

1                    **Iota-carrageenan and Xylitol inhibit SARS-CoV-2 in cell culture**

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19

## 20 **Abstract**

21

22 COVID-19 (coronavirus disease 2019) is a pandemic caused by SARS-CoV-2 (severe acute  
23 respiratory syndrome-coronavirus 2) infection affecting millions of persons around the world. There is an  
24 urgent unmet need to provide an easy-to-produce, affordable medicine to prevent transmission and  
25 provide early treatment for this disease. The nasal cavity and the rhinopharynx are the sites of initial  
26 replication of SARS-CoV-2. Therefore, a nasal spray may be a suitable dosage form for this purpose. The  
27 main objective of our study was to test the antiviral action of three candidate nasal spray formulations  
28 against SARS-CoV-2. We have found that iota-carrageenan in concentrations as low as 6 µg/ mL inhibits  
29 SARS-CoV-2 infection in Vero cell cultures. The concentrations found to be active in vitro against  
30 SARS-CoV-2 may be easily achieved by the application of nasal sprays already marketed in several  
31 countries. Xylitol at a concentration of 5 % m/V has proved to be viricidal on its own and the association  
32 with iota-carrageenan may be beneficial, as well.

33

## 34 **Introduction**

35 SARS-CoV-2 is a single stranded positive sense RNA virus responsible for COVID-19. COVID-  
36 19 has become one of the worst pandemics of our time counting more than 12,232,700 confirmed cases  
37 and more than 554,000 deaths worldwide by July 10<sup>th</sup>, 2020 [1]. In most cases, COVID-19 manifests  
38 itself clinically with flu-like symptoms as a mild or uncomplicated illness, eventually resolving  
39 spontaneously. However, 15% of patients develop severe pneumonia that requires hospitalization and  
40 oxygen support, and 5% of them need admission to an intensive care unit (ICU). More than half of this  
41 patients may die [2]. Even children can be affected although with milder symptoms than adults and can be  
42 transmitters of the disease. [3]. There are no adequate therapeutic or preventive medicines available, so  
43 effective therapeutic approaches are urgently needed to reduce the spread of the virus and its death toll.

44           During the first days of disease the virus is localized mainly in the nasal cavity and the  
45 nasopharynx [4,5]. Recent data show that high viral load and a long virus-shedding period was associated  
46 with severe COVID-19 [6, 7]. Therefore, the use of antiviral nasal sprays would contribute to reduce  
47 nasal and nasopharyngeal viral load, thus slowing down the disease progression in the treated patient and  
48 the disease transmission to others in close contact with him or her.

49           Carrageenans are linear sulfated polysaccharides that are often extracted from red seaweeds.  
50 Carrageenans are commercially available in the form of kappa ( $\kappa$ ), iota ( $\iota$ ) or lambda ( $\lambda$ ). They have been  
51 used for years as thickening agents and stabilizers for food. At present they are extensively used in the  
52 food (cold cuts, cheese, etc.) and in the cosmetic and pharmaceutical industry as suspension and emulsion  
53 stabilizers. Their antiviral capacity has been described decades ago and has been experimentally  
54 confirmed on herpes virus type 1 and 2, human papiloma virus, H1N1 influenza virus, dengue virus,  
55 rhinovirus, hepatitis A virus, enteroviruses, and coronaviruses. Iota-carrageenan inhibits several viruses  
56 based on its interaction with the surface of viral particles, thus preventing them from entering cells and  
57 trapping the viral particles released from the infected cells. [8, 9, 10, 11, 12, 13].

58           Iota-carrageenan formulated into a nasal spray has proved to be safe and effective against virus  
59 causing common cold [14,15,16]. In vitro studies in cell cultures (HeLa and Calu-3) and in primary  
60 respiratory epithelial cells have shown inhibition of rhinovirus, Influenza virus and common-cold  
61 coronavirus. Iota-carrageenan is most active against common-cold coronavirus, inhibiting the infection up  
62 to 90 %. An iota-carrageenan spray reduced mortality by at least 50 % in mice infected with lethal doses  
63 of H1N1 influenza virus [17]. In all cases the antiviral action of iota-carrageenan is more effective, when  
64 administrated preventively or in the early stages of disease and has shown synergy with other antiviral  
65 agents. Studies performed on adults and children with common cold demonstrate effectivity of iota-  
66 carrageenan nasal spray to alleviate the clinical symptoms and shorten their duration, as well as to  
67 decrease the viral load of nasopharyngeal specimens and the relapses during the follow-up period [14, 15,  
68 16, 18, 19, 20]. Iota-carrageenan-containing nasal sprays are already on the market in several countries in  
69 the world.

