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1 Biofilms and coronaviruses reservoirs: a perspective review

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32 **Abstract**

33 Bats are a key reservoir of coronaviruses (CoVs), including the agent of the severe acute  
34 respiratory syndrome, SARS-CoV-2, responsible for the recent deadly viral pneumonia  
35 pandemic. However, understanding how bats can harbor several microorganisms  
36 without developing illnesses is still a matter under discussion. Viruses and other  
37 pathogens are often studied as stand-alone even though it is known that, in nature, they  
38 mostly live in multi-species associations called biofilms - both externally and within the  
39 host. Microorganisms in biofilms are enclosed by an extracellular matrix that confers  
40 protection and improves survival. Previous studies have shown that viruses can  
41 secondarily colonize preexisting biofilms, and viral biofilms have also been already  
42 described. In this review, we raise the perspective that CoVs can persistently infect bats  
43 due to occurrence in biofilm structures. This phenomenon potentially provides an  
44 optimal environment for non-pathogenic and well-adapted viruses to interact with the  
45 host, as well as for viral recombination. Biofilms can also enhance virion viability in  
46 extracellular environments, such as in fomites and aquatic sediments, allowing viral  
47 persistence and dissemination. Moreover, understanding CoVs biofilm lifestyle in  
48 reservoirs might contribute to explain several burning questions that remain unanswered  
49 including persistence and transmissibility by highly pathogenic emerging CoVs.

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51 **Key-words:** Coronaviruses, biofilm, host, bat, microbial interaction, persistent infection

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64 **1. Coronaviruses**

65 Human activities, including urbanization and, increase in global commerce, have  
66 led to drastic ecological changes (1, 2). This phenomenon, allied to social and cultural  
67 behaviors may affect the balance between health and disease (3). As consequence, we  
68 have seen the successful introduction of animal pathogens to the human population,  
69 such as the zoonotic transmission of non-primate viruses (4). Coronaviruses (CoVs)  
70 have been classified as zoonotic pathogens that infect the respiratory, gastrointestinal,  
71 hepatic, and central nervous systems of unrelated hosts. A historical and evolutionary  
72 overview of CoVs (1930-2020) transmission to hosts are summarized in Box 1 and  
73 Figure 1.

**Box 1 – Chronological overview of CoVs**

Probably first observed in the '30s, it was in 1968 that *Nature* published the description of a cluster of viruses found in humans and animals with the appearance of the “solar corona.” These viruses contained a characteristic “fringe”, supported by electron microscopy. The International Committee for the Nomenclature of Viruses (ICNV) named them coronaviruses (5, 6). In the '70s the attention to CoVs was focused on the veterinary field, especially for calves diseases that were causing losses in animal production and economy (7). It was in the 80's we have acquired more scientific information about these viruses according to clinical significance. A book was published summarizing the *status quo* of coronavirus research (8). The next decade was highlighted by advances in molecular and structural biology knowledge and insights about human infections began to appear (9); demonstrating an expansion of the spectrum of animals that could be infected by them. Furthermore, the community-acquired human CoVs strains 229E and OC43 were characterized. Since 2003, several studies have been present in the literature. This occurred as a reflection of the Asia CoV epidemic (SARS) in 2002. In 2012, a novel CoV was reported on the Program for Monitoring Emerging Diseases (ProMED-mail), named later as the Middle East respiratory syndrome-coronavirus (MERS-CoV) (10, 11). MERS was first identified in humans in Saudi Arabia (12). Sporadic outbreaks have been reported until now in African and European countries and the United States of America, but exclusively in individuals traveling back from the Middle East (13). Recently, 2019 raised the “tip of the iceberg” that would become the 2020 declared pandemic by WHO. A respiratory disease of unknown etiology was firstly detected in Wuhan, Hubei Province, China, and started to circulate the world (14). Chinese scientists identified the etiological agent as a new coronavirus, the 2019-nCoV (15) which was renamed SARS-CoV-2. Subsequently, the disease name was recommended as COVID-19 by the WHO. Clinical signs, similar to SARS and MERS, such as fever, cough and difficulties in breathing, with evolution to severe pneumonia and organ failures, were verified in the most drastic cases (16). Only in 2020, more than 37,204 articles were published on this subject (15<sup>th</sup> October 2020 from PubMed) and the emergence of human pathogenic CoVs attend a constant discussion.

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75 CoVs are enveloped viruses that belong to the *Coronaviridae* family (17). The  
76 disease-related CoVs in humans including the severe acute respiratory syndrome  
77 coronavirus (SARS-CoV), the new SARS-CoV-2 and the Middle East respiratorysindromecornavirus  
78 syndrome coronavirus (MERS-CoV) are classified as *Betacoronavirus*. They are  
79 positive-strand RNA viruses owing large genomes (30–32 kb) made of proteins such as  
80 the spike and transmembrane glycoproteins, the envelope protein, the nucleocapsid and,  
81 some of them, the haemagglutinin-esterase (18-20).

82           Although there are different hypotheses about the origin of the virus (21), the  
83 close phylogenetic relationship with bats CoVs provides strong evidence that SARS-  
84 CoV-2 may be originated from these animals and that they are the reservoir host of this  
85 virus. It has been proposed that the SARS-CoV-2 was introduced into a small cluster of  
86 humans from infected animals and, from there, the virus acquired the capacity for  
87 human-to-human transmission. Pangolins could be one of the intermediate amplifying  
88 hosts for the virus, which is surprising since they are solitary animals with relatively  
89 small population sizes, reflecting their endangered status (22). However, human  
90 exposition to these animals exists, since they are recognized as a meat delicacy sold in  
91 the parallel market and their scales are utilized in traditional Chinese medicine.

