Beyond the Gut Bacterial Microbiota: The Gut Virome

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The gastrointestinal tract is colonized with a highly different population of bacterial, viral, ad fungal species; viruses are reported to be dominant. The composition of gut virome is closely related to dietary habits and surrounding environment. Host and their intestinal microbes live in a dynamic equilibrium and viruses stimulate a low degree of immune responses without causing symptoms (host tolerance). However, intestinal phages could lead to a rupture of eubiosis and may contribute to the shift from health to disease in humans and animals. Viral nucleic acids and other products of lysis of bacteria serve as pathogen-associated molecular patterns (PAMPs) and could trigger specific inflammatory modulations. At the same time, phages could elicit innate antiviral immune responses. Toll-like receptors (TLRs) operated as innate antiviral immune sensors and their activation triggers signaling cascades that lead to inflammatory response. *J. Med. Virol. 88: 1467–1472, 2016.* © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

The aim of this review is to focus on the recent knowledge on human gut virome, the method to analyze it, its composition, and its role in the relationship with the human host and its implications in human diseases.

The research of literature was made through Medscape and Pubmed bibliographic database, using key words like "*gut virome, tools for virome detection, virome in health and diseases, virome and immune system,*" including reviews of the literature, abstracts in conferences and workshop, and original papers published from 1990 to 2015.

Viruses inhabit all surfaces of the human body and most of them are bacteriophages [Pride et al., 2012].

Many viral genotypes colonize body surfaces including skin, oral and gastrointestinal tracts, airways, and the bloodstream, that was previously considered a sterile environment.

The human viromes have high levels of genetic and interpersonal diversity that now can be showed by the increased detail afforded by deep sequencing methods. Table I showed summary of viral species detected at various body site.

Generally, there is a lack of correlation between the presence of viruses and pathologic condition, but in patients with chronic diseases they may have a role in the persistence of inflammatory status [Focà et al., 2015].

The collection of microorganism that grows and lives in cooperation in the human gut is the so-called "gut microbiota" [Sekirov et al., 2010]. Beyond bacterial species, viruses, mycetes, and other members of the meiofauna are known as components of this complex system. In fact, human feces contain at least 10^9 virus-like particles (VLPs) per gram. A great part of these have been identified as bacteriophages, but are also included viruses infecting eukaryotic cells, archaea infecting viruses, prophages, endogenous retroviruses, and other endogenous viral elements [Virgin et al., 2009]. Analysis of samples taken from different human individuals shows mostly novel viruses, and only a little part of viral ORFs (Open Reading Frames) matches the ones recognized from previous studies [Minot et al., 2013a].

The gut virome is object of study since 2003 when Breitbart et al. [2003] identified viral sequences from a fecal sample obtained from a single healthy adult. There were 1,200 different virotypes mainly

TABLE I. Virome at Human Body Sites

Body site	Viruses detectables	Possible interaction with host
Oral cavity	Bacteriophages, Herpesviruses	Stable components of the oral ecosystem
Skin	Papillomaviridae (b- and g- papillomaviruses), Polyomaviridae, and Circoviridae	Resident and transient viruses
Airways	More than 175 distinct viral genotypes, almost uncharacterized	Involved in chronic pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis (CF)
Bloodstream	Anelloviruses, Enterovirus/ Rhinoviruses, and Roseoloviruses	Anelloviruses are considered non-pathogenic; Enterovirus/ Rhinoviruses and Roseoloviruses are detected in febrile illnesses in children
Urine	Human Papilloma Virus, $(10)^7$ viral like particles	Could be involved in urological diseases (UTIs)

belonging to the Siphoviridae family. Siphoviridae and temperate phages have subsequently been reported to be the most abundant identifiable viruses in other sampled fecal viromes, followed by members of the family Podoviridae [Reyes et al., 2010].

At the contrary of bacteria, whose conserved bacterial ribosomal genes sequencing are easy to identify, phages do not have a small subunit rRNA and there is no conserved protein or gene to allow a similar characterization. With the newly implemented nextgen sequencing and the ability of better purify virus-like-particles (VLPs) is now possible to define in a relative easy way viral populations [Reyes et al., 2012].