70 Xylitol is a polyol that has been used as a sugar substitute in Finland since the 1960s. It is a  
71 polyol, (formula  $\text{CHOH})_3(\text{CH}_2\text{OH})_2$ ), which is obtained from xylan extracted from hardwood, which has  
72 demonstrated multiple health benefits [21]. It has been extensively used in buccal health care to prevent  
73 caries because of its antibacterial capacity. It is already being used in otorhinolaryngology as a nasal  
74 spray and lavage for the treatment of rhinosinusitis and the prevention of otitis media. [22,23]. Studies “in  
75 vitro” and in animal models has shown antiviral properties of Xylitol against human respiratory syncytial  
76 virus (24).

77 Both iota-carrageenan and xylitol are safe for humans, being used in much larger amounts as food  
78 additive and sweetener, respectively, than those that may be used for nasal delivery. Nasal safety of iota-  
79 carrageenan by nasal and nebulization administration has been already confirmed empirically [25]. The  
80 same holds for 5 % Xylitol water solution both applied as nasal spray and nasal irrigation [26], as well as  
81 applied as a nebulization solution [27]. Both are included in nasal formulations already on the market for  
82 use in children and adults.

83 Based on the above knowledge, an experiment was designed and carried out in a Biosafety Level  
84 3 (BSL3) laboratory to investigate the SARS-CoV-2 inhibition capacity of three different candidate  
85 preservative-free nasal formulations.

86

## 87 **Materials and methods**

88

### 89 **Cells and Virus**

90 Vero E6 cells were purchased from the American Type Culture Collection. Vero E6 cells were  
91 grown in complete minimal essential media (c-MEM) (Corning, NY, USA) which included 5% fetal  
92 bovine serum (FBS)(Gibco, Waltham, MA, USA), 5 mM penicillin/streptomycin (Gibco), and L-  
93 glutamine (Gibco). Cells were incubated at 37°C with 5% CO<sub>2</sub>. SARS-CoV-2 Isolate USA-WA1/2020,  
94 was obtained from BEI Resources (catalogue number NR-52281, Manassas, VA, USA). Virus master

95 seed stock was prepared in T175 flasks of Vero E6 cells using a multiplicity of infection (MOI) of 0.1.  
96 Each flask was harvested on day two post-infection and supernatant was centrifuged twice at 220 x g for  
97 15 minutes to remove cellular debris. Titer of virus stock was determined by plaque assay on Vero E6  
98 cells.

99

## 100 **Preparation of sample formulations**

101 All the formulations and placebos were prepared at Laboratorio Pablo Cassará S.R.L. (Argentina)  
102 under aseptic conditions and provided by Amcyte (US) to the University of Tennessee Health Science  
103 Center. Composition of different formulations is depicted in Tables 1 and 2. Samples 1, 2 and 3 were  
104 diluted at stock concentrations of 1200 µg/mL, 120 µg/mL, 12 µg/mL and 1.2 µg/mL using samples P1,  
105 P2 and P3 respectively as diluents. To determine antiviral efficacy of formulations by titer reduction  
106 assay, sample formulations were used at a final iota-carrageenan concentration of 600 µg/mL; 60 µg/mL,  
107 6 µg/mL and 0.6 µg/mL. Equivalent concentration of placebos (samples P1, P2 and P3) was used for titer  
108 reduction assay as controls.

109

110 **Table 1. Composition of candidate nasal formulations (samples containing iota-carrageenan)**

Component	sample 1	sample 2	sample 3
Iota-carrageenan	1.7 mg/mL	1.2 mg/mL	1.2 mg/mL
Sodium Chloride	9 mg/mL	5 mg/mL	—
Xylitol	—	—	50 mg/mL <sup>a</sup>
pH adjusted to	6.00 – 7.00	6.00 – 7.00	6.00 – 7.00

111

112 <sup>a</sup> Equivalent to 5 % m/V

113

114

115 **Table 2. Composition of placebo samples used as diluents (samples without iota-carrageenan)**

Component	sample P1	sample P2	sample P3
Sodium Chloride	9 mg/mL	5 mg/mL	—
Xylitol	—	—	50 mg/mL <sup>a</sup>
pH adjusted to	6.00 – 7.00	6.00 – 7.00	6.0 – 7.00

