



Biofilms and Coronavirus Reservoirs: a Perspective Review

 Rafael Gomes Von Borowski,^a  Danielle Silva Trentin^b

^aUniversité de Rennes, CNRS, Institut de Génétique et Développement de Rennes (IGDR) UMR6290, Rennes, France

^bDepartamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil

Rafael Gomes Von Borowski and Danielle Silva Trentin contributed equally to this work. Author order was determined by common agreement in order of increasing seniority.

ABSTRACT Bats are a key reservoir of coronaviruses (CoVs), including the agent of the severe acute respiratory syndrome, SARS-CoV-2, responsible for the recent deadly viral pneumonia pandemic. However, understanding how bats can harbor several microorganisms without developing illnesses is still a matter under discussion. Viruses and other pathogens are often studied as stand-alone entities, despite that, in nature, they mostly live in multispecies associations called biofilms—both externally and within the host. Microorganisms in biofilms are enclosed by an extracellular matrix that confers protection and improves survival. Previous studies have shown that viruses can secondarily colonize preexisting biofilms, and viral biofilms have also been described. In this review, we raise the perspective that CoVs can persistently infect bats due to their association with biofilm structures. This phenomenon potentially provides an optimal environment for nonpathogenic and well-adapted viruses to interact with the host, as well as for viral recombination. Biofilms can also enhance virion viability in extracellular environments, such as on fomites and in aquatic sediments, allowing viral persistence and dissemination. Moreover, understanding the biofilm lifestyle of CoVs in reservoirs might contribute to explaining several burning questions as to persistence and transmissibility of highly pathogenic emerging CoVs.

KEYWORDS coronaviruses, biofilm, host, bat, microbial interaction, persistent infection

CORONAVIRUSES

Human activities, including urbanization and increases in global commerce, have led to drastic ecological changes (1, 2). This phenomenon, allied to social and cultural behaviors, may affect the balance between health and disease (3). As a consequence, we have seen the successful introduction of animal pathogens to the human population, such as the zoonotic transmission of nonprimate viruses (4). Coronaviruses (CoVs) have been classified as zoonotic pathogens that infect the respiratory, gastrointestinal, hepatic, and central nervous systems of unrelated hosts. A historical and evolutionary overview of CoVs (1930 to 2020) transmission to hosts is summarized in Box 1 and Fig. 1.

BOX 1: CHRONOLOGICAL OVERVIEW OF CORONAVIRUSES

Probably first observed in the 1930s, 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” (96). 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 1970s, the attention to CoVs was focused on the veterinary field, especially for calf diseases that were causing losses in animal production and economy (7). It was in the 1980s we acquired more scientific information about

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Address correspondence to Danielle Silva Trentin, daniellest@ufcspa.edu.br.

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these viruses according to clinical significance. A book was published summarizing the status quo of CoV 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 CoV strains 229E and OC43 were characterized. Since 2003, several studies have been presented in the literature as a reflection of the Asian CoV epidemic (SARS) in 2002. In 2012, a novel CoV was reported on the Program for Monitoring Emerging Diseases (ProMED-mail), later named 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, but exclusively in individuals traveling back from the Middle East (13). In 2019, the “tip of the iceberg” was raised that would become the 2020 global pandemic declared by the World Health Organization (WHO). A respiratory disease of unknown etiology was first 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 those of SARS and MERS, included fever, cough, and difficulties in breathing, with evolution to severe pneumonia and organ failures in the most drastic cases (16). In 2020 alone, more than 37,204 articles were published on this subject (15 October 2020 from PubMed) and the emergence of human-pathogenic CoVs was a source of constant discussion.

CoVs are enveloped viruses that belong to the *Coronaviridae* family (17). The disease-related CoVs in humans, including the severe acute respiratory syndrome coronavirus (SARS-CoV), the new SARS-CoV-2, and the Middle East respiratory syndrome coronavirus (MERS-CoV) are classified as *Betacoronavirus*. They are positive-strand RNA viruses with large genomes (30 to 32 kb) encoding proteins such as the spike and transmembrane glycoproteins, the envelope protein, the nucleocapsid and, for some CoVs, the hemagglutinin-esterase (18–20).

