a reduced need for lockdowns and associated economic losses.[63]

Distancing and ventilation

SARS-CoV-2 has generally been shown to have higher RNA concentrations, or a higher viral inoculum, at closer distances to an infected source or closer to COVID-19 patient care areas, as well as downstream (versus upstream) of the air flow from an infected source, although these higher concentrations might not correlate to confirmed culturable virus.[64, 65, 66] An air sampling study within a US hospital in the rooms of patients with COVID-19 patients showed higher RNA concentrations with personal air samplers compared with bedroom or hallway air samplers.[64] In another study, in a hospital in Wuhan, China, two (18%) of 11 air samples collected near patients with a COVID-19 infection in the general ward had detectable RNA, compared with none of five samples collected 2·5 m away from patients (sampler positioned upstream of the room's airflow). In the intensive care unit of the same hospital, samples were collected downstream of the room's airflow. Overall, eight (44%) of 18 samples collected 2·5 m away from the patient were positive, while only one (13%) of eight samples collected 4·0 m were positive. Other studies reporting RNA in air samples from COVID-19 care areas did not, unfortunately, measure the distance between sample location and patient.[67, 68]

Ventilation to reduce exposure to viral particles has been well described for respiratory viruses.[217] Encouraging human interactions to happen primarily in outdoor spaces and providing engineering and structural changes to increase ventilation in indoor spaces are important non-pharmaceutical interventions.[69, 70] Handwashing could decrease the viral inoculum by reducing the number of viral particles on the hands, which has been shown to be an efficient transmission route, for instance, of rhinovirus (although not identified as a transmission route of SARS-CoV-2).[71] Finally, it is important to note that the efficacy of non-pharmaceutical interventions will increase when multiple strategies are combined, with no single strategy likely to confer an efficacy of 100% in preventing SARS-CoV-2 transmission. Moreover, one strategy can help compensate for another. We propose the concept of the non-pharmaceutical interventions triangle (appendix), in which an individual intervention (masks, distancing, or ventilation) can be reduced as another intensifies (with host susceptibility to SARS-CoV-2 a central figure in the non-pharmaceutical interventions triangle).

There are several possible study designs that could add additional evidence to the relationship between mask-wearing and susceptibility to SARS-CoV-2. Natural experiments, such as the Swiss military study,[4] or case-contact studies, such as the one done in Catalonia,[38] can be examined in other settings. Econometric methods such as differences-in-differences analyses could be used to study COVID-19 incidence, hospitalisations, and mortality before and after institution of mask-wearing mandates across the world (importantly, controlling for case and testing rates), although adherence to these mandates will also need to be taken into account. Human challenge studies could be attempted with seasonal coronaviruses that do not provoke severe disease. Non-pharmaceutical interventions, such as mask-wearing or physical distancing, could be incorporated into seasonal coronavirus or influenza virus human challenge studies to study their efficacy more rigorously. Furthermore, individuals with SARS-CoV-2 infection could be enrolled into viral culture studies concomitantly with mask-wearing, physical distancing, or both, which could help to quantify the degree of expulsion of viable virus with different non-pharmaceutical interventions.

Non-pharmaceutical interventions and vaccine effectiveness

The effectiveness of a SARS-CoV-2 vaccine could potentially be affected by the population-level burden of COVID-19 disease. The influence of the population-level disease burden on vaccine effectiveness has been well described.[72] Indirect vaccine efficacy (population vaccine efficacy) occurs when a vaccine prevents disease in those who are not vaccinated via sufficient population (herd) immunity. Continuation of non-pharmaceutical interventions will be particularly important for susceptible groups who do not mount a strong immune response to a coronavirus vaccine, and for those who decline a vaccine.[73] Uncontrolled of SARS-CoV-2 in much of the USA could limit the initial efficacy of a SARS-CoV-2 vacc

The recent news of the high efficacy of the Moderna[74] and Pfizer/BioNTech[75] mRNA vaccines for SARS-CoV-2, as well as of the AstraZeneca[76], Novavax[77], Johnson and Johnson[78], and Sputnik V[79] vaccines, are hopeful and exciting. However, the endpoints for the trials of all of these vaccines were preventing symptomatic COVID-19 disease (in which each of the mRNA vaccines showed more than 94% efficacy versus a placebo). Because asymptomatic infection could not be ruled out in patients receiving the vaccine, continued adherence to non-pharmaceutical interventions (even by the vaccinated) will need to be maintained until the pandemic is controlled and widespread vaccination is achieved. During this period, lower priority groups, such as the young, healthy, and people not working in essential services might have delays in being offered vaccines. Non-pharmaceutical interventions will, therefore, remain essential for the near future. While building the infrastructure to stockpile and administer a vaccine at a mass scale, investments should simultaneously be made in the scientific study, production, and promotion of non-pharmaceutical interventions, such as standardised masks, to prevent continued transmission of SARS-CoV-2.

Conclusion

We reviewed the influence of the viral inoculum on disease susceptibility for several human pathogens and the preliminary data available for SARS-CoV-2. We make a plea for continued or enhanced adherence to non-pharmaceutical interventions in combatting SARS-CoV-2 transmission as we await equitable distribution of a safe and effective vaccine. Non-pharmaceutical interventions, including social distancing, mask-wearing, and improved ventilation, especially if associated with higher compliance in settings with unmitigated SARS-CoV-2 transmission, might make an important and positive difference in disease severity and transmissibility worldwide as we approach the second year of the COVID-19 pandemic.

Contributors

MAS and MG did the literature search and initial drafting. DVG, EDG, MB, CB, GR, HC, and EG revised the article for content, clarity, and references.

Declaration of interests

We declare no competing interests.