116

117 <sup>a</sup> Equivalent to 5 % m/V

118

119 **Titer reduction assay**

120 Vero E6 cells were seeded in 12-well plates at density of  $2.5 \times 10^5$ /well and grown overnight at  
121  $37^\circ\text{C}$  under  $5\% \text{CO}_2$ . Next day, cells were washed with PBS (pH 7.2), followed by addition of equivalent  
122 amount of c-MEM with reduced FBS (2%) and sample/placebo formulations. Formulations were  
123 incubated with cells for 2 hr, after which the supernatant was removed. Cells were infected with  $2.5 \times 10^4$   
124 pfu (MOI=0.1) of virus for 1 hr at  $37^\circ\text{C}$ ,  $5\% \text{CO}_2$  with rocking at 15 min intervals. After incubation wells  
125 were washed with DPBS, and sample/placebo formulations were added at same concentrations. After  
126 incubation for 2 days, well contents were collected. For titer reduction, wells with no treatment (only  
127 virus) and cells only were included. Virus titer was determined by performing a  $\text{TCID}_{50}$  assay using MTT  
128 to measure cell viability. Virus endpoint titer was determined using the Reed-Muench formula and  
129 expressed as  $\log \text{TCID}_{50}/\text{mL}$ . Residual virus titer from sample/placebo formulation treated wells was  
130 plotted against virus titer from untreated wells.

131

132

## 133 **Results**

134 To examine the antiviral effects of iota-carrageenan on SARS-CoV-2, three sample formulations  
135 were developed and tested. Each of the three sample formulations were tested in a dose dependent manner  
136 based on the concentration of iota-carrageenan and ranged from 600 µg/mL to 0 µg/mL. SARS-CoV-2  
137 samples treated with 600 µg/mL and 60 µg/mL of sample formulation 1 were reduced > 3.75 Log when  
138 compared to untreated control (Figure 1). The 6 µg/mL concentration of sample formulation 1 also  
139 demonstrated an effect but to a lesser extent, with a 2.5 Log reduction in virus (Figure 1). No activity was  
140 observed with 0.6 µg/mL of Iota-carrageenan (Figure 1). Lastly, there was no reduction in virus with P1,  
141 suggesting that Iota-carrageenan and not the components of sample formulation 1 is inhibiting SARS-  
142 CoV-2 (Figure 1)

143

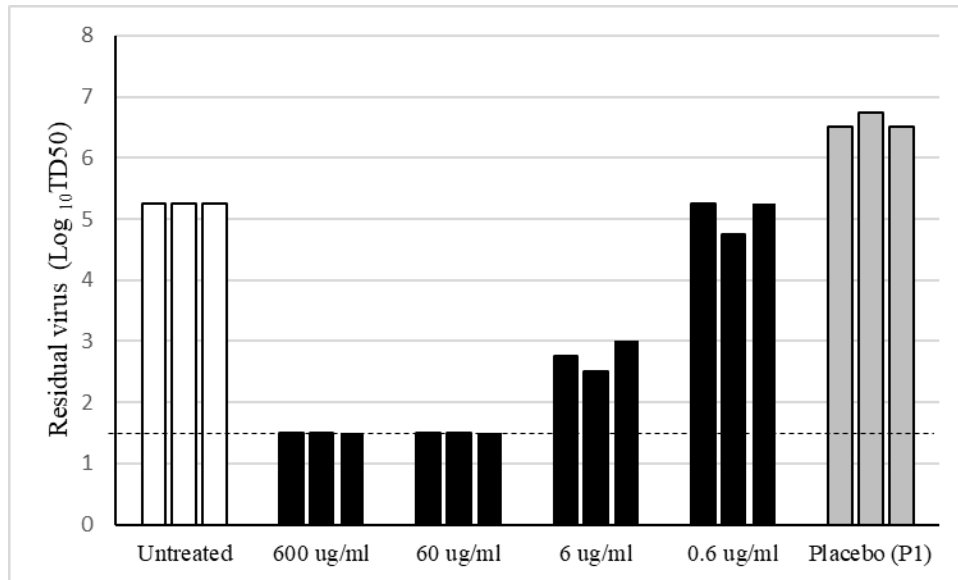
144 SARS-CoV-2 samples treated with dilutions of sample 2 at final iota-carrageenan concentrations  
145 of 600 µg/mL, 60 µg/mL, and 6 µg/mL were reduced > 4.25 Log compared to untreated control (Figure  
146 2). The 0.6 µg/mL concentration of iota-carrageenan was also not effective with sample 2 (Figure 2). No  
147 reduction in virus with P2 was observed, suggesting that iota-carrageenan and not the components of  
148 formulation 2 is inhibiting SARS-CoV-2 (Figure 2).