Although there are different hypotheses about the origin of the virus (21), the close phylogenetic relationship with bat CoVs provides strong evidence that SARS-CoV-2 may have originated from these animals and that they are the reservoir host of this virus. It has been proposed that SARS-CoV-2 was introduced into a small cluster of humans from infected animals and that, from there, the virus acquired the capacity for human-to-human transmission. Pangolins could be one of the intermediate amplifying hosts for the virus, which is surprising since they are solitary animals with relatively small population sizes, reflecting their endangered status (22). However, human exposure to these animals exists

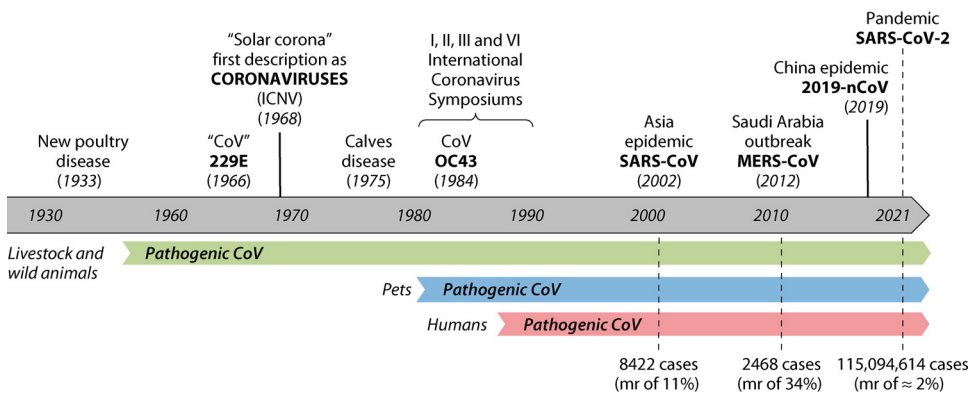


FIG 1 Chronological diagram of the health importance of CoV transmission to hosts (1930 to 2020), based on published reviews using “coronavirus” as a keyword at the PubMed database (accessed early April 2020). Colored lines represent a temporal estimation of the emergence of pathogenic CoVs, divided into three particular affected groups (livestock and wild animals, pets, and humans). ICNV, International Committee for the Nomenclature of Viruses; CoV, coronavirus; SARS-CoV, severe acute respiratory syndrome CoV; MERS-CoV, Middle East respiratory syndrome; 2019-nCoV, 2019 new coronavirus; SARS-CoV-2, severe acute respiratory syndrome CoV-2; mr, mortality rate. The number of cases and mr were updated on 5 March 2021.

because they are recognized as a meat delicacy sold in the parallel market and their scales are utilized in traditional Chinese medicine.

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

CORONAVIRUSES AND HOSTS

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

BOX 2: SARS-COV

The first SARS-CoV patients reported exposure to the 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% 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 fecal samples use the 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 fecal-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).

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 dependent on camels, along with a reliance on these animals for food, medicine, and travel. As reviewed by Cui and collaborators (30), specific antibodies were highly prevalent in camels 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 feces and milk (23), are recognized as possible means of transmission 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 shown 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, feces, saliva, and soiled fruit, indicating the potential for virus transmission via bat excreta in the wild. Emerging bats feed on palms or other crops and those seeds may carry bat feces, possibly possessing MERS-CoVs. When seeds drop to the ground, they may 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 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 SARS-CoV-2 (35). SARS-CoV-2 also uses ACE2 as a cell entry receptor. The virus 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 (35). However, BatCoV RaTG13 might not be the immediate ancestor for SARS-CoV-2 because its genetic sequence coding for the ACE2 receptor-binding domain shares only 89% identity with that of SARS-CoV-2 (95). Recently, another bat-derived CoV (denoted RmYN02) from *Rhinolophus malayanus* was identified. This virus shares 93.3% identity with SARS-CoV-2 across 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 identified (23), and there might possibly be 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 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.