Human fecal samples that have been analyzed so far does not have more than 1,500 virotypes [Reyes et al., 2010]. The size of mammalian virome is not yet known: we should imagine this compartment like an iceberg, where the tip is the 1% explored part at sequence level [Mokili et al., 2012] but the vast majority remains below the surface of the water.

A major difference between the bacterial microbiome and the virome is persistence at various levels (e.g., vascular cells, reticuloendothelial cells) of viral genome, latently integrated into host chromosomes, or as episomes [Foxman and Iwasaki, 2011]. This complex relationship between virome and the human host may have direct effects on health, directly conditioning the presence and replication of pathogens and the organism responses. An example is the ratio of virotypes to bacterial phylotypes that is approximately 10:1 [Kim et al., 2011]. Indeed, integration as a prophage into bacterial cells, provides a protection from superinfection "immunizing" the bacterial host against the same or a closely related phage. Moreover, genes encoded by the phage may expand the capability of bacterial host by enabling, for example, metabolism from new nutrient substrates, providing antibiotic resistance, conveying virulence factors, or influencing bacterial host gene expression [Reyes et al., 2012].

GUT VIROME COMPOSITION

Analysis of gut virome is performed by the characterization of nucleic acid of viruses in feces. Among DNA viruses have been recognized the so called Giant Viruses (>300 kb) (Mimiviridae, Mamaviridae, Marseilleviridae, Poxviridae, Iridoviridae, Ascoviridae, Phycodnaviridae, Asfaviridae) and the Bacteriophages. They act as bacterial "parasites," integrating with its genetic material (prophage state) and inducing other phage particle synthesis with bacterial cell lysis (lytic state) and are divided in single stranded DNA viruses (Microviridae and Inoviridae families) and double stranded DNA viruses (Myoviridae, Siphoviridae, Podoviridae, Tectiviridae, Leviviridae, Inoviridae) [Minot et al., 2013b; Scarpellini et al., 2015]. Bowel mucosal surface has high concentration of viruses and high viral-to-bacteria ratio in comparison with the adjacent environments such as the intestinal lumen and feces [Barr et al., 2013].

Eukaryotic RNA viruses detected in human small bowel and colon include Rotavirus, Astrovirus, Calicivirus, Norovirus, Hepatitis E virus, Coronavirus and Torovirus, Adenovirus (serotypes 40 and 41).

Gut virome composition reflects the evolution of the infant bacterial microbiota, perhaps remaining stable over time and highly personalized [Minot et al., 2011].

Indeed, the infant microbiome is highly dynamic and associated with early life changes in the composition of bacteria, viruses and bacteriophages with age. From birth to 2 years of age, the eukaryotic virome and the bacterial microbiome expanded, but this was accompanied by a contraction of and shift in the bacteriophage virome composition [Lim et al., 2015].

The composition of gut virome is closely related to dietary habits and surrounding environment. As found in a work by Minot et al. [2011], the gut virome is composed by a similar viral population in individuals who share similar dietary habits. In this prospective, food could be considered as common reservoir of viruses or a tool that carries a selective pressure conditioning the mouth colonization.

On the other hand, as appear in a recent study by Breitbart et al. [2008] on colonization of the infant gut in the first weeks of life, the overall viral community composition changed dramatically between 1 and 2 weeks of age and it did not depend on their diet (breast milk or formula). These data support the assumption that the environment is an important initial source of viruses and influence the future virome formation.

Is now well established that there is a persistent or an intermittent shedding of enteric viruses from healthy people. For example, is common the excretion of human enterovirus (HEV) [Witsø et al., 2006] and parechovirus (HPeV) [Kolehmainen et al., 2012] by children under 5 years without any evidence of associated disease. A 1 year longitudinal study performed by Kapusinszky et al. [2012] on samples stool taken at 1 week intervals each others of two healthy brothers demonstrated that viruses were continuously excreted in samples. The most frequently observed viruses were: anellovirus (Torqueteno viruses, TTV, but also TT-like mini viruses, TTMV), picobirnavirus, and HPeV types 1 and 6.