149

150 SARS-CoV-2 samples treated with dilutions of sample 2 at final iota-carrageenan concentrations  
151 of 600 µg/mL, 60 µg/mL, and 6 µg/mL were reduced > 4.25 Log compared to untreated control (Figure  
152 2). The 0.6 µg/mL concentration of iota-carrageenan was also not effective with sample 2 (Figure 2). No  
153 reduction in virus with P2 was observed, suggesting that iota-carrageenan and not the components of  
154 formulation 2 is inhibiting SARS-CoV-2 (Figure 2).

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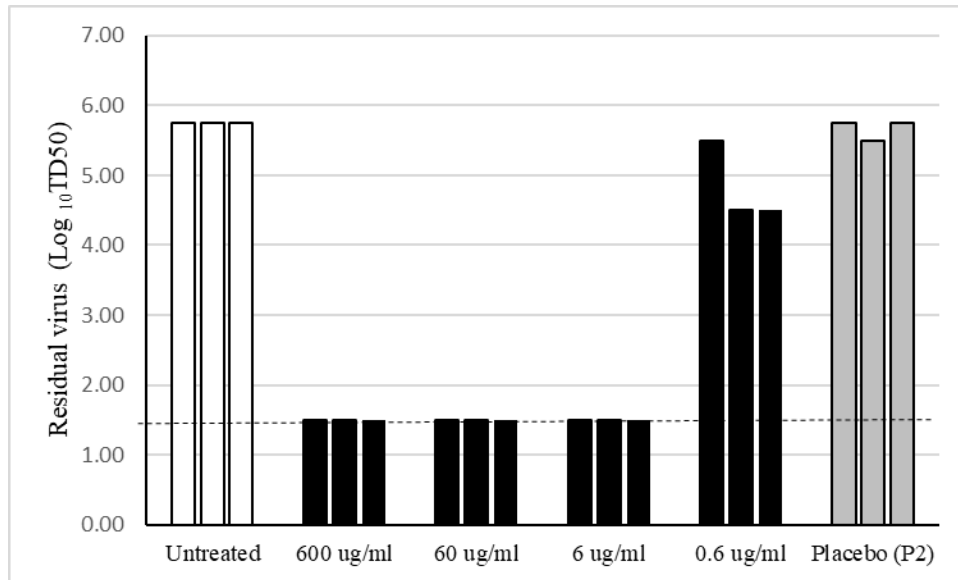
158 **Fig 1. SARS-CoV-2 viral titer after treatment with samples 1 and P1 (3 replicates per treatment).** Sample 1  
159 composition: 1.7 mg/mL iota-carrageenan, 9 mg/mL sodium chloride, pH 6-7. Vero E6 were pre-treated with  
160 dilutions of sample 1 with sample P1 (placebo without iota-carrageenan) to get 600 µg/mL, 60 µg/mL, 6 µg/mL, 0.6  
161 µg/mL iota-carrageenan final concentration for 2 h. After a 2 h pretreatment, cells were infected with SARS-CoV-2  
162 and incubated for 48h in the presence of the same dilutions of sample 1. Supernatants were harvested and virus yield  
163 determined by an end point dilution assay (TCID<sub>50</sub>). Controls consisted of untreated infected cells or infected cells  
164 treated with P1 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed  
165 as log TCID<sub>50</sub>/mL. Dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate.  
166 Underlying data reported in tables S3A and S3B as supporting information.