92           Regarding CoVs, the source of the viruses, their persistence, reservoirs and hosts,  
93 and the transmission to humans are topics under continuous discussion in the scientific  
94 community, and will also be addressed by this review-perspective study. There are  
95 advanced studies that establish the role of biofilms in microbial persistence, resistance  
96 and latent infections but to date, none of them bring up the potential involvement of  
97 biofilms in CoVs reservoirs (bats and environment). Overall, bats have been shown to  
98 possess an evident role in these processes, especially as the natural reservoir of diverse  
99 viruses. However, several attractive questions remain to be answered, such as (i) How  
100 do CoVs and other viruses persistently infect bats? (ii) How do they evade the immune  
101 system of bats? (iii) How are they not pathogenic for bats while leading to lethal human  
102 disease? (iv) How do they undergo several recombination events, favoring the  
103 emergence of distinct genetic variants from bats? And (v) How do they remain  
104 infectious in the environment, at least for hours or days, after being expelled in  
105 secretions by a contaminated host? This perspective review shed new light on complex  
106 host-virus-environmental interactions.

107 **2. Coronaviruses and hosts**

108 For many years the four community-acquired CoVs (i.e. HCoV-229E, HCoV-  
109 OC43, HCoV-HKU1, and HCoV-NL63) circulate in the human population triggering  
110 common cold in immunocompetent subjects, and, likely, these viruses do not depend on  
111 animal reservoir (23). In contrast, highly pathogenic CoVs (SARS-CoV, MERS-CoV,  
112 and SARS-CoV-2) are maintained and propagated in their zoonotic reservoirs and  
113 eventually spillover to susceptible human targets, possibly via one or more intermediate  
114 and amplifying hosts (summarized in Boxes 2-4).

**Box 2 – SARS-CoV**

The first SARS-CoV patients reported exposure to animal trade in wet markets or in restaurants where live animals were kept in Guangdong Province - China. Then, the SARS-CoV-like virus was isolated from Himalayan palm civets (*Paguma larvata*) presenting 99.8% of genomic identity with that from humans (24, 25). Although 80% of other animals from nearby markets possessed anti-SARS-CoV antibodies (26), the massive culling of market civets played a major role in the efficient control of SARS. This indicates that palm civets might serve as the intermediate amplifying host but not as the natural reservoir for SARS-CoV. Multiple species of mammals might also serve as intermediate and dead-end hosts for SARS-CoV (23).

Since 2005 a large number of SARS-related CoVs (SARSr-CoVs) have been detected in horseshoe bat species (*Rhinolophus* spp.) in China (27). A 5-year surveillance study reported distinct sequences of SARSr-CoVs in different genes from multiple species of horseshoe bats. This suggests that bat viruses are considered unlikely to represent the direct progenitor of the human virus. Importantly these bat SARSr-CoV strains present different S proteins but all of them might interact with the cell entry receptor in humans, the angiotensin-converting enzyme II - ACE2 (28). Likewise, another study also showed that SARSr-CoVs from bats faecal samples use ACE2 receptor (from humans, civets, and horseshoe bats) for cell entry, indicating a viral broad species tropism (29).

These findings likely explain that the direct ancestor of SARS-CoVs has arisen from sequential recombination events within bats before spillovers to an intermediate host, such as farmed civets or another mammal, which transmitted the virus to other civets by faecal–oral transmission. So, it is likely that when virus-infected civets were transported to Guangdong markets, the virus spread to market civets and acquired further mutations before spillover to humans (30).

115

**Box 3 – MERS-CoV**

Bats also have been proposed to harbor the progenitor viruses of MERS-CoV, although human infection has been linked to contact with dromedary camels (*Camelus dromedarius*) or other infected-humans (31). The Middle East is a region that possesses religious and cultural practices along with a reliance on camels for food, medicine, and travel. As reviewed by Cui and collaborators (2019), specific antibodies were highly prevalent in these animals from that region and their MERS-CoV strains were almost identical to those from humans (> 99% identity). Furthermore, infectious MERS-CoVs have been isolated from camels (32) and the respiratory route, flesh, and discharges, such as faeces and milk (23) are recognized as possible transmission ways to humans. Nevertheless, several patients did not have an epidemiological link to infected camels or humans (31). In this sense, several animal species including livestock harbor the human MERS-CoV dipeptidyl peptidase 4 (DPP4) receptor, however only alpaca (*Vicugna pacos*) and goat (*Capra hircus*) cells were showed to be permissive for MERS-CoV replication in comparison to sheep, cattle, and rodent cells (33).

MERS-related CoVs (MERSr-CoVs) were found in bat species from two bat families (27, 30) however, none of these viruses is recognized as a direct progenitor of MERS-CoV since their S proteins differ substantially. Overall, all the MERSr-CoVs isolated from bats support the hypothesis that they are originated from these mammals followed by numerous recombination events that implied the exchange of genetic elements between different viral ancestors, including camels and bats (30). Bat viruses have been detected in urine, faeces, saliva, and soiled fruit, indicating the potential for virus transmission via bats excreta in the wild. Emerging bats feed on palms or other crops and those seeds may carry bat faeces, possibly possessing MERS-CoVs. When seeds drop to the ground they might be eaten by camels (34).



