The intestinal virome: lessons from animal models

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Short title: The virome of animal models

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Abstract:

Mucosal surfaces in contact with the environment host specific microbiota. The intestinal tract harbours the most abundant and diverse bacterial and viral populations interacting with each other as well as with the host. Viruses of the microbiota are important components of this ecosystem, as shown by viral alterations associated with various pathologies. However, practical and ethical constraints limit functional studies of the virome in humans, making animal models invaluable experimental tools to understand its impact on intestinal physiology. In this review, we present the recent advances in the study of virome in animal models. We focus on the strategies used to characterise viral changes in disease models and approaches to modulate the microbiota using viruses. In reviewing the interplay between viruses, bacteria, and the animal host, we highlight the potential and limitations of these models in elucidating the role of the virome in determining human health and disease.

Introduction

Several organs and mucosal membranes of animals are colonised by symbiotic microbes, known as microbiota, which include bacteria, archaea, fungi, protozoa and viruses [1]. Viruses of the microbiota are highly diverse and infect either eukaryotic or prokaryotic cells as obligate parasites. Although viruses have mostly been studied as pathogens, they can establish a mutualistic symbiosis with infected cells. This is well illustrated with bacteriophages (phages), viruses that infect bacteria, the most abundant viruses in the microbiota. Their life cycle is often either virulent, leading to phage replication and bacterial death, or temperate, conferring a possible fitness advantage, acting as mobile genetic elements.

Differently from cellular organisms, the genetic information of viruses is stored in DNA or RNA, single or double-stranded, and lacks common genetic markers, considerably limiting their study. Shotgun metagenomics partially overcomes these limitations by being able to access genomic information of different nature and is currently the gold-standard approach to study many microbial environments [2]. Although virome analysis is still challenging, bioinformatics tools are rapidly improving. Indeed, several studies have associated variations in virome composition with human disease, raising questions about their underlying mechanisms. Most have focused on intestinal phages and their overall impact on the bacteriome and gut physiology [3], whereas a few have also examined eukaryotic viruses, which appear to play similar roles as bacteria, affecting the development of host physiology and immunity [4].

Pre-clinical approaches using animal models present several advantages (ethics, costs, and reproducibility) in preliminary investigations on the role of the virome in host physiology and disease. In this review, we present and discuss the most recent advances in the characterisation of the virome in animal models, the factors involved in its variation, its

mechanisms of interaction with the host, and the possible clinical and therapeutic consequences, with a focus on phages.

The intestinal virome of laboratory mice

The use of mice as robust models of virome-associated disorders requires that the baseline viral composition is known and comparable to that observed in clinical studies. Professional providers of laboratory mice raise them under specific pathogen-free (SPF) conditions, which implies that they are only screened for the absence of certain eukaryotic viruses. A major difference in the virome of laboratory mice compared to wild ones is indeed the much lower abundance of eukaryotic viruses, a factor that should be taken into account when discussing the outcome of functional microbiome studies [5].

To date, no facilities control for the presence of phages, as they are *generally recognised as safe* (GRAS) for animals, including humans. The composition of intestinal phages reflects the composition of the intestinal bacteria, which varies between animal facilities and mouse lines. Recent studies have consistently showed that most phages in SPF mice belong to the *Microviridae* family (from 1-10 to 60-85%, depending on the mouse supplier) and Caudovirales order (1-10 to 80-99%), whereas a minority are still unknown or unclassified (1-10 to 25%) [5–7].

These results are coherent with observations in humans, in whom phages, *Microviridae* and Caudovirales, are the major viral component of the intestinal microbiota relative to eukaryotic viruses, as reviewed extensively elsewhere [8,9], further justifying the use of mice in pre-clinical research. Also, certain phage taxa are consistently found in mice from the same vendor, suggesting the existence of a core virome specific to breeding facilities [6].

However, the lack of common standards in virome extraction, sequencing and analysis makes it hard to compare the high variability in composition observed in these studies. Sources of differences include the nature of the starting material (intestinal content [6], faeces [7] or caecal content[5]), the filtration method, the nucleic acid extraction (DNA and RNA viruses [5] or DNA viruses only [6,7]) the sequencing technology (Illumina [5,6] or Ion Torrent [7]). Increasing cycles of DNA amplification during the preparation of Illumina sequencing libraries, was also shown to enrich single-stranded viral DNA, which is consistent with the variability found in the proportion of *Microviridae* [2].