Bocavirus (HBoV-1), adenovirus groups C and F, Aichi virus, astroviruses, and rotavirus were less frequently detected. At the contrary of what is expected, other enteric viruses such as noroviruses, coronaviruses, cardioviruses, cosaviruses, saliviruses, and sapoviruses were not detected, although they are frequently found in stool. The results of this study only give a first indication of the composition of the gut virome, which would benefit from the study of larger samples [Kapusinszky et al., 2012].

In the gut virome, we can find not only fully adapted human viruses, but also the presence of some animal viruses transmitted by the ingestion of contaminated food, with an unclear relationship with human virome. Above all, HEV is a cause of acute hepatitis; genotypes 1 and 2 are human-specific but types 3 and 4 have a reservoir in pigs. Genotypes 1 and 2 are common in humane virome, genotypes 3 and 4, which very frequently infect humans from the animal reservoir, might be adapting to humans [Smith et al., 2013]. Differently from pigs, where the infection is silent, acute infection in humans is frequent and the HEV can be considered zoonotic virus. In this framework arise that the host immune status play a key role in the determination of clinical

features and in the virome composition [Lhomme et al., 2012]. The situation is even less clear for gyroviruses. Within the Circoviridae family, only the chicken anemia virus (CAV) was previously known as a member of this genus until recently. The first gyrovirus in humans (HGyV) was initially found by NGS at the surface of the skin of healthy people [Sauvage et al., 2011]. A very similar virus, AGV2, was found a few months later in chickens, and then a third gyrovirus was detected in human stool, in which CAV and HGyV/AGV2 were also identified [Phan et al., 2012]. A fourth gyrovirus, GyV4, was also found in China in chickens and in human stool [Chu et al., 2012]. Some reports reveal a high frequency of these four gyroviruses in human stool, possibly owing to cross-species transmission of gyroviruses and their replication in humans, or alternatively to passive transit of animal viruses via food intake, as has been observed for plant viruses [Zhang et al., 2006]. Within the Circoviridae family the cycloviruses constitute a new genus, and are found in the feces of humans and other animal species, posing similar questions as gyroviruses [Delwart and Li, 2012]. More recently plant viruses (such as pepper mild mottlevirus (PMMV), oat blue dwarf virus, and others) have been described. They can affect the intestinal bacterial quali-/quantitative composition and its functioning [Scarpellini et al., 2015].

GUT VIROME IN HEALTH AND DISEASE

In healthy population viruses living in eubiosis with their host and gut mucosa is characterized by frequent infections that could influence the host phenotype [Focà et al., 2015]. Phages are able to modulate ecological networks also by infecting bacteria.

However, intestinal phages could lead to a rupture of eubiosis and may contribute to the shift from health to disease in humans and animals [Focà et al., 2015]. De Paepe et al. [2014] have proposed four major models which express how commensal bacteriophages could contribute to the transition from health to disease (see Table II).

On the basis of the "kill the winner" model, the phages act as predators of overgrowed bacteria [De Paepe et al., 2014], although no evidence indicates that "kill-the-winner" dynamics occur in the human

TABLE II. Gut Virome: Viral Population and Models of Interaction With the Human Host

Model of interaction	Supposed mechanisms
Kill the winner	Phages act as predators of overgrowed bacteria
Biological weapon	Phages are used by commensal bacteria to kill bacterial competitor for the intestinal environment
Community shuffling	An host reaction induced phages to act negatively on their host
Emergence of new bacterial strains	Phages operate as reservoirs of genetic diversity, without killing bacteria

intestinal microbiota, whereby a peak in the bacterial (prey) population precedes the increase in bacteriophages (predator), which subsequently decreases bacterial (prey) populations [Lim et al., 2015].

Whereas, in the *"biological weapon"* scheme, the phages are used by commensal bacteria to kill bacterial competitor for the intestinal environment [De Paepe et al., 2014].

In the *Community shuffling* model, a host reaction, such as inflammation or drug use, induced phages to act negatively on their host. In this scenario the result is a global displacement of commensal bacterial population, resulting in intestinal dysbiosis. For example, the use of antibiotics could induce phages in several bacterial species such as *Escherichia coli* [Zhang et al., 2000], *Clostridium difficile* [Meessen-Pinard et al., 2012], *Enterococcus faecalis* [Matos et al., 2013], or *Staphylococcus aureus* [Goerke et al., 2006; Maiques et al., 2006]. An other example of stress is represented by cigarette smoke, which induce Lactobacillus prophages in vitro. In this scenario is possible to correlate smoke with higher risk to contract bacterial vaginosis [De Paepe et al., 2014].