**Box 4 – SARS-CoV-2**

Viral full-length genome sequences obtained from five patients at the early stage of the outbreak were almost identical among them and shared only 79.6% sequence identity to SARS-CoV. These CoV genomes presented 96.2% sequence identity to a bat CoV (BatCoV RaTG13) detected in *Rhinolophus affinis* bats from China, leading to the identification of the SARS-CoV-2 (35). SARS-CoV2 also uses ACE2 as a cell entry receptor. It is capable of using the ACE2 proteins from humans, Chinese horseshoe bats, civets, and pigs and it does not bind to DPP4 receptors, used by MERS-CoV 2 (35). However, BatCoV RaTG13 might be unconsidered the immediate ancestor for SARS-CoV-2 because the genetic sequence encoding for ACE2 shares only 89% of identity with that of SARS-CoV-2. Recently, another bat-derived CoV denoted RmYN02 from *Rhinolophus malayanus* was identified. This virus shares 93.3% of identity with SARS-CoV-2 for the complete genome and 97.2% for the 1ab gene, which is the closest relative of SARS-CoV-2 reported to date. In contrast, RmYN02 might not bind to the ACE2 receptor (15). Presumably, the intermediate animal hosts of SARS-CoV-2 should be among the wildlife species sold at the Huanan Seafood Wholesale Market where many of the initial cases of COVID-19 were associated, indicating an animal-to-human transmission event.

Therefore, the origins of this virus have not been conclusively (23) and possibly has more than one source of infection. SARSr-CoVs were also identified from 25 Malayan pangolins (*Manis javanica*) collected from a wildlife rescue center (36, 37). Because of the sequence divergences over the whole genome, phylogenetic analyses did not support that the SARS-CoV-2 arose directly from the pangolin-CoV (38), but evidence suggests it might have originated from the recombination of a pangolin-CoV-like virus with a Bat-CoV-RaTG13-like virus.

117

118 Pervasive evidence has shown that many of the human and animal CoVs have a  
119 bat origin. They are transmitted to humans via intermediate hosts although the cross-  
120 species pathways are still unknown (27, 39). Bats have been recognized as special  
121 reservoirs for viral pathogens since the ancient phylogenetic relationship between  
122 viruses from bats and a variety of other human viral pathogens has been confirmed (40-  
123 42).

124 Mammalian host-virus relationships demonstrated that bats harbor a significantly  
125 higher proportion of zoonotic viruses than other mammalian orders. Bats account for  
126 more than 20% of all classified mammal species worldwide and they possess wide

127 geographical mobility and distribution, and extensive species diversity (27). They are  
128 the only mammals to have achieved true self-powered flight. This enables them to have  
129 a longer range of migration as well as excrements spread and intimate interactions with  
130 humans and livestock when compared to land ones (27, 43). The migratory ability of  
131 bats likely has particular relevance in the context of disease transmission. Furthermore,  
132 humans might share some ecological niches with bats through butchering or coal  
133 mining; they are consumed as game meat and their excrements, such as faeces, are often  
134 used in traditional Chinese medicine (44). The Chinese food culture beliefs that live  
135 slaughtered animals are more nutritious, potentially enhancing the risks of viral  
136 transmission (27).

137

### 138 **3. Biofilm and viruses**

139 It is established that the majority of microorganisms on earth live in biofilms (45,  
140 46). Viruses play an extensive ecological role and have been reported to exist in diverse  
141 microbial communities such as biofilms. They are involved in several dynamics such as  
142 microbial diversity and biogeochemical cycles due to their prevalence and variation  
143 across diverse ecosystems (47). Therefore, it is essential to comprehend how the viruses  
144 effectively persist in different environments because this process can assist in the  
145 understanding of their transmissibility and cross-infectivity among different hosts. At  
146 this moment, there is limited knowledge about the relationship between viruses and  
147 biofilms, which usually addresses enteric viruses.

148 Biofilm is an ecological cluster of microorganisms, surface-attached or floating,  
149 with the evolutionary purpose given the protection, nutrition, or strengthening survival.  
150 In biofilms, the microorganisms are surrounded by a complex assembled extracellular  
151 matrix. This results in heterogeneous biomolecular and biochemical arrangements that  
152 hinder the entry of exogenous components while facilitates the exchange of genetic

153 elements among microbial cells (48, 49). The consequences of pathogenic biofilm  
154 formation are calamitous for human health, water systems, food, and other industries  
155 and consequently for the economy (50).