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

Mammalian host-virus relationships demonstrated that bats harbor a significantly higher proportion of zoonotic viruses than other mammalian orders. Bats account for more than 20% of all classified mammal species worldwide and they possess wide geographical mobility and distribution, and extensive species diversity (27). They are the only mammals to have achieved true self-powered flight. This enables them to have a longer range of migration, as well as spread of excrement and intimate interactions with humans and livestock compared to land mammals (27, 43). The migratory ability of bats likely has particular relevance in the context of disease transmission. Furthermore, humans might share some ecological niches with bats through butchering or coal mining, as they are consumed as game meat and their excreta, such as feces, are often used in traditional Chinese medicine (44). The Chinese cultural belief that live slaughtered animals are more nutritious potentially enhances the risks of viral transmission (27).

BIOFILM AND VIRUSES

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

Biofilm is an ecological cluster of microorganisms, surface-attached or floating, with the evolutionary purpose of protection, nutrition, or strengthening survival. In biofilms, the microorganisms are surrounded by a complex of assembled extracellular matrix. This results in heterogeneous biomolecular and biochemical arrangements that hinder the entry of exogenous components while facilitating the exchange of genetic elements among microbial cells (48, 49). The consequences of pathogenic biofilm formation are calamitous for human health, water systems, food, and other industries, and consequently for the economy (50).

Reports in the literature about viruses in biofilms are recent, dating from the late 1990s. Specifically, enteroviruses have been detected from biofilms. In 1997, a study with the nonenveloped poliovirus 1, using an artificial water flow distribution system, sparked a discussion about virus persistence and biofilm. They showed a greater amount of viruses recovered from biofilms. Notably, those viruses seemed to be protected against the disinfectant chlorine, in contrast to those in water phase counterparts (51). Nonenveloped viruses belonging to the norovirus and enterovirus groups were also identified in biofilms from drinking water and wastewater samples, and infectious viruses could be released from these biofilms. In addition, virions from wastewater were characterized as being more persistent when associated with biofilm, remaining infectious for up to 30 days. These results warned of the risk of virus-containing biofilms that contribute to temporal dispersion of viral pollution in water environments (52–54). Additionally, biofilm-associated enteroviruses attached to pipeline walls are resistant to flow pressure and disinfectant and may represent 95% of the overall potential contaminant biomass in direct contact with the water (55). In this sense, the article entitled “Enteric virions and microbial biofilms—a secondary source of public health concern?” enquires about the presence of pathogenic virions within biofilms from distribution pipes and their neglected monitoring (56). It is important to mention the detection of SARS-CoV-2 RNA in wastewater (57). Although the isolation of the infectious virus from feces has been described, no cases of fecal-oral transmission have been reported (58).

Viruses, as well as other microorganisms, may come into contact with preexisting biofilms and accidentally adhere and become a part of them, thus constituting secondary colonizers. A study from 2002 supports that biofilms may encompass a set of non-enveloped enteric viruses, including caliciviruses, *Rotavirus* spp., *Astrovirus* spp., and hepatitis A virus, among other microorganisms such as Gram-negative bacteria and filamentous fungi (55). Some authors indicate that the human oral cavity may be an active site of infection and reservoir for SARS-CoV-2, assuming its interaction with the host oral microbiota (59), which is mostly in the form of biofilm. Importantly, both virions and virus-infected eukaryotic cells embedded in biofilms have been reported to retain their infectivity. A study investigated the enveloped virus herpes simplex virus 1 (HSV-1) and the nonenveloped virus coxsackievirus type B5 (CVB5) within the fungal *Candida albicans* biofilms (60). The authors recovered high virus titers from *in vitro* cultures of the virus-exposed biofilms after extensive washes, indicating that virions and viruses are deeply dispersed into the fungi-produced biofilm matrix, and thus protected. The authors related that biofilm reduced virus sensitivity to chemical inactivation and they discussed some mechanisms that may lead to virion inclusion into the fungal biofilm; for example, by sorption sites from the extracellular matrix, by the natural colloidal characteristic of viruses, or by biomolecules present on biological fluids. Since these viruses encompassed on biofilm kept their viability and infectivity, it indicates that biofilm lifestyle does not limit viral dissemination (60) and even can improve it. Therefore, viruses stored in biofilms may be regarded as temporary or long-term reservoirs in the environment (61). As pointed out by Vasickova and collaborators (62), the potential of viral spreading via contaminated surfaces depends particularly on the ability of the virus to maintain infectivity while it is in the environment, and they indicated some benefits for viruses within biofilms, such as protection against desiccation and antimicrobial agents (62).