Overall, the impact of the virome on most animal studies is still unclear. It is possible that experimental reproducibility within and between laboratories could be perturbed by differences in the composition of the virome, as already shown for the bacterial microbiota [10,11]. Exploring virome diversity in these studies would have the double function of improving experimental practice and our understanding of the impact of viruses on host physiology.

The influence of diet on the animal virome

The diet is a source of microbiome variability [12] and a major parameter of several widely used models, such as those employing high-fat (HFD) and low-fat diets (LFD) to study diseases such as obesity or type-2 diabetes. Thus, the effect of diet on the virome is of particular interest.

After dietary intervention with a HFD or LFD, a study of six mice showed no significant differences in the composition of the core faecal virome [6] but a significant change in the alpha diversity (box 1) of phage populations. The bacteria, however, did not vary in alpha diversity in caecal samples after 13 weeks, although the different taxonomic level of analysis

(strains vs genera) prevents a direct comparison between the viral and the bacterial component. [6]. However, Schulfer *et al*. obtained different results for the faeces of 24 mice, 16 weeks after transitioning to a HFD, showing a non-significant increase in alpha diversity of the viral community but a significant reduction in that of the bacterial population [7]. In terms of beta diversity (box 1), there was a significant shift after transitioning to a HFD from a standard diet (SD) in both the bacterial and viral populations in both studies [6,7]. A drop in the ratio of temperate to virulent phages was also observed in mice transitioning to a HFD [7]. The results of these studies are consistent concerning HFD-related changes in the relative abundance of phage, showing an increase in *Microviridae* [6,7] and a decrease in Caudovirales, especially *Siphoviridae*, relative to a LFD [7].

The virome of mice is not only affected by diet but is also altered in several pathological conditions. Moreover, in certain diseases, such as diabetes, the microbiota is a key factor in the development of the pathology [13]. Although current evidence suggests a connection between the microbiota and disease, the role of the virome in this context is still unknown.

Models of disease and their impact on the virome

Mice are often used as a proxy for human diseases to decipher their pathophysiology or test new therapeutics. Virome diversity correlates with several pathological conditions, such as inflammatory bowel disease [14], arthritis [15], and even child growth impairment [16].

In a mouse model, neurotoxic chemicals were administered to induce symptoms of Gulf War illness, a chronic multisystemic disorder characterised by inflammatory bowel disease and neuroinflammation, among other conditions. The authors observed significant changes in the richness and composition of the virome associated with neuroinflammation driven by a decrease in the levels of the proteins which ensure epithelial cell-cell junctions in the gut and

blood brain barrier. Additional antibiotic treatment did not significantly affect these differences but a wide-spectrum anti-viral agent partially reversed them to a level similar to that of the healthy control group [17]. Overall, these results suggest a role of the virome in neuroinflammation and thus in the "gut-brain" axis [17,18].

In another mouse model, Cao *et al*. examined the effect of vaccination with the full-length spike protein on the virome of mice infected with SARS-CoV2. Metagenomics analysis showed a significant increase in alpha diversity in the virome of vaccinated mice relative to controls, without evidence of viral infection in the gastrointestinal tract, suggesting either an undetectable infection or modulation of the immune landscape that altered the gut microbiome [19].

Given the known involvement of the virome in the physiopathology of diseases associated with intestinal dysbiosis, the use of viruses to re-establish the microbial equilibrium has been proposed. Strategies of faecal viral transfer have thus been developed in animal models.

Modifying the microbiota of animal models in virome studies

Faecal microbiota transplantation (FMT) and faecal viral transfer (FVT) (Figure 1) are used to modify the gut microbiota of animal models, either to correct dysbiosis or to study the resulting specific perturbations.

A HFD induces glucose intolerance and weight gain in mice [20]. These symptoms are significantly reduced after FVT from LFD mice. However, depleting gut bacteria with antibiotics neutralises the efficacy of FVT treatment, suggesting a crucial role for the gut bacteria of the recipient in the success of FVT in controlling the symptoms of diabetes [20].

Mice fed a standard diet (SD) and treated by FMT or FVT from mice fed a HFD for 30 days had a significantly different bacterial composition in the small intestine than untreated mice and a beta diversity of bacteria comparable to that of mice fed a HFD. This shows that FMT and FVT can specifically shift the gut microbiota towards the donor composition with similar efficacy [21].