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Supplementary Material

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Supplementary appendix

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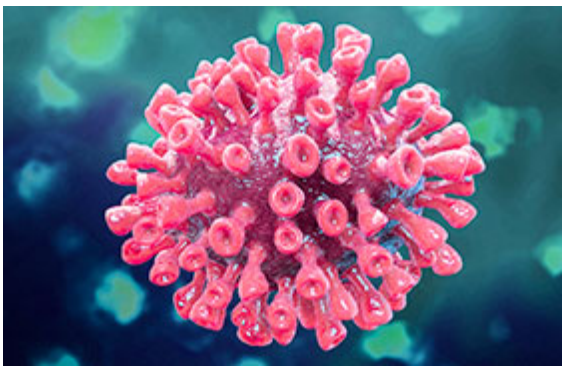

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Effective Nasal Disinfection as an Overlooked Strategy in Our Fight against COVID-19

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Abstract

Although the recent advent of a vaccine and other therapeutic aids in our fight against COVID-19 has brought us a step closer to controlling the pandemic, our fight is far from over. Handwashing, masks, and social distancing practices are considered reasonable measures to control the spread of the disease have been well accepted by government officials and public health officials despite scarce and conflicting scientific evidence. Taking into consideration the aforementioned measures, there is an additional perhaps overlooked practice that warrants our attention-nasal disinfection and hygiene.

Keywords: COVID-19; anti-viral; disinfection; nasal sprays; virucidal.

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Abstract

Although the recent advent of a vaccine and other therapeutic aids in our fight against COVID-19 has brought us a step closer to controlling the pandemic, our fight is far from over. Handwashing, masks, and social distancing practices are considered reasonable measures to control the spread of the disease have been well accepted by government officials and public health officials despite scarce and conflicting scientific evidence. Taking into consideration the aforementioned measures, there is an additional perhaps overlooked practice that warrants our attention—nasal disinfection and hygiene.

Keywords

nasal sprays, disinfection, COVID-19, virucidal, anti-viral

Nearly 200 years ago a Hungarian physician deduced that in the hospital he was offering his services, the high rate of women dying with postpartum infections was caused by doctors coming straight from autopsies without washing their hands.[1] Ignaz Semmelweis did not conduct a randomized controlled trial but rather used his clinical reasoning and anecdotal experience—a scientific observation. Semmelweis strict handwashing policies quickly brought down the mortality rate in the maternity ward from 25% to 30% to less than 1%, but the wider medical establishment rejected his discovery, scoffing at him as

unscientific.[1] It took 150 years for his unprecedented proposal to gain acceptance as the standard of care in hospitals. Today, there is a similar situation, albeit on a global scale, with the coronavirus disease 2019 (COVID-19). Although the recent advent of a vaccine and other therapeutic aids in our fight against COVID-19 has brought us a step closer to controlling the pandemic, our fight is far from over. Handwashing, masks, and social distancing practices are considered reasonable measures to control the spread of the disease have been well accepted by government officials and public health officials despite scarce and conflicting scientific evidence.[2]

Taking into consideration the aforementioned measures, there is an additional perhaps overlooked practice that warrants our attention—effective nasal disinfection.[3] As a simple and obvious yet potentially powerful tool in our medical arsenal, the addition of appropriate nasal disinfection practices might be another turning point in our fight against COVID-19. It is worth mentioning that the nasal epithelium cells have the highest percentage of angiotensin-converting enzyme 2 receptor (ACE2) which is the portal of entry of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[4] Since the nasal epithelium cells have the highest percentage of ACE2 expressing ciliate cells in the proximal airways, it is plausible to suggest that the addition of nasal disinfection practices including using nasal sprays might be optimal candidates for providing effective preventive and therapeutic modalities against COVID-19. Moreover, it is known that 90% of SARS-CoV-2 is in a patient's nose, which is then the point of entry for the lungs where the severe potentially lethal manifestations of the disease occur.[4] Therefore, adding a simple practice of disinfecting the nose with agents displaying anti-viral (preventing the virus from attaching to the cells lining the nose) and virucidal (deactivating the viruses) activities could simply add potential benefits with negligible risk. Fortunately, teams all over the world have been developing such solutions with the potential to improve the effectiveness of nasal disinfection for years. Some of the recent discoveries include the investigation and use of nasal spray solutions with clinical promise, both in vitro and in vivo, including iodine, griffithsin, algae extract, lipid-conjugated peptide, synthetic toll-like receptor 2/6 agonist (INNA-051), grapefruit seed extract with xylitol, and chlorpheniramine maleate.[5-10] It is worth mentioning that the aforementioned substances have shown the ability to deactivate viral particles, and hence may have important benefits in the control of viral load engagement, load, and shedding, an effect that is not apparent with simple saline irrigations.[11]

Interestingly, government regulatory agencies and the scientific community are requesting multiple randomized clinical trials to support nasal disinfection including the use of nasal

sprays in our fight against COVID-19 despite some of these formulations having over 20 years of use.[9] The stakes are high, but the fact is nasal disinfection is a safe cost-effective practice that with the addition of anti-viral and virucidal solutions should give us an additional edge in our fight to control the COVID-19 pandemic. Government officials are reluctant to endorse nasal disinfection practices until there are randomized controlled trials, which could take months. If we consider that current COVID-19 vaccines have been slowly distributed besides the not clear effectiveness in stopping the spread of SARS-CoV-2, the inclusion of nasal disinfection is desperately needed.

Women were dying on Semmelweis' ward, a horrendous death from childbed fever. Semmelweis could not wait; his mission was "to preserve the wife for her husband and the mother for her child." Nor can we wait. Clinical reasoning dictates as we have a simple way to eliminate and control the virus in the most susceptible area of infection—the nose. Many nasal sprays for improving nasal disinfection with virucidal and antiviral properties are available now over the counter. When the risks are extremely low and the potential benefits are enormous, why not recommend improving nasal disinfection and hygiene urgently along with masks and handwashing? The addition of effective nasal disinfection strategies with substances capable of reducing viral engagement, load, and shedding might well be one of the best practices to turn the fight against COVID-19 in our favor for good.

