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EVOLUTIONARY BIOLOGY OF THE VIROME AND IMPACTS IN HUMAN HEALTH AND DISEASE: SUMMARY OF THE 31ST OLD HERBORN UNIVERSITY SEMINAR AND THE STRUCTURED DISCUSSION

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In view of the relatively limited knowledge available about the functional characteristics of the human virome, the 31st Old Herborn University Seminar (OHUS) was planned to acquire a more comprehensive, broad based and interdisciplinary current understanding of the environmental virome. It was also hoped to explore its role in the human homeostatic processes and their biologic functional development during normal physiologic as well as pathologic disease states.

This seminar, based on 10 formal presentations followed by a comprehensive discussion, addressed several important aspects of environmental virome including, the virome of unicellular and multicellular organisms, and the nature of human virome and its impact on human health and disease.

Dr. Mark Riddle from the Uniformed Services University of Health Sciences, Bethesda, Maryland provided a brief overview of the meeting plans relative to the specific areas identified above. He discussed briefly the possible role played by environmental virome in the evolution of life, and in the synthetic aspects of biology, emerging infectious diseases, and the development of some of the relevant tools of modern biotechnology to examine these issues in further detail. He also discussed the framework of the virome proposed recently as an instrument to acquire additional knowledge about human viruses (*Parker*, 2016). This model suggests that virome functions in commensal, parasitic and or in mutualistic interactions with the specific cellular hosts, which may result in, no benefits, deleterious effects, or mutually beneficial effects to the virome as well as the host.

Professor Patrick Forterre from the Institute Pasteur in Paris, France provided a provocative and elegant introduction to the controversies surrounding the definition of the virus, and the development of the environmental virome in unicellular organisms. He started with the complex question of "what is life" and how recent technological advances have challenged the definition of viruses and of life itself. Prof. Forterre reviewed the existing data on viruses in different cellular domains; including archaea, bacteria and eukaryotic life forms, and the discovery of giant viruses whose genomes are bigger than many bacteria and archaea. Based on this information, Prof. Forterre proposed that viruses be defined as cellular organisms producing virions. Virions are products that involve transformation between different transient states: the virus, the virocell, the integrated genome, and others. The concept of virocell is designed to focus on the cellular steps of the viral replication cycle that involves the transformation of all or part of the infected host into a "living" viral organism. He also

provided evidence to suggest that most viral genes originate de novo in the viral genome. Finally he raised the possibility that the interactions between viruses and the host cells are the major engines of biologic evolution.

Dr. Kimberly Seed of the University of California at Berkeley provided a comprehensive discussion of the cellular aspects of the infection of *Vibrio cholerae* with several bacteriophages. Both biotic and abiotic factors critically influence the growth and abundance of *V. cholerae* in many parts of the world. Specific bacteriophages appear to be responsible for the growth patterns and the pathogenesis of this bacterial organism. Based on the studies carried out by Dr. Seed and her colleagues with *V. cholerae* specific phages, ICP1, ICP2 and ICP3, it appears that the bacterium can evolve specific mechanisms of resistance to phage infection. Interestingly enough, the cholera phages can counter-adapt to overcome the defence barriers of the bacterial host organism. She has shown that virtually all *V. cholerae* coexisting with ICP2 phage are resistant to other phage infections. The mechanisms underlying such resistance include induction of phage-inducible chromosomal island like elements (PLE). The induction of PLE appears to be highly specific in its activity to abort phage infections in the bacterial organism. Although, the studies of Dr. Seed are limited to the bacteriophage of *V. cholerae* alone, their implications are clearly applicable to other phage-bacterium interactions**.**

Dr. Joao Margues from the University Federal de Minas Gerais in Brazil provided an overview of the virome of the insect world, and their impact on human health and disease. Insects are one of the most successful life forms, representing over 70% of the animal species and over 50% of all life forms on this planet. Insects can adapt rapidly and are highly successful at colonizing virtually every niche of the earth. Insect virome is as diverse as the vast kingdom of the insects themselves.