Thus, the effect of FVT depends on both the recipient and the donor [21], thus the choice of donors is of utmost importance [22]. As mentioned previously, the composition of the murine virome is highly variable depending on environmental factors, such as the vendors or diet. Differences in the transferred material (faecal of caecal) also lead to grafting different viral populations. To minimise these factors in animal studies, mixing caecal contents or faeces of mice from different origins has been proposed [7,20].

The FVT can also originate from the recipient itself. Autochthonous FVT was shown to restore the antibiotic-treated microbiota of mice to a closer composition to that of the pre-antibiotic status relative to heat-inactivated FVT and increased the ratio between the bacterial phyla *Bacteroidetes* and *Firmicutes*, which was lowered by the antibiotic treatment [23].

FVT can be an effective tool to modulate the microbiota. However, this raises the question of which viruses are necessary or sufficient for the therapeutic action of FVT.

Mechanisms underlying interactions with the virome

The virome, bacteriome, and animal host coexist. Animal models reunite all three actors of this tripartite interaction and allow its study in a controlled environment by separating the myriad of parameters affecting its development (Figure 2).

Virome-host interactions

The virome can affect the host by infecting it or by stimulating its immune system, either directly or *via* the bacteriome. Animal models offer the possibility to study the mechanisms behind such interactions. The advantages include the possibility of using genetic engineering to decipher the molecular mechanisms at play *in vivo* [24] and measuring gene expression of various host cells by RNA sequencing [19].

Phages can also interact directly with the mammalian host. Sweere *et al.* found that a temperate filamentous phage of *Pseudomonas aeruginosa* can impair the murine immune system in a wound-infection model by activating the TLR3 pathway and inhibiting the production of TNF and phagocytosis, thus facilitating infection by its bacterial host [24]. In addition, phages infecting the genera *Lactobacillus*, *Escherichia*, and *Bacteroides*, as well as the phage DNA, triggered a specific inflammatory response in dendritic cells by stimulating the INFγ response via TLR9 and exacerbated the inflammation in a DSS-induced colitis in wild-type but not $IFN\gamma^{-/-}$ or $TLR9^{-/-}$ mice [25]. A recent article demonstrated that a prophage integrated into *Enterococcus hirae* interacts with the class I major histocompatibility complex and enhances the efficacy of anti-tumour immune treatment in mice bearing this bacteria, confirming the hypothesis resulting from metagenomic studies in human patients with a differential response to treatment [26]. Overall, these studies show that the prokaryotic virome can directly interact with the host immune system. [24,26].

Virome-bacteria interactions

The interactions between phages and bacteria *in vivo* have already been reviewed [27–30]. Here, we will focus on a few examples that illustrate the value of animal models in these studies.

Phages can directly modulate the gut microbiota by infecting their bacterial hosts, as well as indirectly, by modulating the diversity of bacterial populations. The addition of four phages, specific to a set of bacteria, profoundly shaped the microbiota of a gnotobiotic mouse model composed of 10 bacterial strains (either phage-hosts or not) coupled with shifts in secreted bacterial metabolites [31].

The host can affect both the virome and the bacteriome. Lourenço *et al.* demonstrated that the spatial heterogeneity of the gut creates refuges that protect bacteria against phage infection in a gnotobiotic mouse model [32]. However, Green *et al.* [33] showed that specific phages can have a strong affinity for glycans, enhancing their effectiveness in the mucus layer. Thus, phage-bacteria interactions vary greatly between phage studies depending on the specificity of the phages, making it difficult to draw general conclusions.

De Sordi *et al.* characterised the phenomenon of “host jump” (change of host) occurring in the murine gut. In this study, the diversity of the microbiota favoured the selection of genetic modifications that allowed phage infection of an originally resistant host strain *via* adaptation to intermediate intestinal hosts. This study shows the importance of the complexity of the environment in phage-bacteria interactions that can be modelled in animals [34].

Conversely, bacteria can modulate the virome. Depletion of the bacterial community with antibiotics in a rhesus monkey model was shown to reduce the alpha diversity and altered the virome composition of both phages and eukaryotic viruses (box 2). The authors proposed a mechanism in which metabolites produced by the bacteria could shift, inhibit, or promote replication of the viruses [35].

