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THE VIROME, COGNITION, AND HUMAN PSYCHIATRIC DISORDERS: A POSSIBLE ROLE FOR VIRUSES WITH HUMAN, BACTERIAL AND ALGAL HOSTS

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SUMMARY

Human psychiatric diseases such as schizophrenia, bipolar disorder, and major depression are important causes of mortality, morbidity, economic hardship, and social disruption worldwide. Cognitive impairments are hallmarks of these disorders and are major contributors to social dysfunction in affected individuals. Although genetic factors have been found which increase the risk of acquiring these disorders, environmental factors are likely to play an important role in disease progression and severity.

We have previously found that serological evidence of exposure to common herpesviruses such as herpes simplex virus type 1 is associated with decreased functioning in memory and other cognitive domains in individuals diagnosed with schizophrenia or bipolar disorder as well as in individuals without a diagnosed psychiatric disorder. Due to the limitations of serological methods, we applied metagenomic sequencing methods to characterize the nasopharyngeal virome of individuals with and without psychiatric disorders. We found surprisingly that DNA mapping to several bacteriophages, including the lactobacillus phage phi-adh was more prevalent in individuals with schizophrenia as compared to controls and was associated with different clinical patterns and responses to medications. We also found that the chlorovirus Acanthocystis turfacea chlorella virus 1 (ATCV-1) was associated with decreased cognitive functioning in individuals without a psychiatric disorder. A possible role for ATCV-1 in animal biology was supported by animal models and the measurement of an immune response to ATCV-1 proteins in humans.

There is ample evidence that exposure to viruses can alter human cognition and behaviour. The human virome is also likely to contain viruses, which can replicate in non-animal hosts and nonetheless have effects on human health and disease.

INTRODUCTION

Serious psychiatric disorders such as schizophrenia, bipolar disorder and major depression are major causes of mortality and morbidity worldwide