Viruses associated with plant insects (phytophagous vectors) include topovirus, cytohabdovirus, nucleohabdovirus, emarovirus, and tenuivirus. Blood-feeding insects of the animal world act as vectors, and carry many viruses. These include, nairovirus, phlebovirus, orthobunyavirus, ephimerovirus and vesiculovirus. Other insect viruses associated with vertebrates include filovirus, alphavirus, bornavirus, flavivirus, hantavirus, influenza virus, paramyxovirus, lyssavirus, and possibly other viruses. Dr. Margues provided an extensive review of the mosquito-borne arbovirus infections, especially in the flavivirus and alphavirus families, which are of major public health importance for humans. At least 100 human arboviruses have been identified to date. These include Eastern, Western and Venezuelan equine encephalitis, chikungunya virus, dengue, Japanese, St. Louis encephalitis virus, West Nile virus, and yellow fever virus. Dr. Margues subsequently provided extensive characterization of the virome of the mosquitoes in Caratinga area in Brazil, as a model for exploring the spectrum of insect virome in parts of the world which are endemic for mosquito and other insect-borne disease.

Following the presentations on bacteriophage, and insect viruses, the virome of corals and the development of immunity in other metazoans were discussed in some detail by Dr. Steven Quistad of the San Diego State University in California. The Cnidarian phyla which includes sea anemones, jellyfish, Hydra, and corals is considered to be

phylogenetically basal and similar to Bilateria (files, worms and humans). Due to their relative position within the tree of life, Cnidarians provide insight into the genetic toolkit of the last common metazoan ancestor. Their simple body plan consists of only two epithelial cell layers connected by a jelly-like mesoglea. Despite their morphological simplicity, the Cnidarian immune system is surprisingly complex, with multiple immune components conserved from the earliest Cnidarian life forms to the modern humans. For example, the tumor necrosis factor receptor superfamily (TNFRSF) is a central mediator of apoptosis in humans. Interestingly, corals have been found to possess the most complex TNFR repertoire ever reported. In addition to TNFRSF, the Acropora genome has been shown to possess all the central components of the canonical apoptotic cascades. These include 3 TRAF (TNF receptor associated factor) proteins, 30 caspases, and 8 members of the Bcl-2 family. Remarkably, exposure of coral cells to human TNF-α was found to cause apoptosis-induced formation of blebs, caspase activation, cell death, and the resultant coral bleaching. Subsequently, exposure of human T-cell lymphocytes to a member of the coral TNFSF was found to cause apoptosis in human cells, suggesting a conservation of TNF-induced apoptotic mechanisms across the 550 million year span of evolution.

In the second portion of his talk, Dr. Quistad outlined a novel approach to identify predicted immune proteins in Cnidarians that are missed by using traditional protein annotation methods alone. Briefly, this approach is based on the identification of similarities between viral gene products and host proteins and can be applied to virtually any system. Viruses are extracted from the non-model organism of interest using a virus-like particle extraction protocol of choice and nucleic acid is sequenced. If a gene model is not available, total RNA is also extracted from host tissue and an assembled transcriptome is prepared. First, viral gene segments are compared to the translated transcriptome through tBLASTn to identify matches to host proteins. These proteins are further analysed through comparison to a well-characterized immune system using BLASTp (e.g., human or mouse) and domain prediction database (e.g., Conserved Domain CDD, Pfam). Proteins that lack hits to either database represent predicted immune genes with unknown function and are selected for further biochemical investigations using *in vitro* and *in vivo* experimentation.

Dr. Tom Lachnit from the Christian-Albrecht's-University at Kiel, Germany presented an elegant discussion of bacteriophage in the fresh water polyp Hydra and their role in the homeostasis of holobiont. A holobiont is a host organism (animal or plant) which is in constant contact with all its associated microorganisms as an entity for selection of evolution. Hydra and possibly humans are model organisms as holobiont. A series of very imaginative studies carried out by Dr. Lachnit, Dr. Thomas Bosch and their colleagues have shown that different species of Hydra are colonized by different sets of bacteria in a highly (species)-specific manner. The bacteria in Hydra exhibit unique bacterial-bacterial interaction between different species via glycocalyx with the associated microbes. This interaction appears to be critically mediated by the presence of bacteriophage in the microbes. The microbial organisms possess specific viral communities. These are predominantly from nine families of eukaryotic viruses, and to a smaller extent from the four families of prokaryotic viruses.