156 Reports in the literature about viruses in biofilms are recent, dating from the late  
157 90s. Specifically, enteroviruses have been detected from biofilms. In 1997 a study with  
158 the nonenveloped *Poliovirus-1*, using an artificial water flow distribution system,  
159 brought up the discussion about virus persistence and biofilm. They showed a greater  
160 amount of viruses recovered from biofilms. Notably, those viruses seemed to be  
161 protected against the disinfectant chlorine in the opposite of those in water phase  
162 counterparts (51). *Enterovirus* and *Norovirus*, nonenveloped viruses, were also  
163 identified into biofilms from drinking water and wastewater samples and infectious  
164 viruses could be released from these biofilms. Besides, virions from wastewater were  
165 characterized as being more persistent when associated with biofilm, remaining  
166 infectious for up to 30 days. These results warned for the risk of virus-containing  
167 biofilms that contribute to temporal dispersion of viral-pollution in water environments  
168 (52-54). Additionally, biofilm-associated enteroviruses developed attached to pipeline  
169 walls are flow pressure and disinfectant resistant and may represent 95% of the overall  
170 potential contaminant biomass in direct contact with the water (55). In this sense, the  
171 entitled article "Enteric virions and microbial biofilms – a secondary source of public  
172 health concern?" enquire about the presence of pathogenic virions within biofilms from  
173 distribution pipes and their neglected monitoring (56). It is important to mention the  
174 detection of SARS-CoV-2 RNA in wastewater (57). Although the isolation of the  
175 infectious virus from faeces was described, no cases of faecal-oral transmission have  
176 been reported (58).

177 Viruses as well as other micro-organisms may come into contact with preexisting  
178 biofilms and accidentally adhere and make part of them, thus constituting secondary  
179 colonizers. A study of 2002 supports that biofilms may encompass a set of  
180 nonenveloped enteric viruses, including *Calicivirus*, *Rotavirus*, *Astrovirus*, and hepatitis  
181 A virus, among other microorganisms such as Gram-negative bacteria and filamentous  
182 fungi (55). Some authors indicate that the human oral cavity may be an active site of  
183 infection and reservoir for SARS-CoV-2 assuming its interaction with the host oral  
184 microbiota (59), which is mostly in the form of biofilm. Importantly, both virions and  
185 virus-infected eukaryotic cells embedded in biofilms have been reported to retain their  
186 infectivity. A study investigated the enveloped virus *Herpes simplex* virus type 1 (HSV-  
187 1) and the nonenveloped virus *Coxsackievirus* type B5 (CVB5) within the fungal  
188 *Candida albicans* biofilms (60). The authors recovered high virus titers from *in vitro*  
189 cultures of the virus-exposed biofilms after extensive washes, indicating that virions  
190 and viruses are deeply dispersed and protected into the fungi-produced biofilm matrix.  
191 Still, the authors related that biofilm reduced virus sensitivity to chemical inactivation.  
192 They discussed some mechanisms that may lead to virions inclusion into the fungal  
193 biofilm; for example, by sorption sites from the extracellular matrix, by the natural  
194 colloidal characteristic of viruses, or by biomolecules present on biological fluids. Since  
195 these viruses encompassed on biofilm kept their viability and infectivity, it indicates  
196 that biofilm lifestyle does not limit viral dissemination (60) and even can improve it.  
197 Therefore, viruses may be stored in biofilms regarding temporary or long-term  
198 reservoirs in the environment (61). As pointed by Vasickova and collaborators (2010),  
199 the potential of viral spreading via contaminated surfaces depends particularly on the  
200 ability of the virus to maintain infectivity while it is in the environment, and they

201 indicated some benefits for viruses within biofilms, such as protection against  
202 desiccation and antimicrobial agents (62).

203         A pioneering study conducted by French scientists using the enveloped human T-  
204 cell leukemia virus type 1 (HTLV-1) showed that the biofilm-like extracellular viral  
205 assemblies mediate the HTLV-1 cell-to-cell transmission (63). Notably, the  
206 extracellular state represents a crucial step for viral dissemination since intracellular  
207 formed virions have to spread the surface of other target cells to re-start viral  
208 replication. At this moment they are exposed to physicochemical and host immune  
209 system challenges. To overcome it the authors proposed that the observed “extracellular  
210 viral assemblies” would correspond to a “viral biofilm.” Viral biofilms were then  
211 defined as a protective form of “virion community embedded in a matrix of highly  
212 glycosylated proteins” with enhanced persistence, infectious capacity, and  
213 dissemination (64). They also detail the similarities of these extracellular viral  
214 assemblies and the composition, organization, and dissemination to bacterial biofilms.  
215 To conclude, the authors pointed that it is likely a similar mechanism can be used also  
216 by other enveloped viruses, and CoVs possess enveloped capsid.

217         A growing number of studies reports that enveloped and non-enveloped viruses  
218 can spread in groups in so-called collective infectious units (65-67). The vehicles  
219 mediating collective spread vary widely and include lipid vesicles, protein matrices,  
220 diverse forms of aggregation, and binding to the surface of host or non-host cells (66).  
221 In accordance, Tozzi and co-workers (2020) described the collective arrangements of  
222 multiple SARS-CoV-2 particles based on microscopic image analysis of viruses  
223 emerging from the surface of cultured cells. They suggest that when the virions exit the  
224 host cells, single particles tend at first to aggregate in spherical assemblies, then to  
225 develop interconnected network-like branches and nodes and this time called collective

226 clustering dynamics (68). However, further investigations are needed to characterize the  
227 presence of the matrix for SARS-CoV-2, such as in the study developed by Thoulouze  
228 and colleagues (2011) for HTLV-1. In this sense, the ability of *Betacoronaviruses* to  
229 bind carbohydrates has been mapped due to their high saccharides or carbohydrate  
230 domains and glycosylation patterns (69-71). Likewise, we speculate that the affinity of  
231 CoVs virions to polysaccharides presented on the matrix of biofilms probably would  
232 play a role in secondary colonization. Moreover, would this high saccharide profile be  
233 involved in the formation of viral biofilms?