Most of this work relies on reductionist approaches using defined couples of phages and bacteria that can be monitored within intestinal microbiota of different complexity. The necessity of simple model is partly driven by the fact that predicting the bacterial hosts of gut bacteriophages is a challenging task in natural communities. However, new advances in experimental and computational approaches show promise in tackling these limitations. As an example, meta3C, derived from the chromosome conformation capture method (3C), was used in mice microbiomes to assign putative bacterial hosts to specific phages using the physical proximity between their genomes [36].

Animal models offer the possibility to unravel the complex relationships in which each population influences the other two. They reunite all three actors, viruses, bacteria, and the host, and each can be specifically modified to aid in the deciphering of this tripartite association (Figure 2). Models with a controlled microbiota, also called gnotobiotic models, facilitate the study of the virome using a simpler, characterised microbial environment.

Non mouse models

Although mice are the most widely used laboratory animals, other mammals are also suitable for the study of the intestinal microbiota. Macaques are genetically closely related to humans and are therefore a pertinent model to study parameters that influence the human virome. Cynomolgus macaques have recently been used to analyse the effect of aging on the gut virome [37]. Aging is already known to have an effect on the bacteriome [38], however, the consequences of aging on the composition of the virome are mostly unknown. The macaque virome has been found to be dominated by phages and alpha diversity is not significantly altered with aging. However, the viral species change with age, which was also shown to be the case for the KEGG pathways present in the virome, suggesting a role for the virome in

regulating metabolism [37]. The bacteriome and virome thus appear to be linked and to evolve together as the animals age.

Zhao *et al.* characterised the enteric virome of rhesus macaques with chronic diarrhoea [39]. They found the virome to be dominated by phages of the Caudovirales order and *Microviridae* families, much like the intestinal virome of mice and humans, as also confirmed in a second study [35]. They also used biogeographical analysis, to find that the rectal virome is similar to the one of the colon but distinct to the virome of the ileum. A recent study also confirmed the distinction in virome composition between the large and the small intestine in rhesus monkey and pigs with a higher phage diversity in the lumen and predominance of eukaryotic viruses predominate in mucosal samples [40]. Recently, the faecal, oral, blood, and skin virome of laboratory rabbits were described leading to the identification of a novel polyomavirus [41]. Finally, swine models have also been used for virome studies. For example, FVT was shown to protect preterm pigs against necrotising enterocolitis [42].

Overall, these results show that different animals have a similar virome structure, with the presence of key eukaryotic viruses and quantitative domination by phages. However, further and more detailed characterisation of the components is required for a better understanding their differences and roles.

Conclusions

The virome has been increasingly linked to health and disease. Although animal models have been spearheading new discoveries in this field, the study of their virome is still in its infancy. As for humans, phages are the most abundant component of the animal virome and new mechanisms of their physiological roles remain to be characterised in relation to their direct effect on the host and indirect effect as a modulator of bacterial populations. Further studies,

taking advantage of the flexibility of animal models, are required to investigate the interactions of the virome with both other elements of the microbiome and the host. In addition, future research needs to tackle the issue of determining the active actors in the microbiota in physiology and pathophysiology. The challenge lies in dissociating the actions of viruses from those of bacteria and between individual viruses, with the objective of identifying key players in human health.

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Boxes and Figure legends

Box 1: Definitions of viral diversities from virome data

In ecology, **diversity** reflects the relative abundance of different species while the **richness** represents the number of different species. Within a specific ecosystem, the diversity is defined as **alpha diversity**, whereas **beta diversity** measures differences in diversity between two or more ecosystems. For the microbiota, the local ecosystem is the intestinal tract of a specific individual, therefore the alpha diversity represents the intra-individual diversity while the beta diversity represents the inter-individual diversity.

Box 2: Roles of the eukaryotic virome in animal models

SPF mice have a low abundance of eukaryotic viruses. When detected, the most represented are DNA viruses of families *Phycodnaviridae* and *Mimiviridae* [7,43] or the RNA viruses of the *Astroviridae* family [5]. However, viral annotation might be a limiting step in these analysis

since two studies detected high amounts of giant *Mimiviridae* (with capsid of approximately 0.7µm in diameter) that should have been excluded by filtration at 0.45 and 0.22µm [7].