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Exhibit P

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Iota-carrageenan and xylitol inhibit SARS-CoV-2 in Vero cell culture

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Abstract

Last year observed a global pandemic caused by SARS-CoV-2 (severe acute respiratory syndrome-coronavirus 2) infection affecting millions of individuals worldwide. There is an urgent unmet need to provide an easily producible and affordable medicine to prevent transmission and provide early treatment for this disease. Since the nasal cavity and the rhinopharynx are the sites of initial replication of SARS-CoV-2, a nasal spray may be an effective option to target SARS-CoV-2 infection. In this study, we tested the antiviral action of three candidate nasal spray formulations against SARS-CoV-2 in vitro. We determined that iota-carrageenan in concentrations as low as 6 μg/mL inhibits SARS-CoV-2 in vitro. The concentrations of iota-carrageenan with activity against SARS-CoV-2 in vitro may be easily achieved through the application of nasal sprays as commonly used in several countries. Recently a double-blind, placebo-controlled study showed that iota-carrageenan in isotonic sodium chloride reduces ca. five times the risk of infection by SARS-CoV-2 in health care personnel. Further, xylitol at a concentration of 50 mg/mL (ca. 329 mM) was found to exert some antiviral action, though this preliminary finding needs further confirmation.

Figures

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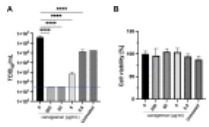

Fig 1. SARS-CoV-2 viral titer after treatment...

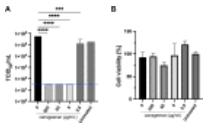

Fig 2. SARS-CoV-2 viral titer after treatment...

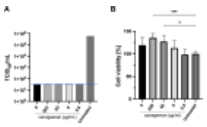

Fig 3. SARS-CoV-2 viral titer after treatment...

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Abstract

Last year observed a global pandemic caused by SARS-CoV-2 (severe acute respiratory syndrome-coronavirus 2) infection affecting millions of individuals worldwide. There is an urgent unmet need to provide an easily producible and affordable medicine to prevent transmission and provide early treatment for this disease. Since the nasal cavity and the rhinopharynx are the sites of initial replication of SARS-CoV-2, a nasal spray may be an effective option to target SARS-CoV-2 infection. In this study, we tested the antiviral action of three candidate nasal spray formulations against SARS-CoV-2 *in vitro*. We determined that iota-carrageenan in concentrations as low as 6 μg/mL inhibits SARS-CoV-2 *in vitro*. The concentrations of iota-carrageenan with activity against SARS-CoV-2 *in vitro* may be easily achieved through the application of nasal sprays as commonly used in several countries. Recently a double-blind, placebo-controlled study showed that iota-carrageenan in isotonic sodium chloride reduces ca. five times the risk of infection by SARS-CoV-2 in health care personnel. Further, xylitol at a concentration of 50 mg/mL (ca. 329 mM) was found to exert some antiviral action, though this preliminary finding needs further confirmation.

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Competing interests: I have read the journal's policy and the authors of this manuscript have the following competing interests: I have a commercial affiliation to Laboratorio Pablo Cassará S.R.L, who provided the formulations for the study and to Amcyte Pharma Inc.. This submission is related to US Patent No. 11.013.687 "PREVENTIVE AND THERAPEUTIC TREATMENT FOR COVID 19 AND ANY OTHER DISEASE CAUSED BY SARS COV 2", whose inventor is Julio César Vega. This does not alter our adherence to PLOS ONE's policies on sharing data and materials.

Introduction

SARS-CoV-2 is the single-stranded positive-sense RNA virus responsible for COVID-19, causing one of the most significant pandemics of our time, with more than 244,980,203 confirmed cases and more than 4,971,409 deaths worldwide as of October 27th, 2021 [1]. In most cases, COVID-19 manifests with flu-like symptoms and results in manageable symptoms that resolve without intervention. However, 15% of patients develop severe pneumonia that requires hospitalization and oxygen support. This includes 5% requiring admission to an intensive care unit (ICU), and among these cases, half result in death [2]. Older adults and those with pre-existing conditions are most susceptible to adverse outcomes. Children are also affected but display milder symptoms than adults, nonetheless, they remain active transmitters of COVID-19 [3].

Currently, there are no adequate therapeutic or preventive medicines available for COVID-19, except for vaccines. However, by the time of this publication, only 3.1% of people in low-income countries have received at least one dose [4]. Therefore, effective approaches are urgently needed to reduce the spread of the virus and its death toll. Recent data have shown that a high viral load and a long virus-shedding period were associated with severe COVID-19 [5, 6]. Furthermore, in the early stage of pathogenesis, the virus is localized mainly in the nasal cavity and the nasopharynx [7, 8]. Therefore, the use of antiviral nasal sprays may help reduce nasal and nasopharyngeal viral load, thereby slowing down disease progression and transmission.

Iota-carrageenan formulated into a nasal spray has proven to be safe and effective against coronavirus virus causing common cold [9–11]. Moreover, iota-carrageenan-containing nasal sprays are currently available in several countries in the world. Carrageenans are linear sulfated polysaccharides that are often extracted from red seaweeds, and commercially available in the form of kappa (κ), iota (ι), or lambda (λ). They have been used for years as thickening agents and stabilizers for food and in the cosmetic and pharmaceutical industry as suspension and emulsion stabilizers. Their antiviral capacity was discovered decades ago and has been experimentally confirmed on herpes virus type 1 and 2, human papilloma virus, H1N1 influenza virus, dengue virus, rhinovirus, hepatitis A virus, enteroviruses, and coronaviruses. Iota-carrageenan inhibits several viruses based on its interaction with the surface of viral particles, thus preventing viral entry and viral budding. [12–17]. *In vitro* studies examining HeLa cells and primary respiratory epithelial cells have shown inhibition of rhinovirus and influenza. Further, in one study, iota-carrageenan spray reduced mortality by at least 50% in mice infected with lethal doses of the H1N1 influenza virus [18]. In all cases, the antiviral action of iota-carrageenan is more effective, when administrated prophylactically or in the early stages of disease and has shown synergy with other antiviral agents. Studies performed on adults and children with a common cold demonstrate the effectiveness of an iota-carrageenan nasal spray to alleviate clinical symptoms and shorten their duration, as well as to decrease the viral load of nasopharyngeal specimens and relapses during the follow-up period [9–11, 19–21].