234 This set of evidence allows us to propose that CoVs also could be present in  
235 biofilm patterns, either as viral biofilm or as a secondary colonizer of a microbial  
236 biofilm (**Figure 2**). Moreover, previous studies addressed in this review indicate that the  
237 (i) viral particles comprising a viral biofilm are embedded in an extracellular matrix  
238 from the host cell microenvironment, eventually, induced by the viral infection;  
239 otherwise, (ii) viral particles that are inside a microbial biofilm are surrounded by an  
240 extracellular matrix produced and externalized by bacterial and/or fungal cells.

241 Since viruses are strict intracellular parasites, we hypothesize that they will be  
242 unable to proliferate in biofilms, but they would persist infectious in a reservoir host due  
243 to the advantages conferred by the biofilm structure. Furthermore, the maintenance of  
244 viral particles inside biofilms facilitates the co-infection process due to physical  
245 proximity. When viruses spread collectively, they should locally increase the number of  
246 viral genomes that initiate a cell infection, which may contribute to increasing  
247 infectivity and may favor co-infections (66, 67). To co-infect the same cell is beneficial  
248 for microorganisms since it may provide diverse genetic materials, favoring mutations  
249 and recombinations, biological diversity, and drug resistance (72) (**Figure 2**, step 3b).  
250 Subsequently, we expect that CoVs-containing biofilms may also act as a viral

251 environmental reservoir, including under fomites, and contribute to viral persistence and  
252 dissemination (61).

253         Importantly, these circumstances may increase viral infectivity and favor co-  
254 infections (73). A retrospective study from Jiangsu Province showed that 242 (94.2 %  
255 of 257) SARS-CoV-2 positive patients were co-infected with one or more pathogens.  
256 Bacterial co-infections were dominant and *Streptococcus pneumoniae*, *Klebsiella*  
257 *pneumoniae*, and *Haemophilus influenzae* were the most common species. Additionally,  
258 viral, fungal, and bacterial-fungal co-infections were present in the highest severe  
259 SARS-CoV-2 cases (74). In accordance, another study from Renmin Hospital of Wuhan  
260 University including 354 SARS-CoV-2 positive patients concluded that 24.4% of  
261 critical cases were co-infected with other respiratory pathogens. These conclusions  
262 suggest that co-infections are associated with the severity of SARS-CoV-2 (75).

263

#### 264         **4. Can microbial biofilms favor viral reservoirs in bats?**

265         Bats harbor a rich pool of virus species, increasing the probability of cell co-  
266 infections. It enables the interspecies exchange of genetic fragments and the emergence  
267 of new viruses. Longevity, densely packed colonies, close social interaction, and strong  
268 ability to fly are all favorable conditions for bats to be an ideal virus spreader. It has  
269 been proposed that bats coexist with pathogens due to adaptations inadvertently  
270 acquired during the evolution of flight (76). Moreover, multiple pieces of evidence  
271 support an evolutionary origin of all human CoVs from bats.

272         In 2016, Plowright and colleagues described three hypotheses that dominate the  
273 current research on emerging bat infections. They pointed that pulses of viral excretion  
274 could be generated by episodic shedding from persistently infected bats through a  
275 combination of physiological and ecological factors. In this scenario, the viral acute

276 infection is resolved without clearance of viruses which allows pulses of transmission.  
277 This is triggered by viral reactivation and the stable carriage of the pathogen in space  
278 and time, even if viral shedding is episodic. For example, enveloped *Lyssaviruses* and  
279 *Flaviviruses* persistently have infected bats and shed viruses for extensive periods  
280 without evidence of disease (77). In 2017, Jeong and co-workers demonstrated that  
281 persistent infections support the maintenance of CoVs in a bat population. It was  
282 assumed that temporally synchronized stressors such as pregnancy or poor nutrition  
283 may weaken the immune system of bats facilitating the viral reactivation, the pulses of  
284 viral shedding, and the spillover of bat-borne viruses (78, 79). Several hurdles including  
285 the necessity of biosecurity level 4 conditions, the difficulty in reproducing natural  
286 infections in bat through experimental infections, and in isolating infectious virus from  
287 bats have constrained the understanding of emerging bat viruses (80). However, the  
288 mechanisms including physiological and ecological factors which drive infectious  
289 disease dynamics in bats such as viral persistence, clearance, and transmission without  
290 apparent disease remain poorly characterized.

291       Based on this set of evidence and the features regarding biofilms, we raise the  
292 perspective that some viruses, such as CoVs, can persistently infect reservoir hosts,  
293 including bats, due to biofilm lifestyle. This assumption may contribute to response  
294 some important questions and is supported by the following observations and  
295 discussions:

296       *1 - In persistent infections, a virus is not cleared from the host, but remains*  
297 *associated with specific cells for long periods (79).*

298       Biofilms are recognized as an important way to maintain microbial cells or  
299 particles in metabolic latency while they are protected by an external matrix. In this  
300 sense, as pointed by Thoulouze and Alcover (2011) viral biofilms could represent “viral



301 communities” with enhanced infectious capacity and improved spread compared with  
302 “free” viral particles. This might constitute a key reservoir for chronic infections.  
303 Additionally, as discussed by Dawley and Gibson (2019) specific associations of  
304 viruses with bacteria or other micro-organisms are likely to allow the easier entry of  
305 virions into the biofilm resulting in a reservoir of viruses. These virions are as difficult  
306 to remove and inactivate as their bacterial counterparts, turning infections persistent for  
307 a long time (81).