In the *"emergence of new bacterial strains"* model, phages operate as reservoirs of genetic diversity, without killing bacteria [De Paepe et al., 2014]. For example *E. faecalis* is one of the prevalent member of the human intestinal microflora and high degree of variation among its genomes are registered in the mammalian intestine. Some of this genomic variation can be the expression of the introduction phage genes into the bacterial genome [Duerkop et al., 2012].

Similar to that observed for the association between lean andobese gut bacterial microbiota and their respective metabolic patterns, a viral–metabolic association is supposed according to the evidence that twin pairs and their mothers have phage and bacterial community similarities depending with the alimentary behavior.

Norman et al. [2015] registered changes in the enteric virome occur in both major forms of IBD, Crohn's disease, and ulcerative colitis. In patients with IBD, their virome shows a significant expansion of *Caudovirales* bacteriophages, then, one of the potential therapeutic approach could be the manipulation of enteric microbiota by fecal transplantation. However, early attempts have not proven successful.

In a recent published paper, Reyes et al. [2015] studied the interactions between the maturation of gut microbiota and its perturbation during severe acute malnutrition (SAM). Normal postnatal maturation of the gut microbiota is perturbed in SAM; children living in Malawi and in Bangladesh have gut microbiota with bacterial configurations that appear younger (more immature) than the microbiota of chronologically age-matched individuals with healthy growth phenotypes. The authors identified age-discriminatory viruses that define a growth model of phage and eukaryotic components of the gut

virome, which is delayed when subjects developed SAM. Such growth model may be useful for forecasting normal versus perturbed postnatal maturation of the gut virome component in populations at risk for malnutrition.

THE VIROME AND IMMUNE SYSTEM

Host and their intestinal microbes live in a dynamic equilibrium and viruses stimulate a low degree of immune responses without causing symptoms (host tolerance) [De Paepe et al., 2014].

Viral nucleic acids and other products of lysis of bacteria serve as pathogen-associated molecular patterns (PAMPs) and could trigger specific inflammatory modulations by stimulating low-level immune response, with potential consequences for host resistance to other infections and susceptibility of diseases [Virgin, 2014]. At the same time, phages could elicit innate antiviral immune responses. Toll-like receptors (TLRs) operated as innate antiviral immune sensors and their activation triggers signaling cascades that lead to inflammatory response. Bacteriophages have the immunogenic potential to generate antibodies in human infants and endotoxin-free bacteriophage particles are reported to stimulate production of interleukin-1b (IL-1b) and tumor necrosis factor alpha (TNF-a) by macrophages [Virgin, 2014].

On the other hand, immunodeficiency also may influence the composition of gut virome. Effectively, immunodeficiency occurring during lentivirus infection is associated with significant expansion of the enteric virome, such as in pathogenic simian immunodeficiency virus (SIV) [Handley et al., 2012]. The same author showed that the nature of the enteric virome might be a prognostic indicator of HIV progression, and might contribute to AIDS pathogenenesis [Handley et al., 2012].

TOOLS FOR VIROME DETECTION

The diffusion of next-generation sequencing (NGS) is now revolutionizing the field of genomics providing a significant contributions to multiples area including virus discovery, molecular epidemiology, studies of pathogenesis of infections and viruses-host immune system interactions.

Next-generation sequencing, also known as "second generation" or "deep sequencing," is a type of DNA sequencing technology that uses parallel sequencing of multiple small fragments of DNA to determine sequence.

In the last 10 years, an increasing number of NGS technologies have been available at the market, all using slightly different methodologies to achieve clonal amplification and sequencing [Quiñones-Mateu et al., 2014].