In addition, xylitol is a polyol (formula $(CHOH)_3(CH_2OH)_2$) that has been used as a sugar substitute in Finland since the 1960s. Obtained from xylan, which is first extracted from hardwood, xylitol has demonstrated multiple health benefits [22]. It has been extensively used in oral health care to prevent cavities because of its antibacterial capacity. It is already being used in otorhinolaryngology as a nasal spray and irrigation for the treatment of rhinosinusitis and the prevention of otitis media [23, 24]. Both *in vitro* and animal model studies have demonstrated the antiviral properties of xylitol against the human respiratory syncytial virus [25].

Both iota-carrageenan and xylitol are safe for humans, being used in much larger amounts as food additives than what may be used for nasal delivery. The safety of iota-carrageenan has already been tested intranasally in New Zealand rabbits in daily doses up to 448 μg/kg/day for up to 28 days and by inhalation in F344 rats for seven days in doses up to 1.2 mg/kg/day [26]. These studies showed neither local nor systemic toxicity. No immunotoxicity or immunogenicity was observed either. On the other hand, a 50 mg/mL xylitol aqueous solution was well tolerated when administered as a nasal irrigation to chronic rhinosinusitis patients [27] and when administered by inhalation to naïve and atopic mice, as well as to healthy human volunteers [28]. Both are included in nasal formulations currently available for use in children and adults.

Based on the above knowledge, an experiment was designed and carried out in a Biosafety Level 3 (BSL3) laboratory to investigate the SARS-CoV-2 inhibition capacity of three different candidate preservative-free formulations. It is postulated that the antiviral pathways of iota-carrageenan are due to the electrostatic attraction between its negatively charged molecules and positively charged viral particles [29]. Therefore, by increasing the ionic strength of the medium, the *in vitro* antiviral action of iota-carrageenan should decrease, as has been previously observed [30]. For this reason, we tested formulations with two different concentrations of sodium chloride (9 and 5 mg/mL) and one that contains xylitol (50 mg/mL) with almost no addition of electrolytes.

Materials and methods

Working conditions and installations

All work involving SARS-CoV-2 was performed at the University of Tennessee Health Science Center (UTHSC) Regional Containment Facility using BSL3 practices under protocols reviewed and approved under Institutional Biosafety Committee (IBC) 17/531.

Cells and virus

African green monkey kidney Vero E6 cells were purchased from the American Type Culture Collection (ATCC® CRL-1586™). Vero E6 cells were grown in complete minimal essential media (c-MEM) (Corning, NY, USA), which included 5% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA), 5 mM penicillin/streptomycin (Gibco, Waltham, MA, USA), and L-glutamine (Gibco, Waltham, MA, USA). Cells were incubated at 37°C with 5% CO_2.

SARS-CoV-2 isolate USA-WA1/2020, was isolated from an oropharyngeal swab from a patient with a respiratory illness who had recently returned from travel to the affected region of China and developed clinical disease (COVID-19) in January 2020 in Washington, USA, it was obtained from BEI Resources, established by the National Institute of Allergy and Infectious Diseases (NIAID) to provide reagents (catalogue number NR-52281, Manassas, VA, USA). Viral master seed stock was prepared by infecting T-175 flasks of Vero E6 cells using a multiplicity of infection (MOI) of 0.1. Each flask was harvested on day two post-infection. The supernatant of each flask was centrifuged twice at 220 *x g* for 15 minutes to remove cellular debris in a benchtop centrifuge using buckets with biocontainment. Titer of virus stock was determined by plaque assay on Vero E6 cells and expressed as plaque-forming units per ml (pfu/ml) [31].

Preparation of sample formulations

All the samples were prepared at Laboratorio Pablo Cassará S.R.L. (Argentina) under aseptic conditions and provided by Amcyte Pharma Inc. (US) to the University of Tennessee Health Science Center. The composition of the samples is depicted in Tables 1 and 2.

Table 1. Composition of samples containing iota-carrageenan.
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Table 2. Composition of samples used as diluents (samples without iota-carrageenan).
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Samples P1, P2, and P3 are referred to as Diluents P1, P2, and P3, respectively, throughout the rest of the text.

Preparation of formulations from Samples 1 and P1.

A 1,200 μg/mL iota-carrageenan stock solution was obtained by taking 7 mL of Sample 1 and bringing it to 10 mL with Sample P1. This stock solution was used to obtain the highest final concentration of iota-carrageenan in the corresponding wells (600 μg/mL). This was achieved by applying 0.5 mL with the same volume of culture media.

Stock solutions with 120, 12, and 1.2 μg/mL iota-carrageenan concentrations were obtained from the 1,200 μg/mL stock solution by 1:10 serial dilutions using Sample P1 as diluent. The corresponding final concentrations of iota-carrageenan in the wells (60, 6, and 0.6 μg/mL) were obtained by applying 0.5 mL of each of these dilutions with the same volume of culture media.

All concentrations and control were tested in triplicate.

Preparation of formulations from Samples 2 and P2.