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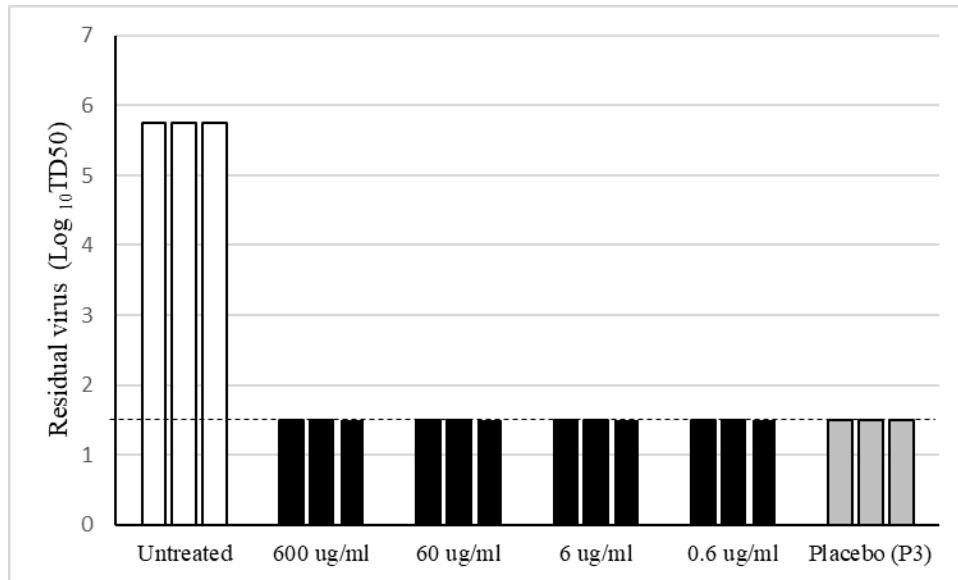


170

171 **Fig 2. SARS-CoV-2 viral titer after treatment with samples 2 and P2 (3 replicates per treatment)** Sample 2  
172 composition: 1.2 mg/mL iota-carrageenan, 5 mg/mL sodium chloride, pH 6-7. Vero E6 were pre-treated with  
173 dilutions of sample 2 with sample P2 (placebo without iota-carrageenan) to get 600  $\mu$ g/mL, 60  $\mu$ g/mL, 6  $\mu$ g/mL, and  
174 0.6  $\mu$ g/mL final iota-carrageenan concentration for 2 h. After a 2 h pretreatment, cells were infected with SARS-  
175 CoV-2 and incubated for 48h in the presence of the same dilutions of sample 2. Supernatants were harvested and  
176 virus yield determined by an end point dilution assay (TCID50). Controls consisted of untreated infected cells or  
177 infected cells treated with P2 (no iota-carrageenan). Results were determined using the Reed and Muench formula  
178 and expressed as log TCID50/mL. Dotted line shows the limit of detection (LOD). Testing of samples was  
179 performed in triplicate. Underlying data reported in tables S2A and S2B as supporting information.

180

181 All concentrations tested (600 - 0.6  $\mu$ g/mL) with sample 3 demonstrated antiviral activity  
182 including the P3 control that did not contain iota-carrageenan (Figure 3). Xylitol was present in this  
183 sample formulation and not in sample 1 or 2. The result suggests this component might also exert an  
184 antiviral effect.



185

186 **Fig 3. SARS-CoV-2 viral titer after treatment with samples 3 and P3 (3 replicates per treatment).** Sample 3  
187 composition: 1.2 mg/mL iota-carrageenan, 50 mg/mL xylitol, pH 6-7. Vero E6 were pre-treated with dilutions of  
188 sample 3 with sample P3 (placebo without iota-carrageenan) to get 600 µg/mL, 60 µg/mL, 6 µg/mL, and 0.6 µg/mL  
189 final iota-carrageenan concentration for 2 h. After a 2 h pretreatment, cells were infected with SARS-CoV-2 and  
190 incubated for 48h in the presence of the same dilutions of sample 3. Supernatants were harvested and virus yield  
191 determined by an end point dilution assay (TCID<sub>50</sub>). Controls consisted of untreated infected cells or infected cells  
192 treated with P3 (no iota-carrageenan). Results were determined using the Reed and Muench formula and expressed  
193 as log TCID<sub>50</sub>/mL. Dotted line shows the limit of detection (LOD). Testing of samples was performed in triplicate.  
194 Underlying data reported in tables S3A and S3B as supporting information.

195

196 A comparison of all three samples tested indicate that iota-carrageenan (600 µg/mL, 60 µg/mL,  
197 and 6 µg/mL) in samples 1 and 2 are effective at inhibiting SARS-CoV-2 (Table 3). Sample 3, which  
198 contained xylitol, was the most effective and demonstrated an antiviral effect at all concentrations tested  
199 (Table 3).