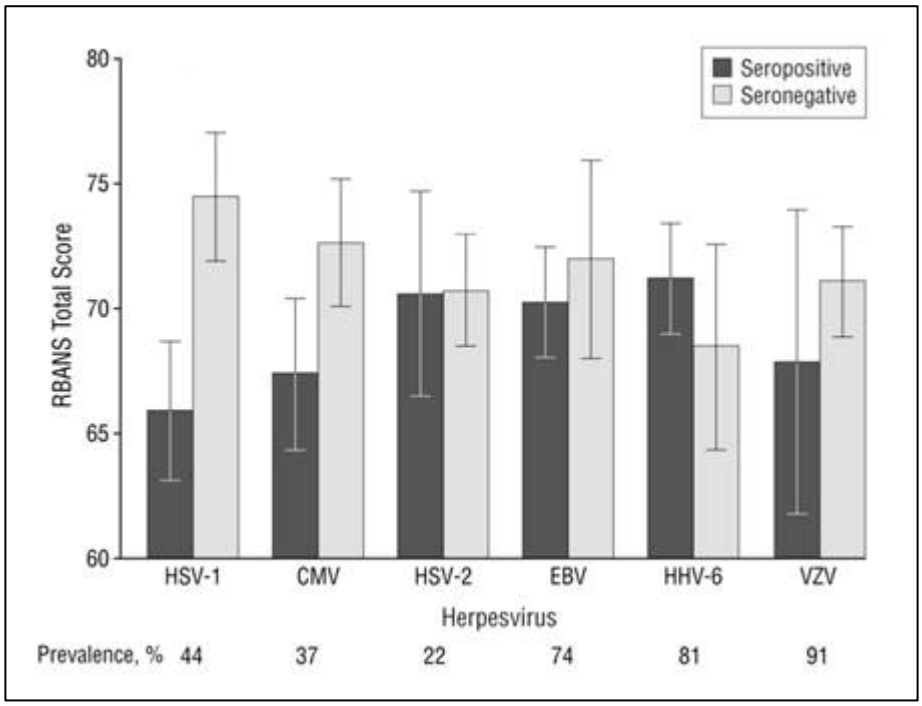

Figure 1: Cognitive functioning in individuals with schizophrenia related to serologic evidence of infection with specific herpesviruses. Levels of IgG class antibodies to herpesviruses were measured by means of an enzyme immunoassay, and cognitive functioning was measured by the total score of the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) in individuals with schizophrenia (N = 229). Bars indicate the mean scores (±95% confidence intervals [CIs]) of individuals who were seropositive or seronegative for the indicated herpesvirus. The percentage of individuals in the total study population who were seropositive for the indicated herpesvirus is shown in the lower line. The asterisk indicates p<.001 between the RBANS total score for herpes simplex virus 1 (HSV-1) seropositive and seronegative individuals as calculated by 1-way analysis of variance; the differences between the RBANS total scores in terms of seroreactivity to the other viruses were not statistically significant (at the level of α= .008). CMV indicates cytomegalovirus; EBV, Epstein-Barr virus; HSV-2, herpes simplex virus 2; HHV-6, human virus 6; and VZV, varicella-zoster virus. (Figure reproduced from *Dickerson* et al. (2003).

(*Millier* et al., 2014). Numerous studies point to the importance of family history and genetic factors in the aetiology of these disorders (*Bergen* et al., 2014). However, epidemiological studies also point to environmental factors as contributors to disease risk. In particular, studies have documented immune activation in many individuals with psychiatric disorders (*Khandaker* et al., 2015). These studies have intensified interest in understanding the instigating factors for this inflammation as well as for the mechanisms by which immune activation might lead to development of psychiatric disorders in some individuals. Most of this research has focused on exposure to infectious agents and to food antigens in light of the major role of these factors in the generation of immune activation in humans and animal models (*Severance* et al., 2016).

Altered behaviour, mood, and perception are cardinal features of human psychiatric disorders. However, psychiatric disorders are also associated with varying degrees of cognitive

impairment. This is particularly the case in schizophrenia where cognitive impairment is often present and can remain as a significant problem when other symptoms have been alleviated following the administration of antipsychotic medications (*Buchanan* et al., 2005). This residual cognitive impairment is often one of the main barriers to the ability of individuals with schizophrenia to function within work or social environments (*Tas* et al., 2013). Numerous studies in humans and experimental animals have pointed to a role for infectious agents in inducing changes in cognitive functioning particularly in the memory domain (*Williamson* et al., 2011). We thus have explored the relationship between exposure to infectious agents and cognitive functioning in individuals with psychiatric disorders as well as individuals without a psychiatric diagnosis.

HERPESVIRUSES AND COGNITIVE FUNCTIONING

Our initial studies focused on individuals with schizophrenia because this diagnosis is generally associated with a high rate of cognitive impairment. We also focused on exposure to common infectious agents, including human herpesviruses, because of their high prevalence and their ability to infect the human central nervous systems in some situations (*Dickerson* et al., 2003). As depicted in Figure 1, we found that exposure to herpes simplex virus type 1 (HSV-1), as revealed by the presence of IgG class antibodies, was associated with decreased cognitive functioning as measured by the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS). This association correlated with the quantitative level of antibodies, involved several domains including both immediate and delayed memory and was independent of demographic factors such as age, race, gender, and socio-economic status as determined by the level of parental education. The association between decreased cognitive functioning and HSV-1 was also somewhat virus specific in that significant associations were not found with exposures to other herpesviruses such as herpes simplex virus type 2 (HSV-2), Epstein Barr virus (EBV), varicella zoster virus (VZV) or human herpes virus type 6 (HHV-6). There were some associations between cognitive deficits and exposure to cytomegalovirus (CMV), although the strength of these associations was lessened when controlling for demographic factors. Subsequent studies indicated that the association between HSV-1 and cognitive impairment in individuals with schizophrenia is increased in individuals who are cigarette smokers (*Dickerson* et al., 2016) and individuals who have immune activation as evidenced by increased levels of C-reactive protein (*Dickerson* et al., 2012). The association between exposure to HSV-1 and cognitive functioning in individuals with schizophrenia was subsequently found in several other populations in the United States, Europe, and Asia (*Yolken* et al., 2011; *Prasad* et al., 2013; *Thomas* et al., 2013; *Watson* et al., 2013; *Hamdani* et al., 2017)

The possible role of HSV-1 in cognitive functioning in individuals with schizophrenia is supported by additional experimental data. Imaging studies have documented alteration in brain structure and function associated with exposure to HSV-1 in individuals with schizophrenia (*Prasad* et al., 2007; *Schretlen* et al., 2010). It is not clear