308 *2 - If bats do not clear infections and viruses may persist within bat hosts, it is*  
309 *assumed that bat hosts may tolerate viruses. In this case, a tempered inflammatory*  
310 *response would minimize immunopathology and viral replication may fluctuate as a*  
311 *function of the immunological competence of the host (78).*

312 It has been reported that bats host intracellular microorganisms such as protozoa  
313 (*Plasmodium* spp.), bacteria (*Bartonella* spp.), and fungi (*Histoplasma capsulatum*),  
314 without ostensible disease symptoms. By contrast, pathogens that predominantly occupy  
315 the extracellular space present considerable challenges for bat immune systems (82).  
316 The immune system of bats appears to be different in some features from those of other  
317 mammals and avoid an over-induction of inflammation (83). In this point, is important  
318 to mention the inflammatory process also may be inhibited by biofilms, since the  
319 surrounding matrix hinders the recognition of microorganisms by the immune system.  
320 Additionally, once viruses are encompassed on biofilms, they will be unable to  
321 replicate. These biofilm characteristics may also explain the controlled rate of viruses,  
322 the persistent infection, the lower activation of the immune system, and the absence of  
323 active disease in bats.

324 *3 - Genomic recombination events have been shown for several viruses, including*  
325 *CoVs, that are harbored by bats (23). Compared to other RNA viruses, the expanded*

326 genome size of CoVs facilitates the acquisition of genes encoding accessory proteins  
327 that are beneficial for CoVs adaptation to a specific host and as a result, genome  
328 changes caused by recombination, gene interchange, and insertion or deletion are  
329 common among CoVs (27). This genetic plasticity is important to inter-species  
330 persistence-survival mechanisms (84).

331 Biofilms may contribute to viral recombination events especially for supporting  
332 an environment in which distinct and infectious viruses are physically close and in the  
333 presence of extracellular enzymes and genetic fragments. These viruses would be  
334 released from the biofilm and would co-infect the same host cell leading to gene  
335 exchanges and recombination. Therefore, the facilitated cell co-infection by diverse  
336 viruses allows the evolution and emergence of new viral particles that can undergo  
337 spillover events to other animals.

338 In this sense, it was demonstrated that secondary infection with the white-nose  
339 syndrome fungus (*Pseudogymnoascus destructans*) increased CoV replication in bats  
340 (85). This study opens up a new perspective about infection dynamics and microbial  
341 interactions. It demonstrates that a complex regulation possibly modulates bat responses  
342 to virus replication through their translocation from the biofilm (related to persistent  
343 infections) to virion free-living state (related to active infection) and vice-versa.

344 4 - It has been reported the difficulty of isolating infectious viruses from bats,  
345 which may yield false-negative samples. Moreover, it has been suggested that persistent  
346 pathogens within their hosts may be sequestered in tissues while viremia or shedding in  
347 excreta may be periodic, justifying the false negatives status (78, 86).

348 It is important to point out that this technical obstacle also may be imposed if  
349 viruses are enclosed within biofilm structures.

350

351 **5. Do biofilm mediate CoVs extracellular viability?**

352 Viruses dissemination closely depends on hosts and environmental interactions.  
353 SARS-CoV and -CoV-2 human transmission can occur with both direct (air-borne and  
354 droplets) and indirect contact (contaminated surfaces) between susceptible and infected  
355 individuals (87, 88). For this, viruses must be shed into the environment and  
356 contaminate biotic (mucosa) or abiotic (non-living structures like fomites) surfaces.  
357 Notably, they must be capable to remain infectious on that for at least a time (**Figure 3**).

358 In 2017, a review addressed the different types of viruses' collective spread and  
359 pointed the advantages of viral dissemination through "complex multi-virion  
360 structures", such as viral biofilms and viruses as secondary colonizers, in comparison to  
361 free individual virions (65, 66). In this context, we point out two leading biofilm  
362 features that could directly or indirectly afford and maintain virus viability through  
363 some intrinsic abilities:

364 1 - Biofilm may act as viral storage where virions remain infectious within the  
365 hosts (wild, livestock or domestic animals and humans) and on the environment (food,  
366 water, excrements, plants, pastures, and fomites);

367 2 - Biofilm may support virions persistence through matrix physicochemical  
368 protection against stresses such as desiccation in the extracellular medium, against  
369 immune responses (immunological modulation) and resistance to chemical substances,  
370 keeping viral particles infective over time;

371 Surfaces can serve as a vehicle for virus dissemination because they can be  
372 contaminated by direct contact with the animal or human excretions, fluids, and through  
373 transfer by mechanical vectors (**Figure 3**) (87). The degree to which  
374 environmental/surface-based transmission of SARS-CoV-2 is actually occurring is still  
375 unclear. Considering that CoVs are enveloped viruses, it is expected a structural

376 fragility when facing environmental conditions. However, scientists have investigated  
377 the capacity of CoVs to survive on several surfaces (plastics, latex, metals, and glasses)  
378 and the results show that they remain infectious during long periods (hours-days for  
379 CoVs, while weeks-months for others) (89). In this sense, SARS-CoV, SARS-CoV-2,  
380 and MERS-CoV would be infectious in environmental reservoirs like water, foods, and  
381 in sewage time sufficiently to facilitate onward transmission. The viability and  
382 persistence of CoVs depend on several variables and the exact mechanisms are still  
383 unknown (90, 91). Although these studies consider some characteristics of the surface  
384 substrate (porous and non-porous) the biofilm condition has not been contemplated until  
385 now (87, 91-93). It was shown that SARS-CoV and MERS-CoV could survive for  
386 extended periods when suspended in human secretions while the influenza virus could  
387 survive longer in mucus (91, 94). These observations contribute to biofilm-theory since  
388 organic materials are highly hydrated and rich in macromolecules which in turn can act  
389 as the extracellular matrix.