The highest iota-carrageenan concentration in the wells (600 μg/mL) was obtained by combining 0.5 mL of Sample 2 with the same volume of culture media. Stock solutions with 120, 12, and 1.2 μg/mL iota-carrageenan concentrations were obtained from this stock solution through 1:10 serial dilutions using Sample P2 as diluent. The corresponding final concentrations of iota-carrageenan in the wells (60, 6, and 0.6 μg/mL) were obtained by applying 0.5 mL of each of these dilutions with the same volume of culture media.

All concentrations and control were tested in triplicate.

Preparation of formulations from Samples 3 and P3.

The highest iota-carrageenan concentration in the wells (600 μg/mL) was obtained by applying 0.5 mL of Sample 3 with the same volume of culture media. Stock solutions with 120, 12, and 1.2 μg/mL iota-carrageenan concentrations were obtained from this stock solution by 1:10 serial dilutions using Sample P2 as diluent. The corresponding final concentrations of iota-carrageenan in the wells (60, 6, and 0.6 μg/mL) were obtained by applying 0.5 mL of each of these dilutions with the same volume of culture media.

All concentrations and control were tested in triplicate.

To determine antiviral efficacy of formulations through titer reduction assay, sample formulations were used at a final iota-carrageenan concentration of 600 μg/mL, 60 μg/mL, 6 μg/mL, and 0.6 μg/mL. Equivalent amounts of diluents (Samples P1, P2, and P3) were used for titer reduction assay as controls for the corresponding sample formulations.

Titer reduction assay

Vero E6 cells were seeded in 12-well plates at a density of $2.5x10^5$/well and grown overnight at 37°C under 5%CO_2.

The next day, cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS), pH 7.2, followed by addition of equivalent amount of c-MEM supplemented with reduced fetal bovine serum (2%) and sample formulations/diluents. Formulations and diluents were incubated with cells for two hours, after which the supernatant was removed.

Cells were infected with $2.5x10^4$ pfu (MOI = 0.1) of virus for 1 hour at 37°C, 5%CO_2 with rocking at 15-minute intervals. After incubation, wells were washed with DPBS, and sample formulations / diluents were added at the same concentrations.

After incubation for two days, well contents were collected, and titer was determined.

Residual virus was measured in the different supernatants, by TCID50 assay in 96-well plate format with 3 wells per dilution of virus. 10-fold serial dilutions (10^{-1} to 10^{-7}) of collected samples (treated, control, or virus only) were used to infect Vero E6 cells in a 96-well plate. The cell plates were incubated at 37°C, 5% CO2, with humidity for an additional 2 days. After 2 days, the virus endpoint titer was determined using the Reed-Muench formula and expressed as log TCID50/mL using MTT proliferation assay to measure cell viability. Virus endpoint titer was determined using the Reed-Muench formula and expressed as log $TCID_{50}$/mL [32].

Statistical analysis of data

Medians of virus titer obtained after each treatment were calculated. Each set of formulations containing the same diluent were analyzed separately. Virus titers expressed in $TCID_{50}$/mL were determined after each treatment and with no treatment (untreated or 'virus only' wells) for each diluent and compared using the Kruskal Wallis non-parametric test. Pairwise post hoc comparisons were assessed with the Conover test using Bonferroni correction.

All statistical tests were two-sided, and the null hypothesis was rejected at a significance level of 5% (a = 0.05). No mathematical transformation of data was applied. Values of $TCID_{50}$/mL below the limit of detection were considered equal to this limit (31.6 $TCID_{50}$/mL) for all statistical tests and descriptive statistics.

In the cellular viability assays, the results were analyzed statistically by One-way ANOVA followed by Tukey's multiple comparisons test, using GraphPad Prism version 9.1.0, GraphPad Software, San Diego, California USA, www.graphpad.com.

Results

To examine the antiviral effects of iota-carrageenan on SARS-CoV-2, three sample formulations were developed and tested. Each of the three sample formulations were tested in a dose dependent manner based on varying concentrations of iota-carrageenan ranging from 600 μg/mL to 0 μg/mL.

The medians of the virus titer found after each treatment with iota-carrageenan solutions in diluent P1 (sodium chloride 9 mg/mL adjusted to pH 6–7) showed that iota-carrageenan markedly inhibits SARS-CoV-2 production in a dose-dependent manner, Fig 1A. In other set of experiments, Vero E6 cells were treated with different iota-carrageenan concentrations (600 μg/mL to 0.6 μg/ml), and cell viability was quantified, No difference in cell viability was observed in iota-carrageenan treated cells compared to vehicle-treated control cells, without the addition of virus, Fig 1B.