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

A growing number of studies report that enveloped and nonenveloped viruses can spread in groups in so-called "collective infectious units" (65–67). The vehicles mediating collective spread vary widely and include lipid vesicles, protein matrices, diverse forms of aggregation, and binding to the surface of host or non-host cells (66). In accordance, Tozzi and coworkers (68) described the collective arrangements of multiple SARS-CoV-2 particles based on microscopic image analysis of viruses emerging from the surface of cultured cells. They suggest that when the virions exit the host cells, single particles tend at first to aggregate in spherical assemblies, then to develop interconnected network-like branches and nodes, which they called collective clustering dynamics (68). However, further investigations are needed to characterize the presence of the matrix for SARS-CoV-2, such as in the study developed by Thoulouze and colleagues (64) for HTLV-1. In this sense, the ability of betacoronaviruses to bind carbohydrates has been mapped due to their high saccharides or carbohydrate domains and glycosylation patterns (69–71). Likewise, we speculate that the affinity of CoV virions for the polysaccharides presented on the matrix of biofilms probably would play a role

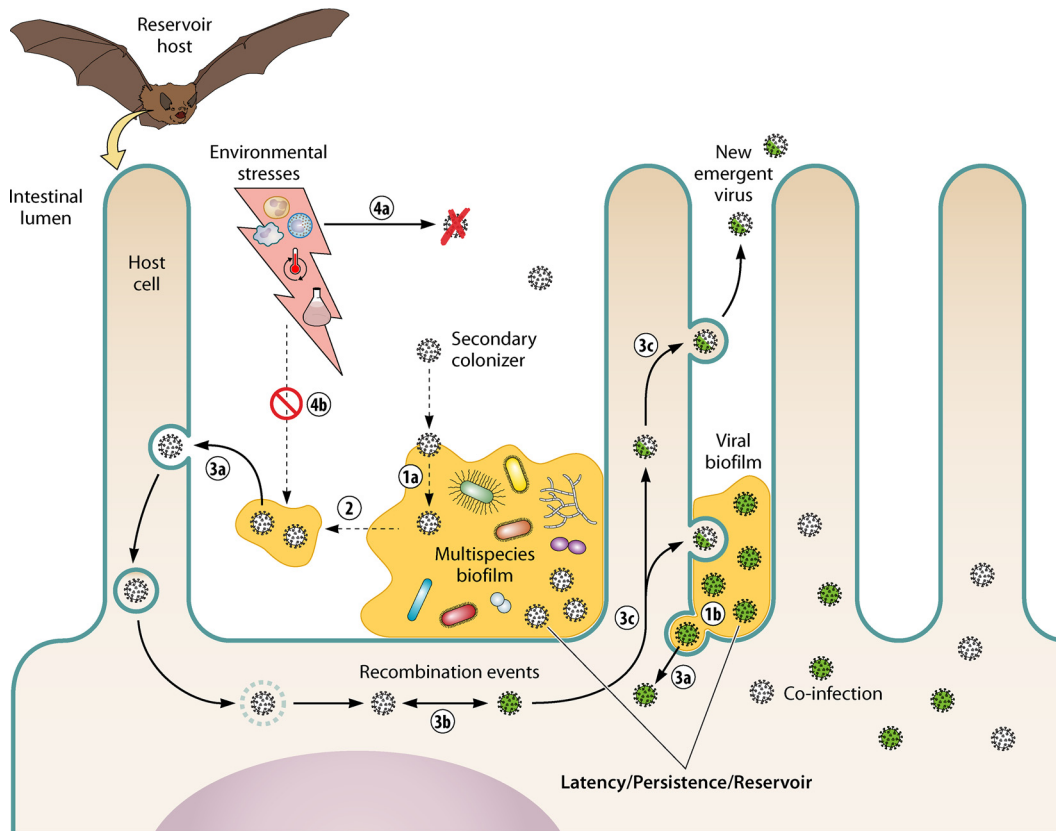


FIG 2 Biofilms and viruses. Viruses are shown in the extracellular space in planktonic or biofilm forms. (1a) Some viruses come into contact with preexisting biofilms (multispecies biofilm) and adhere to them, constituting secondary colonizers. (1b) Some viruses are enclosed in a single viral biofilm. Once these structures are established, they become reservoirs and could persist like a “state of latency,” keeping viral particles infective over time. (2) Biofilm detachment allows viruses to spread and can release virions that will colonize new sites and infect new host cells. (3a) A virion detached from multispecies or viral biofilms infecting the host cell. (3b) Viral gene exchanges and recombination events are possible in the presence of coinfections (exemplified by the green and blank viruses) and can lead to the emergence of new viruses (3c). (4) Environmental stresses such as chemicals, immune responses (immune modulation), and desiccation in the extracellular medium can inactivate/eliminate planktonic virions (4a), while virions in biofilm, protected by the matrix, are more tolerant to these agents and may remain infectious over time (4b).

in secondary colonization. Moreover, would this high saccharide profile be involved in the formation of viral biofilms?

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

Since viruses are strict intracellular parasites, we hypothesize that they will be unable to proliferate in biofilms, but they would persist as infectious agents in a reservoir host due to the advantages conferred by the biofilm structure. Furthermore, the maintenance of viral particles inside biofilms facilitates the coinfection process due to physical proximity. When viruses spread collectively, they should locally increase the number of viral genomes that initiate a cell infection, which may contribute to increasing infectivity and may favor coinfections (66, 67). To coinfect the same cell is beneficial for microorganisms since it may provide diverse genetic materials, favoring mutations and recombinations, biological diversity, and drug resistance (72) (Fig. 2, step 3b). Subsequently, we expect that CoV-containing biofilms may also act as a viral environmental reservoir, including under fomites, and contribute to viral persistence and dissemination (61).