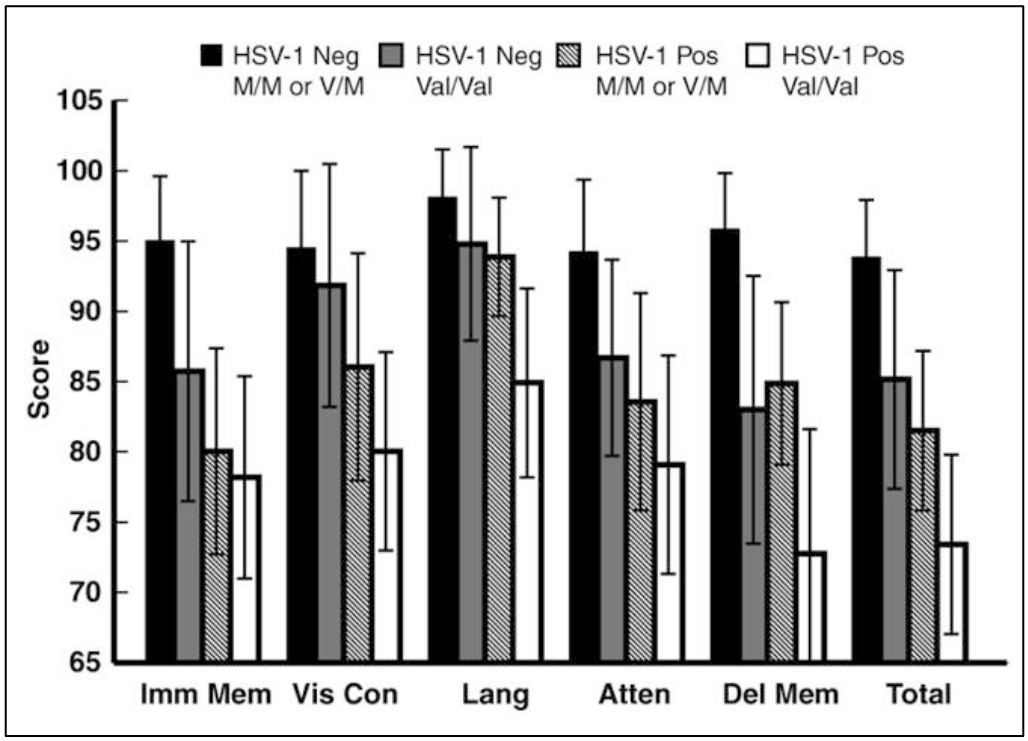

Figure 2: Relationship between COMT Val158Met genotype, HSV-1 serological status and RBANS scores. The bars indicate the mean and 95% confidence intervals of the RBANS cognitive scores measured in individuals with bipolar disorder with the indicated HSV-1 and COMT Val158Met polymorphism status. The component scores measure Immediate Memory (ImmMem), Visuospatial Constructional (VisCon), Language (Lang), Attention (Atten), and Delayed Memory (DelMem). All of the components are expressed as age-adjusted scaled scores. The HSV-1 and COMT polymorphism status are depicted as follows: individuals who are HSV-1 seropositive and who have the COMT Met/Met or COMT158 Met/Val genotype (solid bars), individuals who are HSV-1 seronegative and who have the COMT158 Val/Val genotype (shaded bars), individuals who are HSV-1 seropositive and who have the COMT158 Met/Met or COMT158 Met/Val genotype (striped bars), and individuals who are HSV-1 seronegative and who have the COMT158 Val/Val genotype (open bars). (Figure reproduced from *Dickerson* et al. (2006).

whether these effects are related to the replication of HSV-1 in the brain as occurs in encephalitis (*Hahn* et al., 2012), activation of microglia and other immune cells within the central nervous system (*Patterson*, 2015), the development of autoantibodies to brain receptors (*Westman* et al., 2016) or some combination of these and other processes. It is noteworthy in this regard that HSV-1 induces changes in gene expression of neurons derived from human induced pluripotent stem cells (iPSCs), which are consistent with alterations seen in the brains of individuals with schizophrenia (*D'Aiuto* et al., 2012). Furthermore, a pilot study found significant improvement in some cognitive domains but not others or psychiatric symptoms in individuals with schizophrenia treated with the anti-herpes medication valacyclovir (*Prasad* et al., 2013). Follow up studies testing the efficacy of this medication in larger populations of individuals with schizophrenia and serological evidence of infection with HSV-1 are ongoing.