Fig 1. SARS-CoV-2 viral titer after treatment with Samples 1 and P1.
A) Infection assay. Sample 1 composition: 1.2mg/mL iota-carrageenan, 9 mg/mL sodium chloride, pH 6–7. Vero E6 were pre-treated for two hours with dilutions of Sample 1 with Sample P1 (diluent without iota-carrageenan) to obtain 600 μg/mL, 60 μg/mL, 6 μg/mL, and 0.6 μg/mL iota-carrageenan final concentration. After this pretreatment, cells were infected with SARS-CoV-2 and incubated for 48 hours in the presence of the same dilutions of Sample 1. Supernatants were harvested and virus yield was determined using an end point dilution assay ($TCID_{50}$). Controls consisted of untreated infected cells or infected cells treated with P1 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed as log $TCID_{50}$/mL. The dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate, and the p-values are $p \leq 0.00025$ (****). B) Cellular viability assays. Vero-E6 cells were treated with iota-carrageenan or vehicle (600 μg/mL to 0 μg/mL) for 48 h at 37°C. After incubation, cellular viability was analyzed, and no statistically significant difference was found between the groups compared to the untreated control group (Group 600 μg/ml, $p = 0.7464$, Group 60 μg/ml, $p = 0.0908$, Group 6 μg/ml, $p = 0.1208$, and Group 0.6 μg/ml, $p = 0.8938$). Data are expressed as mean ± SD. Therefore, these compositions do not adversely affect cell viability. For this reason, cell lysis and death detected in the reported experiments after infection must be attributed to the action of the virus.
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SARS-CoV-2 samples treated with 600 μg/mL,60 μg/mL and 6 μg/mL of Sample 1 significantly reduced the residual virus titer when compared to untreated control and Diluent P1 at a concentration not below 6 μg/mL. No statistically significant reductions of the virus were detected after treatment with 0.6 μg/mL of iota-carrageenan. Treatment with Diluent P1 demonstrated a statistically significant increase in residual virus titer when compared with untreated control. This could be due to either better growth conditions for the virus or to an analytical artifact but does not influence the main conclusion, as Diluent P1 does not have any antiviral activity, Fig 1A. Taking the untreated residual virus titer as a reference, SARS-CoV-2 titer is reduced ca. 4 logarithmic units (more than 3.75, in fact) after treatment with 600 or 60 μg/mL iota-carrageenan solutions containing 9 mg/mL sodium chloride adjusted to pH 6–7. In the same diluent SARS-CoV-2 titer is reduced more than 2 logarithmic units after treatment with a solution containing 6 μg/ml iota-carrageenan.

The medians of the virus titer found after each treatment with iota-carrageenan solutions when using diluent 2 (sodium chloride 5 mg/mL adjusted to pH 6–7) are shown in Fig 2A. The statistical analysis of data comparing virus titers after each treatment against those of untreated wells (virus only wells) and those treated with Diluent 2 without iota-carrageenan. No difference in cell viability was observed in iota-carrageenan treated cells compared to vehicle-treated control cells, without the addition of virus, Fig 2B.

Fig 2. SARS-CoV-2 viral titer after treatment with Samples 2 and P2.
A) Infection assay. Sample 2 composition: 1.2mg/mL iota-carrageenan, 5 mg/mL sodium chloride, pH 6–7. Vero E6 were pre-treated with dilutions of Sample 2 with Sample P2 (diluent without iota-carrageenan) to get 600 μg/mL, 60 μg/mL, 6 μg/mL, and 0.6 μg/mL final iota-carrageenan concentration for two hours. After this pretreatment, cells were infected with SARS-CoV-2 and incubated for 48 hours in the presence of the same dilutions of Sample 2. Supernatants were harvested and virus yield was determined using an end point dilution assay ($TCID_{50}$). Controls consisted of untreated infected cells or infected cells treated with P2 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed as log $TCID_{50}$/mL. The dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate, and the p-values are $p \leq 0.00074$ (***) and $p \leq 0.00001$. B) Cellular viability assays. Vero-E6 cells were treated with iota-carrageenan or vehicle (600 μg/mL to 0 μg/mL) for 48 h at 37°C. After incubation, cellular viability was analyzed, and no statistically significant difference was found between the groups compared to the untreated control group (Group 600 μg/ml, $p = 0.9880$, Group 60 μg/ml, $p = 0.0683$, Group 6 μg/ml, $p = 0.9993$, and Group 0.6 μg/ml, $p = 0.1957$). Data are expressed as mean ± SD.
https://doi.org/10.1371/journal.pone.0259943.g002

SARS-CoV-2 samples treated with dilutions of Sample 2 at final iota-carrageenan concentrations of 600 μg/mL, 60 μg/mL, and 6 μg/mL, demonstrated statistically significant reductions in virus titers when compared with untreated controls and controls treated with Diluent P2. However, concentrations of not less than 6 μg/mL showed a much higher logarithmic reduction in virus titer (more than 4 logarithmic units), than 0.6 μg/mL, which showed a reduction below 1 logarithmic unit. As shown in Fig 2A, which plots the logarithm of the residual virus titer in $TCID_{50}$/mL after each treatment. No significant differences were detected between diluent and untreated samples showing that it has no effect on virus titer. This means that the iota-carrageenan is the component that inhibits SARS-CoV-2. In summary, solutions of iota-carrageenan in Diluent P2 (sodium chloride 5 mg/mL and pH adjusted to 6–7) revealed a marked antiviral activity against SARS-CoV-2 in concentrations not below 6 μg/mL and to a lesser extent at lower concentration.

Regarding cell viability, Fig 2B, shows no significant effect on cell viability of diluent P2 and solutions obtained using sample 2 at final iota-carrageenan concentrations of 600 μg/mL and 6 μg/mL. Only two compositions differ significantly from the value obtained on untreated cells. The only case in which cell viability decreased significantly after this treatment is at a concentration of 60 μg/mL of iota-carrageenan. This finding is atypical and does not show any relation to the remaining experimental data. Neither 0.5% sodium chloride (diluent P2) nor iota-carrageenan show any potential to damage Vero cell cultures in any other case. Therefore, this atypical result does not affect the general conclusion about these compositions in that they do not seem to affect cell viability. For this reason, cell lysis and death detected in the reported experiments after infection must be attributed to the action of the virus.

The medians of the virus titer found after each treatment with iota-carrageenan solutions in Diluent 3 (xylitol 50 mg/mL adjusted to pH 6–7) are shown in Fig 3A. The statistical analysis of data comparing virus titers after each treatment clearly indicates significant differences between the untreated control and the iota-carrageenan treated groups. No post hoc analysis could be performed.