Also, there appears to be on-going interactions between the prokaryotic and eukaryotic associated bacteria and the host. Studies carried out with prophage of Curvibacter species in this model system have suggested that environmental stress may potentiate induction of bacteriophage, and Curvibacter phages can also influence bacterial colonization *in vivo*. Genome sequencing of 13 Hydra species- associated bacteria have revealed the presence of phages as, intact prophage, partial prophage or no prophage in, 54%, 23% and 27% of the bacterial species respectively. These observations suggest that phages are an important part of the metaorganism. The host appears to influence bacterial-bacterial interaction via the induction of prophages. Finally, it was suggested that mechanisms of host immune defence can specifically regulate the activation of prophages in this model holobiont species.

The final component of this seminar focused on the interaction of the virome in human health and disease. Professor Frederick Bushman from University of Pennsylvania at Philadelphia discussed via a previously recorded video, several important aspects of human virome. These included composition and targeted hyper-variation of the virome and the mechanisms underlying the individual variations in harbouring distinct and different viral communities by different subjects. Professor Bushman provided a very comprehensive database to suggest that human virome is composed of three sets of distinct viral communities:

1) **Persistent and latent human viruses**.

These include, among others, Epstein Barr virus (EBV), Varicella-Zoster virus (VZV), Herpesviruses including Herpes simplex virus, HSV1, papillomaviruses, Cytomegalovirus (CMV), and possibly other viruses. Up to 60-100% of humans are seropositive for these viral agents during their lifetime. Other human viruses such as HSV2, Human Immune deficiency virus (HIV), Hepatitis C virus (HCV) are less common and exhibit lifetime infection rates ranging from up to 22% for HSV and to about 1% for HIV respectively.

2) Endogenous retroviruses.

These agents constitute up to 8% of human DNA.

3) Bacteriophages.

Predators of bacteria and archaea associated with human skin and mucosal surfaces (especially in gut). It is estimated that over $10^{10\text{-}11}$ viral particles are present in each gram of human faeces. Based on Solexa/Illumina HiSeq data analysis of viral sequences, it appears that as many as 500-1000 virus species may be present in each individual. Most viral sequences identified to date probably represent new viruses, with little or no resemblance to isolates from other subjects.

The composition of faecal virome in healthy subjects is mostly derived from the bacteriophage. These resident phages have profound influence on the functional attributes of the bacterial host. These include development of toxins (*Shigella, V. cholerae*), virulence determinants, metabolic capacity and development of antibiotic resistance. Furthermore, bacteriophages function as parasites and the host bacteria devote much of their coding capacity toward the assembly of the phage. During diseased or immunologically compromised states, the virome appears to be quite different. In one such situation, metagenomics sequencing have identified a virus, a new bocavirus in the parvovirus family, from several patients with severe combined immunodeficiency disorder.

Targeted hypervariation appears to be another distinct feature of human gut bacteriophage. This phenomenon is mediated by an abundant class of reverse transcriptase enzyme. Another bacteriophage driven process described recently is the CRISPER (Clusters of Regularly Interspaced Short Palindromic Repeats) system, which targets viruses for the generation of escape mutants. Other genomic alterations result in rapid evolution of ssDNA phages in the gut referred to as microviridae. These biologic phenomena in the gut environment appear to be responsible for generation and harbouring of newly evolved viral communities in the human virome.

Dr. Uri Laserson from the Mount Sinai Medical Center, New York provided a comprehensive report on an important recently developed immunological tool, the reading of antibody repertoire to environmental and human microbiome and auto-immune (self) antigens. This approach is based on Phage immunoprecipitation sequencing (PhIP-seq). It can evaluate binding to several hundred epitopes of given antibodies with unknown specificity. The PhIP-seq system requires antigen library cloned into phage, and the antibodies to be profiled. The system has been employed to, identify short synthetic peptides, scan for mutagenesis, and more recently to identify known variants of viruses and, personalize malignancy induced neopeptides.