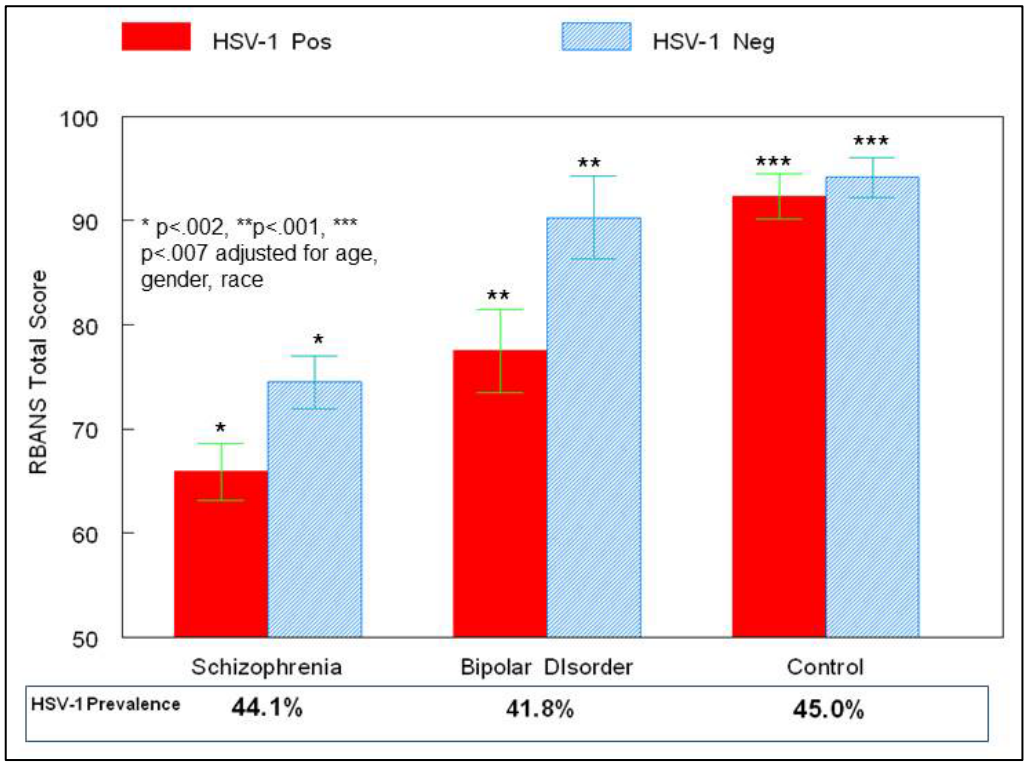

Figure 3: The association between serological evidence of exposure to HSV-1 and cognitive functioning as measured by the RBANS Total Score. The numbers in the box indicate similar levels of prevalence to HSV-1 in the 3 populations.

We have also examined the relationship between exposure to common infectious agents and cognitive functioning in individuals with bipolar disorder. We found an association between exposure to HSV-1 and decreased scores on tests of memory in these individuals (*Dickerson* et al., 2004). Overall the level of cognitive impairment in individuals with bipolar disorder is less than that in individuals with schizophrenia. However, the relative effect of exposure to HSV-1 is similar. We also uncovered an example of gene-environmental interaction in the form of polymorphisms in the gene encoding catechol-*O*-methyltransferase (COMT) (*Dickerson* et al., 2006). As shown in Figure 2, there was an additive effect of exposure to HSV-1 and the genotype of COMT. Individuals who had serological evidence indicating exposure to HSV-1 and the high-risk val/val genotype of COMT had the most cognitive impairment. On the other hand, individuals who were not exposed to HSV-1 and who had the low risk Met/Met genotype of COMT had relatively low levels of cognitive impairment. The scores measured in individuals in this group did not differ substantially from the scores of control individuals. HSV-1 has also been reported to be associated with lower levels of cognitive functioning in other populations of individuals with bipolar disorder (*Gerber* et al., 2012; *Hamdani* et al., 2017).