Fig 3. SARS-CoV-2 viral titer after treatment with Samples 3 and P3.
A) Infection assay. Sample 3 composition: 1.2mg/mL iota-carrageenan, 5% m/V xylitol, pH 6–7. Vero E6 were pre-treated with dilutions of Sample 3 and Sample P3 (placebo without iota-carrageenan) to get 600 μg/mL, 60 μg/mL, 6 μg/mL, and 0.6 μg/mL final iota-carrageenan concentration for two hours. After this pretreatment, cells were infected with SARS-CoV-2 and incubated for 48 hours in the presence of the same dilutions of Sample 3. Supernatants were harvested, and virus yield was determined by an endpoint dilution assay ($TCID_{50}$). Controls consisted of untreated infected cells or infected cells treated with P3 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed as log $TCID_{50}$/mL. The dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate and the p-value indicates that the groups are significantly different $p = 0.0045$, nevertheless no post-hoc analysis could be performed. B) Cellular viability assays. Vero-E6 cells were treated with iota-carrageenan or vehicle (600 μg/mL to 0 μg/mL) for 48 h at 37°C. After incubation, cellular viability was analyzed. No statistically significant difference was found between compositions at low iota-carrageenan concentrations, 6 μg/mL ($p = 0.6904$) and 0.6 μg/mL ($p > 0.9999$) compared to the untreated control group (Group 6 μg/ml, $p = 0.6904$, and Group 0.6 μg/ml, $p > 0.9999$). Compositions with higher concentrations of iota-carrageenan tend to show a significant difference increasing cell viability (Group 600 μg/ml, $p = 0.0031$ (***), Group 60 μg/ml, $p = 0.0417$ (*), They are certainly not toxic, but may exert some cytoprotective effect.). Data are expressed as mean ± SD.
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All final concentrations tested (600–0.6 μg/mL) with iota-carrageenan solutions in Diluent 3 (xylitol 50 mg/mL adjusted to pH 6–7) demonstrated statistically significant antiviral activity including Diluent P3, which did not contain iota-carrageenan. In all these groups the residual viral titer was below the limit of detection. It is important to point out that SARS-Cov-2 residual titer determined in these untreated wells is similar to those obtained in untreated controls in the previous test runs with Diluents P1 and P2. These unexpected results suggest that xylitol has an antiviral activity of its own, as it is the only diluent component that is not present in Diluents P1 and P2. Fig 3 shows a plot of the logarithm of residual virus titers after each treatment.

Regarding cell viability, diluent P3, as well as compositions obtained with sample 3 at low iota-carrageenan concentrations, 6 μg/mL ($p = 0.6904$) and 0.6 μg/mL ($p > 0.9999$) do not show any significant effect. However, higher iota-carrageenan concentrations tend to show consistently higher optical densities. These compositions are certainly not toxic to the cell culture but may exert some cytoprotective effect. Therefore, it can be concluded that these compositions do not damage cells, and some of them may have some cytoprotective effect. For this reason, cell lysis and death detected in the reported experiments after infection must be attributed to the action of the virus.

A comparison of all three formulations tested containing 600 μg/mL, 60 μg/mL, and 6 μg/mL iota-carrageenan in solutions with Diluents P1, P2, and P3 show a marked antiviral activity against SARS-CoV-2. In Diluent 2, even a 0.6 μg/mL showed a minor antiviral activity, rendering a residual virus titer significantly less than untreated samples. These data are consistent with previous reported in literature observing that the antiviral action of iota-carrageenan is due to the electrostatic attraction between its negatively charged molecules and positively charged viral particles. The electric attraction force between opposite charges decreases as ionic strength increases. For this reason, iota-carrageenan is speculated to be a more potent antiviral in media with lower ionic strength. Solutions in Diluent 3, which contained xylitol, were the most effective and demonstrated an antiviral effect at all concentrations tested. The only composition which showed statistically significant reduction in cell viability was a solution containing 0.5% w/v sodium chloride and 60 μg/mL of iota carrageenan but without relation to any other experimental data. Even though this solution is hypotonic, other solutions with lower and higher iota carrageenan concentrations did not show any reduction in cell viability. This atypical result could be due to some variation in well location or other experimental factors and does not affect the overall conclusion about iota carrageenan and 0.5% sodium chloride in that they do not adversely affect cell viability. Compositions containing xylitol and high concentrations of iota carrageenan (600 μg/mL and 60 μg/mL) may have some cytoprotective effect.

Discussion

Results from our study indicate that iota-carrageenan significantly inhibits SARS-CoV-2 *in vitro*. Our results are in line with those already published in the literature [33, 34] and show promise for the clinical use of an iota-carrageenan nasal spray for the prevention and early treatment of COVID-19. Iota-carrageenan nasal spray formulations, already effective *in vitro* against rhinovirus [15], proved to be clinically effective in preventing and reducing the symptoms and duration of the common cold [9–11, 19]. Moreover, the concentrations of iota-carrageenan tested in this study were expected to be similar to the immediate concentration achieved after the administration of a nasal spray. The estimated amount of airway surface liquid volume in the nasal cavity is in the range of 50–375 μL, based on data reported by different sources. On one hand, these reported values of the surface area of the nasal mucosa range from 100 to 250 cm^2 [35–38]. On the other hand, reported values of the airway surface liquid height are within the range of 5–15 μm [39, 40]. If we take an average of 200 μL of airway surface liquid in the nose (i.e., an average airway surface liquid height of 10 μm and an average nasal mucosa surface area of 200 cm^2) plus 200 μL of formulation after delivering one 100-μL of a 1.2 mg/mL iota-carrageenan solution in each nostril, the immediate concentration of iota-carrageenan in the nasal cavity would be 600 μg/mL. This coincides with the highest concentration tested *in vitro* and capable of reducing virus titer to the LOD in our assay. Furthermore, considering that even 1/100 of this concentration is still active *in vitro* and that iota-carrageenan may stay for up to four hours [20] in the nasal cavity, we can reasonably surmise that this nasal spray may significantly help in the prevention and early treatment of COVID-19. Expected concentrations of iota-carrageenan in the nasal cavity would be even higher if we consider a nasal formulation containing 1.7 mg / mL (0.17% m/V) as some marketed nasal sprays already have.