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

CAN MICROBIAL BIOFILMS FAVOR VIRAL RESERVOIRS IN BATS?

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

In 2016, Plowright and colleagues described three hypotheses that dominate the current research on emerging bat infections. They pointed out that pulses of viral excretion could be generated by episodic shedding from persistently infected bats through a combination of physiological and ecological factors. In this scenario, the viral acute infection is resolved without clearance of viruses, which allows pulses of transmission to be triggered by viral reactivation. Moreover, the carriage of the pathogen is stable in space and time, even if viral shedding is episodic (78). For example, enveloped *Lyssavirus* spp. and *Flavivirus* spp. have persistently infected bats and shed viruses for extensive periods without evidence of disease (77). In 2017, Jeong and coworkers demonstrated that persistent infections support the maintenance of CoVs in a bat population. It was assumed that temporally synchronized stressors, such as pregnancy or poor nutrition, may weaken the immune system of bats and thus facilitate viral reactivation, the pulses of viral shedding, and the spillover of bat-borne viruses (79). Several hurdles, including the necessity of biosecurity level 4 conditions, the difficulty in reproducing natural infections in bat through experimental infections, and in isolating infectious virus from bats, have constrained the understanding of emerging bat viruses (80). Therefore, the mechanisms, including physiological and ecological factors, which drive infectious disease dynamics in bats, such as viral persistence, clearance, and transmission without apparent disease, remain poorly characterized.

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

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

Biofilms are recognized as an important means to maintain microbial cells or particles in metabolic latency while they are protected by an external matrix. In this sense, as pointed out by Thoulouze and Alcover (64), viral biofilms could represent “viral communities” with enhanced infectious capacity and improved spread compared with “free” viral particles. This might constitute a key reservoir for chronic infections. Additionally, as discussed by Dawley and Gibson (81), specific associations of viruses with bacteria or other microorganisms are likely to allow the easier entry of virions into the biofilm, resulting in a reservoir of viruses. These virions are as difficult to remove and inactivate as their bacterial counterparts, turning infections persistent for a long time (81).

(ii) If bats do not clear infections and viruses may persist within bat hosts, it is assumed that bat hosts may tolerate viruses. In this case, a tempered inflammatory response would

minimize immunopathology and viral replication may fluctuate as a function of the immunological competence of the host (78).

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

(iii) Genomic recombination events have been shown for several viruses, including CoVs, that are harbored by bats (23). Compared to other RNA viruses, the expanded genome size of CoVs facilitates the acquisition of genes encoding accessory proteins that are beneficial for CoV adaptation to a specific host and, as a result, genome changes caused by recombination, gene interchange, and insertion or deletion are common among CoVs (27). This genetic plasticity is important to interspecies persistence and survival mechanisms (84).