We have also examined the relationship between exposure to HSV-1 and

cognitive functioning in individuals who do not have a psychiatric disorder. Initial studies using small sample sizes did not demonstrate an association. However, when we tested larger sample sizes we found that individuals who had serological evidence of exposure to HSV-1 had small but statistically significant decreases in functioning particularly in the domain of delayed memory (*Dickerson* et al., 2008). We also found an interaction with the COMT gene as described above in the population of individuals with bipolar disorder. These associations were independent of demographic factors such as age, gender, race, and maternal education. Thus as depicted in Figure 3, exposure to HSV-1 is associated with performance on tests of memory in individuals with schizophrenia or bipolar disorder and individuals without a psychiatric disorder in our study population. However the relative levels of cognitive performance differ greatly among these populations. HSV-1 has been associated with decreased cognitive functioning in several other populations including healthy young adults (*Fruchter* et al., 2015; *Hamdani* et al., 2017), middle aged adults (*Gale* et al., 2016), children with a liability to substance use disorder (*Vanyukov* et al., 2017a,b) and elderly individuals (*Itzhaki*, 2014). Recent progress has been made on the development of new medications (*D'Aiuto* et al., 2017) and vaccines (*Chentoufi* et al., 2012) for the prevention of HSV-1 infection and the effective treatment of latent infection. Our studies support the need to develop methods for the prevention of the serious effects of HSV-1 infection on many different populations worldwide.

There are many possible explanations for the HSV-1 related associations with cognitive dysfunction. Foremost is the criticism that many of the reports described above are correlative in nature; moreover, the cognitive changes or the changes in brain imaging variables could be attributed to another variable, such as a coincidental infection or even a demographic variable such as low socio-economic status that correlates well with most infections. Therefore, we have attempted to evaluate whether the aforementioned studies are sufficient to invoke causal relationships between HSV-1 infection and cognitive impairment using the Bradford-Hill criteria (*Hill*, 1965). Dr. Bradford Hill enunciated the eponymous criteria to evaluate causal links in the context of common exposures such as cigarette smoke and common, chronic conditions, such as cancers. We evaluated HSV-1 infection and cognitive dysfunction in an earlier review (*Prasad* et al., 2012) and have updated them in Table 1. We surmise that moderate to strong evidence supporting five of the nine criteria are available, namely the criteria related to strength, consistency, plausibility, temporality and coherence. It should be noted that the criterion for 'biological gradient' cannot be tested in this context because the 'dose' of HSV-1 virions during the initial or the on-going infection cannot be calculated and proxies such as the host antibody response reflects the severity of infection imprecisely, particularly in the chronic stage. Unlike the classic Koch's postulates that require rigid confirmation to all its requirements, Bradford Hill emphasized: 'What I do not believe – and this has been suggested – is that we can usefully lay down some hard-and-fast rules of evidence that *must* be obeyed before we accept cause and effect.' Thus, overall, the HSV-1 related effects described in this monograph cannot be dismissed merely as trivial or chance associations.

Table 1: HSV-1 associated cognitive impairments evaluated in relation to Bradford Hill criteria

Criterion	Explanation	Evidence
Strength	A strong association is more plausible, but it is not a *sine qua non*.	Association between HSV-1 exposure and cognition has small to medium effect size (*Dickerson* et al., 2003; *Thomas* et al. 2013; *Watson* et al., 2013).
Consistency	Causal effects need to be replicable.	The association has been detected in over ten studies (*Dickerson* et al., 2003, 2004, 2008, 2012; *Prasad* et al., 2007, 2012a; *Schretlen* et al., 2010; *Shirts* et al., 2008; *Strandberg* et al., 2003; *Tarter* et al., 2014; *Thomas* et al., 2013; *Watson* et al., 2013; *Yolken* et al., 2011; *Vanyukov* et al., 2017a,b).
Specificity	Need to consider alternative explanations, including potential confounding factors.	The association is detectable after accounting for potential confounding factors such as age, gender and socio-economic status. However, other herpes viruses are also associated with cognitive impairments, e.g., cytomegalovirus (*Nimgaonkar* et al., 2016) and herpes simplex virus, type 2 (*Watson* et al., 2013).
Plausibility	Plausible biological mechanisms for the associations should be available.	(i) The cognitive impairment could be due to immune reactions to recurrent infection (*Steiner* et al., 2007; *Dantzer* et al., 2008; *Li* et al., 2006) (ii) it could be due to an initial infection in childhood that impairs neurodevelopment (*Meyer* et al., 2009; *Vanyukov* et al., 2017a,b); (iii) it could be due to latent infection in the brain (*Becker*, 1995).
Experiment	Another line of evidence supporting the association, such as a treatment trial.	A randomized double blind trial of adjunctive treatment with acyclovir, a specific antiviral drug for HSV-1 infection led to improved cognitive function among HSV-1 seropositive patients with schizophrenia (*Prasad* et al., 2012), a similar trial in patients with chronic schizophrenia seropositive for cytomegalovirus did not show beneficial effects (*Dickerson* et al., 2009).
Temporality	HSV-1 exposure should predate cognitive impairments	Evidence from longitudinal follow up studies is mixed, but the majority of studies have been conducted in adults in whom duration of exposure is uncertain (*Strandberg* et al., 2003; *Aiello* et al., 2008; *Prasad* et al., 2012; *Barnes* et al., 2014; *Nimgaonkar* et al., 2015). The only prospective study among children found supportive evidence linking prior HSV-1 exposure with cognitive impairment (*Vanyukov* et al., 2017a,b).
Coherence	Congruence between epidemiological and laboratory findings	Latent infection has been modelled in neuron-like cells derived from human induced pluripotent stem cells (hiPSCs) that have features of cortical glutamatergic neurons, suggesting that latent HSV-1 infection can be established in the brain (*D'Aiuto* et al., 2014). This provides a mechanism linking persistent HSV-1 infection directly to cognitive dysfunction.
Biological Gradient	Demonstrable link between 'dose' of risk factor such as smoking, and 'effect', such as lung cancer.	It is not possible to test in the context of viral infections and subsequent cognitive impairment, as the 'dose' of initial infective viral load cannot be assayed.
Analogy	Other lines of evidence, such as animal studies.	One study in a rodent model of HSV-1 infection showed cognitive impairment in animals following HSV-1 infection *Beers, et al.* (1995).