As already commented in the introduction, a study in New Zealand rabbits tested the intranasal safety of iota-carrageenan up to daily doses of 448 g/kg. The estimated maximum daily dose of a nasal spray, when applied one actuation in each nostril 6 times a day, would be: 12 x 0.17 mg/actuation = 2.04 mg/day, which is equivalent to 29 μg/kg, and 15 times less than the maximum intranasal dose already tested in New Zealand rabbits for 28 days [26].

Another important finding from our study is that xylitol may exert an antiviral effect on SARS-CoV-2. Xylitol can reduce Human Respiratory Syncytial Virus titers in Hep-2 cell culture and infected mice [25]. Xylitol solutions at 50 mg/mL (ca. 329 mM) are practically isotonic [28] and have proved to be safe for use in the nasal and inhalation administration routes [24, 27, 28], suggesting that using iota-carrageenan and xylitol in combination might be a good strategy for a nasal spray formulation. Further testing would be needed to better characterize the antiviral action of xylitol and its combination with iota-carrageenan.

Despite the implementation of significant personal protection measures, the COVID-19 pandemic continues to affect a significant proportion of health care workers with severe consequences for them, their patients, and the wider community. At the same time, most COVID-19 patients remain at home, thus increasing the likely exposure of other household members and caregivers. Providing these groups with simple interventions, such as nasal sprays with either iota-carrageenan and/or xylitol in nasal devices, may lower the risk of infection progression and transmission.

An inhalation solution of the same composition may be effective in severe cases of COVID-19. While there are studies showing the safety of both carrageenan and xylitol use through nebulization [26, 28], clinical trials would be needed to fully confirm these hypotheses. Risk of spreading the virus should be considered in this form of administration and due protection should be used to contain it [41].

Randomized, double-blind, placebo-controlled clinical trials are needed to confirm that the *in vitro* results obtained in our experiments correlate *in vivo*. A pilot study has been recently published showing a significant reduction in the risk of transmission in healthy hospital personnel dedicated patients when using an iota-carrageenan nasal spray corresponding to the composition of sample 1 of our experiment (1.7 mg/mL iota-carrageenan in 0.9% sodium chloride) in addition to standard preventive measures (ClinicalTrials.gov Identifier: NCT04521322) [42].

Conclusions

Iota-carrageenan inhibits SARS CoV-2 *in vitro* at concentrations easily achievable by nasal and nebulization formulations. Furthermore, xylitol may also exhibit antiviral activity on SARS-CoV-2, but further testing would be needed to characterize its antiviral action. A combination of iota-carrageenan and xylitol may increase the benefit of a formulated nasal spray. A randomized, double-blind, placebo-controlled clinical trial has recently been published, showing the efficacy of the formulation containing 1.7 mg/mL iota carrageenan in 0.9% sodium chloride in the prevention of transmission of COVID 19 in hospital personnel dedicated to care of COVID-19 patients when taken in addition to standard preventive measures. Further clinical trials are needed to confirm other potential benefits of these formulations in the prevention and early treatment of COVID-19.

Supporting information

S1 Table. Residual virus titer (TCID50/mL) after treatment with iota-carrageenan solutions in Diluent P1 (sodium chloride 9 mg/mL adjusted to pH 6–7).
https://doi.org/10.1371/journal.pone.0259943.s001
(PDF)

S2 Table. Statistical analysis of residual virus titers determined after each treatment with different concentrations of iota-carrageenan in Diluent P1 (sodium chloride 9 mg/mL adjusted to pH 6–7).
https://doi.org/10.1371/journal.pone.0259943.s002
(PDF)

S3 Table. Cell viability found by MTT assay after treatment with diluent P1 and and solutions of iota carrageenan obtained from sample 1 without the addition of virus expressed as optical density and statistical analysis compared to untreated cells.
The original data of the residual SARS-CoV-2 viral titer after treatment with iota-carrageenan solutions in Diluent P2 and the viability assay, related to Fig 2A and 2B. respectively.
https://doi.org/10.1371/journal.pone.0259943.s003
(PDF)

S4 Table. Residual virus titer ($TCID_{50}$/mL) after treatment with iota-carrageenan solutions in Diluent P2 (sodium chloride 5 mg/mL adjusted to pH 6–7).
https://doi.org/10.1371/journal.pone.0259943.s004
(PDF)

S5 Table. Statistical analysis of residual virus titers determined after each treatment with different concentrations of iota-carrageenan in Diluent P2 (sodium chloride 5 mg/mL adjusted to pH 6–7).
https://doi.org/10.1371/journal.pone.0259943.s005
(PDF)

S6 Table. Cell viability found by MTT assay after treatment with diluent P2 and solutions of iota carrageenan obtained from sample 2 without the addition of virus expressed as optical density and statistical analysis compared to untreated cells.
The original data of the residual SARS-CoV-2 viral titer after treatment with iota-carrageenan solutions in Diluent P3 and the viability assay, related to Fig 3A and 3B. respectively.
https://doi.org/10.1371/journal.pone.0259943.s006
(PDF)

S7 Table. Residual virus titer (TCID50/mL) after treatment with iota-carrageenan solutions in Diluent P3 (xylitol 50 mg/mL adjusted to pH 6–7).
https://doi.org/10.1371/journal.pone.0259943.s007
(PDF)

S8 Table. Statistical analysis of residual virus titers determined after each treatment with different concentrations of iota-carrageenan in Diluent P3 (xylitol 50 mg/mL adjusted to pH 6–7).
https://doi.org/10.1371/journal.pone.0259943.s008
(PDF)

S9 Table. Cell viability found by MTT assay after treatment with diluents and solutions of iota carrageenan without the addition of virus expressed as optical density and statistical analysis compared to untreated cells.
https://doi.org/10.1371/journal.pone.0259943.s009
(PDF)

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