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

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

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

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

DO BIOFILMS MEDIATE CORONAVIRUS EXTRACELLULAR VIABILITY?

Virus dissemination closely depends on host and environmental interactions. SARS-CoV and SARS-CoV-2 transmission to humans can occur with both direct (airborne and droplets) and indirect contact (contaminated surfaces) between susceptible and infected individuals (87, 88). For this, viruses must be shed into the environment and contaminate biotic (mucosa) or abiotic (nonliving structures like fomites) surfaces. Notably, they must be capable of remaining infectious for at least some time (Fig. 3).

Reviews of the literature have addressed different types of virus collective spread. They highlighted the advantages of viral dissemination through “complex multivirion structures,” such as viral biofilms and viruses as secondary colonizers, in comparison to free individual virions (65, 66). In this context, we point out two leading biofilm features that could directly or indirectly afford and maintain virus viability through some intrinsic abilities.

(i) Biofilm may act as viral storage, where virions remain infectious within the hosts (humans and wild, livestock, or domestic animals) and in the environment (food, water, excreta, plants, pastures, and fomites).

(ii) Biofilm may support virion persistence through matrix physicochemical protection against stresses, such as desiccation in the extracellular medium, and against immune

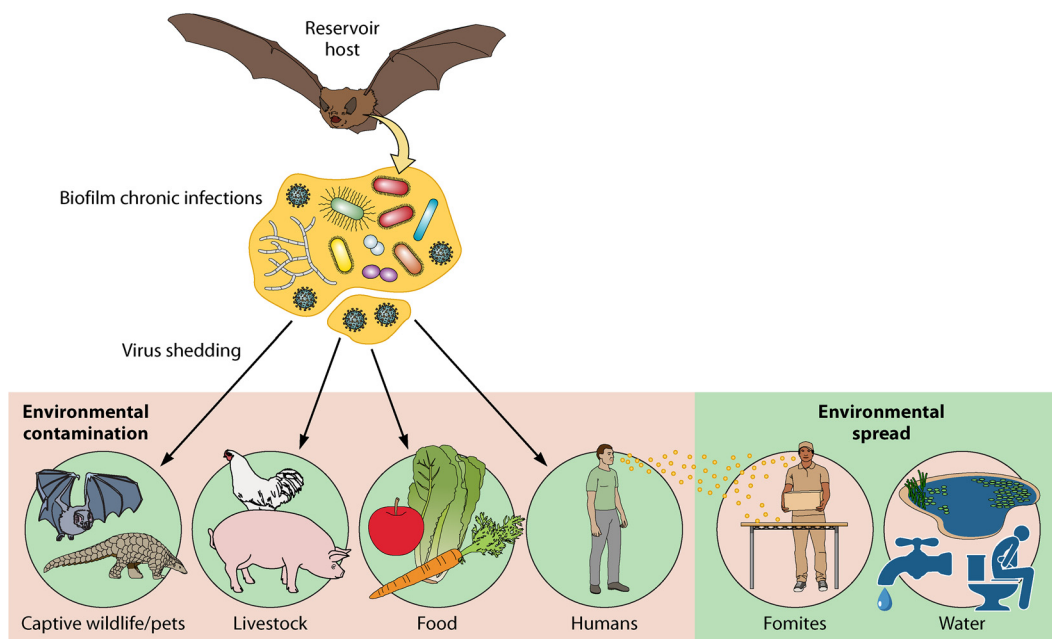


FIG 3 Proposed role of biofilms in host-CoV-environmental interactions and viral dissemination. Bats are the reservoir hosts persistently infected by several viruses, potentially in the form of biofilm chronic infections (multispecies and viral biofilms are depicted in enlarged view). Viruses are shed from the host into the environment in biofilms present on excretions. The environmental viral spread can contaminate organisms (e.g., humans, wildlife, livestock, and pet animals, plants, and fruits) or abiotic components (e.g., fomites and water). Viral transmission is possible via direct (airborne and droplets) or indirect (contaminated surfaces) contact between susceptible and infected individuals/hosts.

responses (immunological modulation) and also through resistance to chemical substances, keeping viral particles infective over time.