The final presentation considered the role of viral infections on the longitudinal cognitive functioning in man. Professor Robert Yolken from the Johns Hopkins School of Medicine, Baltimore, Maryland reviewed psychiatric disorders such as schizophrenia, bipolar disorder, and major depression, as causes of mortality and serious morbidity worldwide, particularly in younger individuals. These disorders are associated with both alterations in mood and cognitive impairment. Several genetic, epidemiological and pathophysiological studies indicate a possible role for viruses and other microbial agents in the pathogenesis of some of these disorders. However no specific causative agents have been identified to date. He and his colleagues have employed a number of techniques in an attempt to identify any possible viral agents associated with such psychiatric disorders and cognitive impairment. Using standard enzyme immunoassays they have found that increased levels of antibodies to the neurotropic herpesvirus, Herpes simplex Virus Type 1 (HSV-1) are associated with decreased levels of cognitive functioning in individuals with schizophrenia or bipolar disorder, as well as in individuals without any defined psychiatric disorder. This dysfunction was seen largely in the domains assessing memory and was not seen in association with antibodies to other herpesviruses.

In a series of recent studies, Professor Yolken and his colleagues have explored the role of viruses in the aetiology of these conditions by the use of metagenomics sequencing of throat swab samples. These studies also did not find any recognized human virus to be associated with cognitive impairment or a psychiatric diagnosis. However, a number of phage and other non-human viruses were differentially present in many samples. In particular, the Lactobacillus phage Phiadh was significantly increased in samples obtained from individuals with schizophrenia and was associated with an increased rate of autoimmune disorders and a differential response to medications. It was also found that an algae virus, Acanthocystis turfacea chlorella

virus 1 (ATCV-1), was associated with significantly lower performance in cognitive tests of motor ability in individuals without any defined psychiatric disorder. Feeding this virus to mice resulted in altered cognitive performance and changes in the expression of relevant transcripts within the brain. These observations indicate that both human and non-human viruses may affect human behaviour and cognition. A greater understanding of the role of these viruses and other infectious agents may lead to new methods for the prevention and treatment of psychiatric disorders and cognitive dysfunction.

The formal session of scientific presentations concluded with a presentation by Professors Peter Heidt and Volker Rusch. This special presentation was a tribute to Professor Dirk van der Waaij who passed away in 2016 after an illustrious academic career.

Professor van der Waaij was the Professor and Chairman of the Department of Bacteriology and Serology at the University of Groningen from 1975 until his retirement in 1992. However, he remained a true scholar even after his retirement. During his lifetime, Professor van der Waaij made seminal contributions to the understanding of human microbiome in human health and disease (*van Bekkum* et al., 1974; *van der Waaij*, 1977; *Heidt* et al., 1983; *Vossen* et al., 2014). These include, the introduction of concepts of complete gastrointestinal decontamination, reverse isolation, use of human donor flora for reconstitution after complete decontamination (Julia flora), microbial intervention in immunodeficiency states, characterization of microflora by morphometry and many other innovative contributions which are still in use in current patient management.

This tribute to Professor van der Waaij during the 31st Seminar is especially personal to the OHUS organization. He was one of the founders of the OHUS seminar series. He was instrumental as a major planner and as co-editor of the first twenty-one volumes of Seminar publications. For his scientific contribution and for his personal commitment, we are profoundly grateful to our late friend.

Concluding Remarks

Based on the information summarized during this seminar, it is clear that our understanding of the environmental virome and its impact on human health and disease is still in its infancy, but continues to evolve rather rapidly. A modest consensus is emerging about the definition of the virus as an intracellular organism that involves transformation between different transient states, the virion, the virocell, the integrated genome and possibly other cellular events. Most viral genes seem to originate *de novo* in the viral genomes. The origin of viruses and their interaction with the host cells may represent the single most important trigger for the selection in the on-going evolutionary biology.