Like for phages, the proportions of intestinal eukaryotic viruses are impacted by the diet, as shown by HFD-fed mice showing significant relative increase in the most abundant intestinal families [7]. Eukaryotic viruses also do interact with the immune system of the host. Dallari *et al*. systematically assessed the immune response to six viruses belonging to different enteric families. The authors found that these viruses have an immunomodulatory properties independent of their capacity to induce gastroenteritis [44]. In another study, an intestinal murine astrovirus could confer protection from norovirus and rotavirus infections by complementing the animal immunodeficiency via the stimulation of IFN-λ-mediated signalling in epithelial cells [45]. Here, the authors identify the active viral component by metagenomic comparison of active and inactive FVT in transferring protection to viral pathogens. Viral immunomodulation can also alter the physiopathology of bacterial infections. Both murine norovirus and astrovirus were shown to confer protection from to pathogenic *E. coli* in the gastrointestinal tract [46,47]. Conversely, mice treated with antibiotics are less susceptible to poliovirus infection, suggesting a facilitating role of bacteria in the infection process [48]. Eukaryotic viruses are less abundant than phages in the microbiota, but their influence on the host and its immune system is major. Whether a connection exists between the immune response elicited by eukaryotic viruses and the diversity of phage populations is still unexplored territory.

Figure 1: Faecal Microbial and Viral Transplantation.

Faecal Microbial Transplantation (FMT) is the procedure used to transfer the faecal microbiota of an individual to himself or to another individual. It is used to correct dysbiosis and has proven to be effective to treat recurrent infections from *Clostridioides difficile* [49]. Faecal Viral Transplantation (FVT) is a similar approach that includes an additional filtration step during the preparatory procedure. This method is aimed at eliminating intestinal bacteria while keeping only viruses and small molecules. It is sometimes also referred to as sterile Faecal Filtrate Transfer (FFT). FVT can be exposed to UV treatment or heat shock to inactivate viruses and determine if the effect of the FVT is due to viral activity or other small metabolites. Conversely, the effect of small molecules can be eliminated by a supplementary filtration step that will retain viral particles while excluding other small compounds. FVT has shown similar efficacy as FMT in treating *C. difficile* infection in a small clinical assay with 5 patients [50] while alleviating potential secondary effects such as the implantation of pathogenic bacteria. FVT is used in animal models to decipher the importance of the virome in the mechanisms governing physiology and pathology, as described in section “Modifying the microbiota of animal models in virome studies”.

Figure 2: Virome, bacteriome, host: three actors and a myriad of interactions

Schematic representation of the interactions between the viral, the bacterial compartments and the host. Most of the studies on the microbiota have focused on the bacteria-host interactions as reviewed by Ahern and Maloy [51] (dashed arrow). Viruses (blue arrows) have a strong connection to bacteria (green arrows): both populations regulate each other, directly (e.g., by phage predation or mutualism) or indirectly (*via* the immune system of the host). In turn, the virome can shift the metabolome by modulating bacterial populations and indirectly

impacting the barrier and immunity (production cytokine is shown as coloured circles) of the host [31]. Antibiotic treatments can alter the alpha diversity of eukaryotic viruses, demonstrating that bacteria can also affect the diversity of eukaryotic viruses. The host (orange arrows), is interacting with eukaryotic and prokaryotic viruses that modulate its immune landscape and the epithelial barrier. The host offers a particular environment for phage-bacteria interactions and modulates their populations by secreting small regulatory molecules or by its spatial heterogeneity affecting their interactions. All displayed connections were drawn from studies in animal models, as described in this review.

Figure1

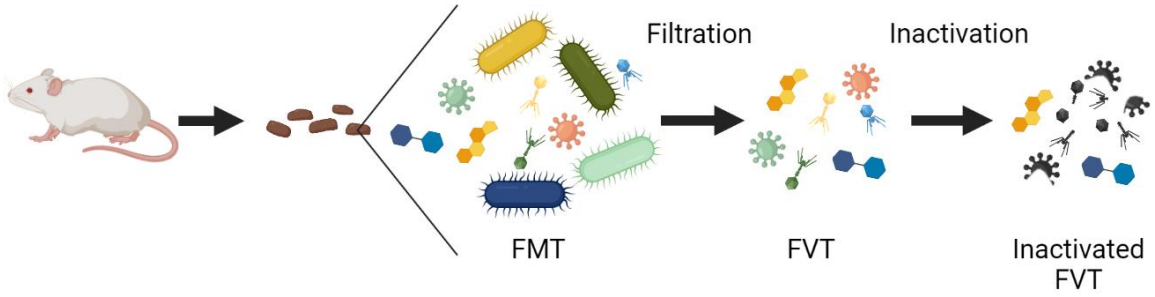

Figure 2