Surfaces can serve as a vehicle for virus dissemination because they can be contaminated by direct contact with the animal or human excretions, fluids, and through transfer by mechanical vectors (Fig. 3) (87). The degree to which environmental/surface-based transmission of SARS-CoV-2 is actually occurring is still unclear. Considering that CoVs are enveloped viruses, there is expected to be a structural fragility when facing environmental conditions. However, scientists have investigated the capacity of CoVs to survive on several surfaces (plastics, latex, metals, and glasses) and the results show they remain infectious over long periods (hours to days for CoVs, while weeks to months for others) (89). In this sense, SARS-CoV, SARS-CoV-2, and MERS-CoV would be infectious in environmental reservoirs like water, foods, and in sewage for sufficient time to facilitate onward transmission. The viability and persistence of CoVs depend on several variables and the exact mechanisms are still unknown (90, 91). Although these studies consider some characteristics of the surface substrate (e.g., porous versus nonporous), the biofilm condition has not been contemplated until now (87, 91–93). It was shown that SARS-CoV and MERS-CoV could survive for extended periods when suspended in human secretions, while the influenza virus could survive longer in mucus (91, 94). These observations contribute to the biofilm theory, since organic materials are highly hydrated and rich in macromolecules, which in turn can act as the extracellular matrix.

SUMMARIZING REMARKS

The scientific literature allows us to propose that biofilms may play a role in pathogenicity modulation of CoVs, in their ability to persist in reservoir hosts and in the environment, and in their transmissibility. Assuming that CoVs can also occur in biofilms, new perspectives could be provided for important questions about (i) the absent or low pathogenicity of CoVs within reservoir hosts, like bats; (ii) a more depressed level of immune system stimulation; (iii) viral persistence and a chronic infection profile in reservoir hosts, like bats; (iv) the viral genetic plasticity and emergence of new viral entities; (v) the viral environmental spreading

through excretions; (vi) the persistence of the viruses outside host cells; and (vii) indirect cross-species transmissibility by handling and consuming contaminated products. The SARS-CoV-2 pandemic has exposed the enormous inequalities of our societies and how these negatively impact our lives. Given the public health and economic gravity of this pandemic, SARS-CoV-2 is currently the hot topic and significant financial investment has been designated by several countries. These investments support basic and applied research to understand the biology, pathogenesis, origin, and dynamics of the virus and to accelerate the development of drugs and vaccines. We strongly feel this is a significant interdisciplinary contribution connecting microbiology, ecology, and public health. It will aid the advancement of alternative approaches for the control of these pathogens and the management of the current and potential future crises.

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We declare no competing interests.

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Rafael Gomes Von Borowski is a pharmacist and Ph.D. in Pharmaceutical Sciences (Federal University of Rio Grande do Sul, Brazil) and Microbiology (University of Rennes 1, France). His doctoral thesis received honorable mention and a Rennes 1 Foundation Theses Award. After an experience in a public hospital in the south of Brazil, he has been working in the Research and Development departments of public and private laboratories. He is particularly interested in new strategies and natural antimicrobials to control infectious disease. Currently, he is responsible for the development of a diagnostic test at a pharmaceutical biotechnology company specializing in human phage therapy.

Danielle Silva Trentin is a pharmacist and holds an M.Sc. (2009) and Ph.D. (2013) in Pharmaceutical Sciences from the Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil. Her thesis received honorable mention via the Brazilian national award "CAPES Theses Award," field of Pharmacy. From 2013 to 2016, she completed postdoctoral fellowships at UFRGS. Since 2017, she has held the adjunct professor position at the Department of Basic Health Sciences at the Federal University of Health Sciences of Porto Alegre (Porto Alegre, Brazil), in Microbiology. She was awarded grant funding by the Serrapilheira Institute and is leader of the research group "Bacteriology and Alternative Experimental Models." Her research interests are focused on microbial virulence factors, including biofilms, as an approach to control infectious disease. Currently, she is working with *Galleria mellonella* larvae as an experimental host to evaluate *in vivo* virulence-attenuating compounds and as an animal model to study plastic biodegradation.