200

201

202

**Table 3. Log reduction of TCID<sub>50</sub>/mL found after 48 hs.**

<b>Iota-carrageenan</b>	<b>Sample 1</b>	<b>Sample 2</b>	<b>Sample 3</b>
600 µg/mL	≥ 3.75	≥ 4.25	≥ 4.25
60 µg/mL	≥ 3.75	≥ 4.25	≥ 4.25
6 µg/mL	2.50	≥ 4.25	≥ 4.25
0.6 µg/mL	0.17	0.92	≥ 4.25
0 µg/mL <sup>a</sup>	None	None	≥ 4.25

203

204 The values reported are calculated as mean of untreated replicates minus mean of treated replicates. Mean  
205 values used for this calculation are reported in Tables S1B, S2B and S3B as supporting information.

206 <sup>a</sup>These are the placebo samples P1, P2 and P3 having the same components as samples 1, 2 and 3 except  
207 for iota-carrageenan.

208

## 209 Discussion

210 Results from our study indicate that iota-carrageenan significantly inhibits SARS-CoV-2 in vitro.  
211 Our results are encouraging for clinical use of iota-carrageenan nasal spray for the prevention and early  
212 treatment of COVID-19. Clinical studies have demonstrated that iota-carrageenan nasal spray  
213 formulations already effective in vitro against rhinovirus [11] proved to be clinically effective in  
214 preventing and reducing symptoms and duration of common cold [14, 15, 16, 18]. Moreover, this study  
215 was designed so that the concentrations tested in vitro resemble the immediate concentration in nasal  
216 cavity and lower concentrations expected as iota-carrageenan is cleared from it. To do that we estimated  
217 airway surface liquid volume to be in the range 50 – 375 µL in nasal cavity based on a surface area of the  
218 nasal mucosa of 100 – 250 cm<sup>2</sup> [28, 29, 30, 31] and airway surface liquid height estimated as 5 – 15 µm

219 [32. 33]. If we take an average content of 200  $\mu\text{L}$  of airway surface liquid in the nose plus 200  $\mu\text{L}$  of  
220 formulation after delivering one 100- $\mu\text{L}$  of a 1.2 mg/mL iota-carrageenan solution in each nostril, the  
221 immediate concentration of iota-carrageenan in the nasal cavity would be 600  $\mu\text{g}/\text{mL}$ , coinciding with  
222 the highest concentration tested in vitro and capable of reducing virus yield to the LOD in our assay.  
223 Furthermore, considering that even 1/100 of this concentration is still active in vitro and that iota-  
224 carrageenan may stay for 4 hours [19] in the nasal cavity, there is a reasonable chance that this nasal spray  
225 may significantly help in the prevention and early treatment of COVID-19. Expected concentrations of  
226 iota-carrageenan in the nasal cavity will be even higher, if we consider a nasal formulation containing 1.7  
227 mg / mL (0.17 % m/V) as some marketed nasal sprays.

228

229 The other remarkably interesting result is that xylitol exhibits antiviral activity on SARS-CoV-2  
230 based on the results obtained with sample P3. Xylitol has been demonstrated to reduce titers of Human  
231 Respiratory Syncytial Virus in Hep-2 cells culture and in infected mice [24].

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

238 An inhalation solution of the same composition may be effective in case of severe cases of  
239 COVID 19. Even though there are studies showing safety of the use of both carrageenan and xylitol in  
240 nebulization [25,27], clinical trials would be needed to fully confirm these hypotheses. Risk of spreading  
241 the virus should be considered in this form of administration and due protection should be used to contain  
242 it [34].

243 We are starting multicenter randomized controlled trials to evaluate the efficacy of iota-  
244 carrageenan nasal sprays in health care staff assisting COVID-19 patients and in patients suffering from  
245 COVID 19 and other persons in close contact with them. However, it must be stressed that this and other  
246 similar nasal sprays are on the market and their safety profile is remarkable. The current COVID-19  
247 emergency warrants the urgent development of potential strategies to protect people even if more robust  
248 data on antiviral therapies is yet to come.

249

## 250 **Conclusions**

251 Iota-carrageenan inhibits SARS CoV-2 in vitro at concentrations easily achievable by nasal and  
252 nebulization formulations. Furthermore, xylitol exhibits antiviral activity on SARS-CoV-2, as well. An  
253 association with iota-carrageenan may be beneficial. There are already nasal sprays on the market having  
254 similar formulations to some of those tested in this in vitro study with adequate safety profile. Clinical  
255 trials are in progress to evaluate that nasal sprays based on the tested formulations are useful in the  
256 prevention and treatment of COVID 19. The data presented here are certainly encouraging in this  
257 direction.

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261

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