Virtually all cellular life forms are colonized or parasitized by a specific viral entity. Yet, relatively little is known about the impact of the virome on the development and cellular homeostasis of the host. The interaction between the human host and the virome environment must include the relative impact of viruses on the particular strategies of the life forms which have adapted to their environments and reproductive success. For example, the impact of a particular virus on a multicellular regeneration-capable sponge is quite different than the impact on a human with a neuron that, once damaged, may be catastrophic to that organism. As such, one might expect that the host response repertoires and pathways to

deal with viruses may be similarly divergent in their tolerance to such external threats. Thus, to understand the different adaptive responses to viruses, host interactions from the context of evolutionary principles should be considered. For example, one must consider the question of how bacterial and phage interactions provide any fitness benefits to the host.

Little information is currently available about the possible use of any intervention measures directed at different components of human virome including bacteriophage to prevent or treat human disease. During the open discussion session period of the seminar, Prof. Yolken introduced some exciting additional studies from his laboratory on the role of microbiome in psychiatric disorders relative to gut-brain-immune interaction and opportunities for prevention and treatment. Prof. Yolken suggested, based on large nationwide survey, that infections and repeated use of anti-infective agents exhibit a strong correlation with the risk of severe mental disorders especially affective disorders and possibly schizophrenia (*Kohler*, et al, 2017). A significant association was observed between the use of antibiotics and bipolar mania in hospitalized patient settings. These observations suggest in patients hospitalized with mania a possible role of altered microbiome, associated with mucosal inflammation of the gut secondary to the use of antibiotics. Based on these observations, a longitudinal study employing orally administrated Lactobacillus GG and *Bifidobacterium lactis* ($>10^8$ CFU) for a period of 6 months was undertaken in a large number of subjects. Preliminary analysis of the results suggests that such treatment improved the clinical course of patients with mania, but did not significantly alter the psychotic symptoms in patients with schizophrenia.

Other recognized concepts of importance, but also uncertainty, relate to understanding the effect of predatory phage in the aquatic environment. Few preliminary studies have begun to examine this relationship in the human host. However, large gaps remain in our understanding about the nature of specific interactions in the marine estuarial environments. Could such events also shape factors that are involved in human disease? Furthermore, very little is known about virome-virome interactions, as well as about specific virome dynamics in insects which are vectors in a host of human diseases. The adaptive immune system and impacts on host-virome interactions is an additional area of future research needs.

During the past two decades the incidence of microbial antibiotic resistance and emergence of new infectious disease states has increased at an alarming rate. Significant effort is currently underway to utilize bacteriophage components of human virome as a potential tool to preserve a healthy human microbiome. Although the evidence of possible phage-induced antibacterial activity in bacteria-free filtered water of river Ganges in India was observed by Hankin as early as 1896, the concepts of phage therapy are now being re-examined in order to develop alternate approaches to the use of antibiotics. Currently, several potential bacteriophage-based therapeutic products are being explored for their antibacterial activity and for possible use in humans. These include phage lysine for *Staphylococcus aureus* bacteraemia, and natural phage cocktails for *E. coli* and Shigella enteric infections, chronic ulcers, skin infections and prosthetic infections (*Madhusoodan*, 2016).

The human virome is relatively specific in each individual and significant hypervariation is associated with the constant generation of novel viral parti-

cles in the human gut. However, it remains to be determined if the introduction of foreign phages as probiotics, or via faecal transplants will be safer and more physiologic than the use of antibiotics.

Finally since their discovery, viruses have been viewed negatively, mostly as nasty disease-producing organisms. Unfortunately this perception is not always based on facts. Of the trillions of viruses and viral particles which form the resident environmental virome, only a few hundred produce a symptomatic infection and or a fatal clinical disease. Viruses have been recovered from all mucosal surfaces and the skin, and are acquired in a very distinct pattern before and immediately after birth. The role of viral infections in the anatomical development of mammalian mucosal surfaces (such as intestinal villi), regulation of mucosal inflammation and maturation and functional development of immune system are well known. However, it is also well recognized that a small minority of viruses are pathogenic and the immune system is designed to promote tolerance towards the vast majority of non-threatening antigens that it sees on a daily basis. Thus, the precise roles of the virome in the functional development of the diverse homeostatic mechanisms of the human host are critical to understand, and still remain to be clearly defined.

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