Many articles have described on detail and compared the different deep sequencing methodologies, with four dominating platforms: 454TM (454 Life Sciences/Roche, Branford, CT), Illumina1 (Illumina,

Inc., San Diego, CA), Ion TorrentTM (Ion Torrent/ Life Technologies, South San Francisco, CA), and PacBio1 (Pacific Biosciences, Menlo Park, CA). All these technologies allow to generate valuable sequencing information; however, there are significant difference about the types of diagnostic applications and the number of different tests to set up, the accuracy, sequencing protocol, throughput, speed, and costs [Quiñones-Mateu et al., 2014].

Despite all deep sequencing technologies continue to improve, 454TM and Illumina1 systems are the most widespread systems used in virology study. Identification of viral sequences is generally achieved by comparison of microbial sequences to reference genomes. BLAST and BLASTX [Altschul et al., 1990] are the main programs that analyse used data by ABI 3730 and Roche 454 pyrosequencer or for sequences assembled from shorter Illumina reads.

Identification of new viral pathogens is one of the most interesting application of NGS; it allow to identify new or unsuspected viruses, including commensal or non-pathogenic ones. NGS is also a good instrument to perform a metagenomics analysis of the human virome. These technologies allow to understand the composition of the human virome in health and disease and the complex interplay between viruses and their human host. The contribution of NGS to the analysis of virus–host interaction and pathogenesis includes RNA-sequencing analysis of viral gene expression and the analysis of viral and host transcriptome and epigenome.

Anyway, new methods are under investigation to better recognize viral population and the relationship with host response. Recently, Xu et al. [2015] pre-] presented a new method called VirScan.

This is a high-throughput method to comprehensively analyze antiviral antibodies using immunoprecipitation and massively parallel DNA sequencing of a bacteriophage library displaying proteome-wide peptides from all human viruses. They analyzed over 108 antibody-peptide interactions in 569 humans across four continents, nearly doubling the number of previously established viral epitopes. Indeed, they detected antibodies to an average of 10 viral species per person and 84 species in at least two individuals. Although rates of specific virus exposure were heterogeneous across populations, antibody responses targeted strongly conserved "public epitopes" for each virus, suggesting that they may elicit highly similar antibodies [Xu et al., 2015].

Recently, to overcome bias in viral population detection, Conceiçao-Neto purposed a method of analysis based on homogenization, centrifugation, filtration, chloroform treatment and random amplification on a mock-virome (containing nine highly diverse viruses) and a bacterial mock-community (containing four fecal bacterial species) using quantitative PCR and next-generation sequencing. This protocol, called NetoVIR (Novel enrichment technique of VIRomes) allows for a fast, reproducible and high throughput sample preparation for viral metagenomics studies, introducing minimal bias. This procedure is optimized mainly for fecal samples and increases the capability of removal of non-viral nucleic acids [Conceição-Neto et al., 2015].

CONCLUSION—FUTURE PERSPECTIVES

We are actually in the beginning of applied metagenomics era. The study of such a huge and complex system like the human virome is posing enormous challenges that could only be faced through the interaction between new acquisitions in DNA (and also RNA) deep sequencing and the implementation of a powerful biological database to effectively store and compare data about the viral component of microbiome. Particular attention is given to metagenomics to the extent that have been proposed metagenomics-modified Koch's postulates where the microorganism is replaced by a particular metagenomic trait. This particular approach is very useful when more than one viral agents are supposed to be involved in a disease.

Many diseases are actually defined as of "unknown origin" but in the near future a sequence analysis of everyone's viral metagenome could answer to open questions in obscures medical condition comparing data between ill patients and healthy people. The creation of a "human virome project" just like the human genome project could lay the basis in understanding not only diseases pathophysiology but also to know how viral populations that inhabits the human body could interfere with therapies and vice versa how therapies could change the viral ecosystem.

The possibility to modulate gut virome, making it able to affect intestinal bacteria and to impact on human metabolism, seems to be one of the most important targets. "Engineered" gut virome may act in preventing viral gastroenteritis through a competition for the gut microbiome ecosystem. The ability of bacteriophages in regulating the immune response and influencing the inflammatory response in IBD, is the basis for future phage-specific treatments. Another potential role for bacteriophages is their use against antibiotic resistance by their ability to downregulate the proliferation of pathogenic multiresistant bacteria and the introduction of genes against these pathogens.

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