390

## 391 **6. Concluding remarks**

392 The scientific literature allows us to propose that biofilms may play a role in  
393 CoVs pathogenicity modulation, the ability to persist in reservoir hosts and the  
394 environment, and viral transmissibility. Assuming that CoVs can also occur in biofilms,  
395 new perspectives could be provided for important questions (i) the absent or low  
396 pathogenicity of CoVs within reservoir hosts, like bats; (ii) a more depressed level of  
397 immune system stimulation; (iii) viral persistence and chronic infection profile in  
398 reservoir hosts, like bats; (iv) the viral genetic plasticity and emergence of new viral  
399 entities; (v) the viral environmental spreading through excretions; (vi) the persistence of  
400 the viruses outside host cells; and (vii) indirect cross-species transmissibility by

401 handling and consuming contaminated products. SARS-CoV-2 pandemic exposes  
402 enormous inequalities of our societies that negatively impact our lives. Given the public  
403 health and economic gravity of this pandemic, SARS-CoV-2 is currently the hot topic  
404 and significant financial investment has been destined by several countries. It is  
405 supporting basic research, to understand the biology and pathogenesis of the virus, as  
406 well as applied research, to obtain epidemiological data to understand the origin and  
407 dynamics of the virus and accelerate the development of drugs and vaccines. We  
408 strongly feel this is a significant interdisciplinary contribution connecting microbiology,  
409 ecology, and public health. It will aid the advancement of alternative approaches for the  
410 control of these pathogens, and the management of the current and potential future  
411 crises.

412

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417

#### 418 **Competing interests**

419 The authors declare no competing interests.

420

#### 421 **Author contribution**

422 Both authors contributed equally to this study.

423

#### 424 **Biographical sketch**

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444 Currently, she is working with *Galleria mellonella* larvae as an experimental host to  
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446 plastic biodegradation.

447

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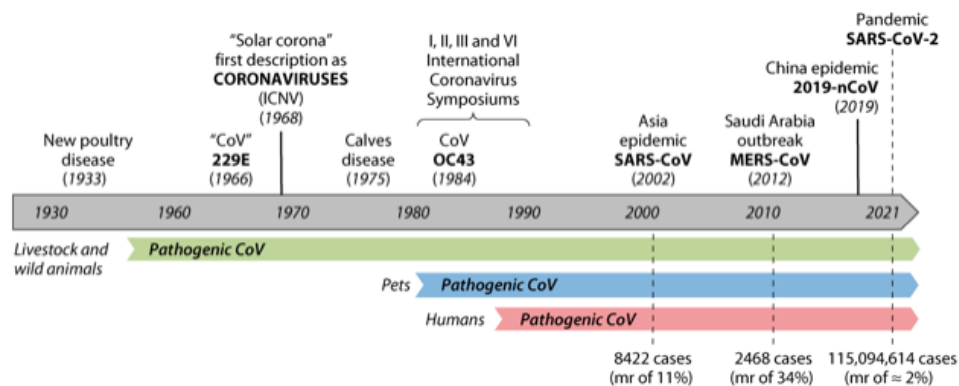
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748 **Figures**

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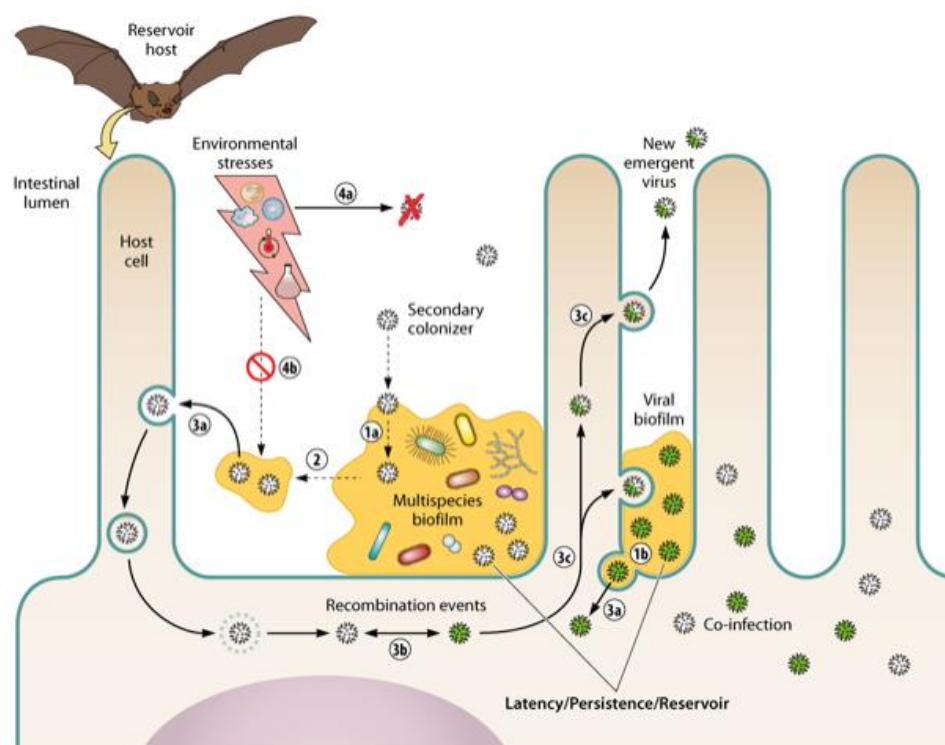
750 **Figure 1:** Chronology diagram of health importance-CoVs transmission to hosts (1930  
 751 – 2020), based on published reviews using "coronavirus" as a keyword at PubMed  
 752 database (accessed on the first week of April 2020). Color lines represent a temporal  
 753 estimation of the emergence of pathogenic CoVs, divided into three particular affected  
 754 groups (livestock and wild animals, pets and humans). ICNV: International Committee  
 755 for the Nomenclature of Viruses; CoV: Coronavirus; SARS-CoV: Severe Acute  
 756 Respiratory Syndrome CoV; MERS-CoV: Middle East Respiratory Syndrome; 2019-  
 757 nCoV: 2019 – new Coronavirus; SARS-CoV-2: Severe Acute Respiratory Syndrome  
 758 CoV-2 and mr: mortality rate. The number of cases and mr were updated on 5<sup>th</sup> March  
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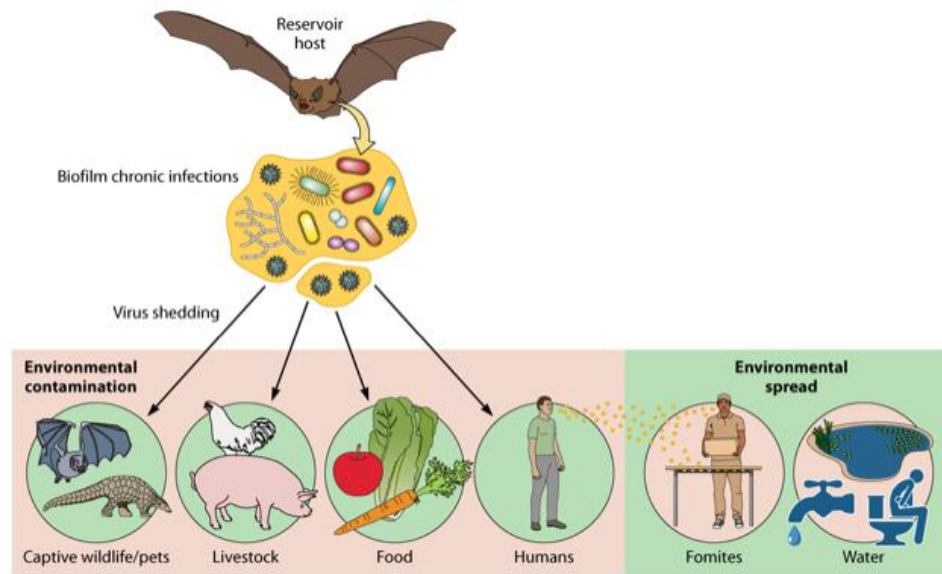


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773 **Figure 2:** Biofilms and viruses. Viruses are shown in the extracellular space in  
774 planktonic or biofilm forms. **1a)** Some viruses come into contact with preexisting  
775 biofilms (multispecies biofilm in yellow) and adhere to them, constituting  
776 secondary colonizers, or **1b)** they are enclosed in a single viral biofilm (orange). Once these  
777 structures are established, they become reservoirs and could persist like a “state of  
778 latency”, keeping viral particles infective over time. **2)** Biofilm detachment allows  
779 viruses to spread and can liberate virions that will colonize new sites and infect new  
780 host cells. **3a)** A virion detached from multispecies or viral biofilms infecting the host  
781 cell; **3b)** Viral gene exchanges and recombination events are possible in the presence of  
782 co-infections (exemplified by the green and blank viruses) and can lead to the



783 emergence of new viruses (3c). 4) Environmental stresses such as chemicals, immune  
784 responses (immune modulation) and desiccation in the extracellular medium can  
785 inactivate/eliminate planktonic virions (4a) while virions in biofilm, protected by the  
786 matrix, are more tolerant to these agents and may remain infectious over time (4b).  
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790 **Figure 3:** Proposed role of biofilms in host-coronavirus-environmental interactions and  
791 viral dissemination. Bats are the reservoir hosts persistently infected by several viruses,  
792 potentially in the form of biofilm chronic infections (multi-species and viral biofilms  
793 depicted in enlarged view). Viruses are shed from the host into the environment in  
794 biofilms present on excretions. The environmental viral spread can contaminate  
795 organisms (e.g. humans, wildlife, livestock and pet animals, plants, and fruits) or abiotic  
796 components (e.g. fomites and water). Viral transmission is possible via direct (air-borne  
797 and droplets) or indirect (contaminated surfaces) contact between susceptible and  
798 infected individuals/hosts.  
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