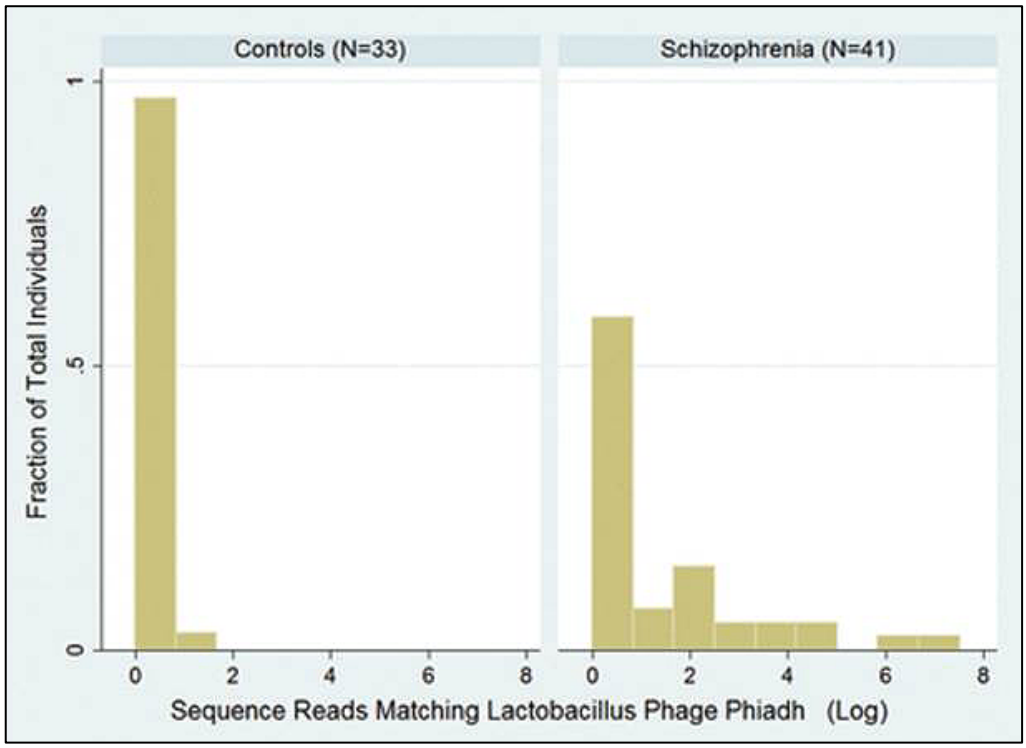

Figure 4: Histogram depicting the number of sequence reads matching Lactobacillus phage φ-adh (NC_000896) obtained from 41 individuals with schizophrenia and 33 controls. Sequence matches were originally identified by the use of the CLC homology algorithm and confirmed by Blastn homology searches against the entire non-redundant nucleotide database. (Figure reproduced from *Yolken*, et al. (2015).

METAGENOMIC SEQUENCING

The above studies indicate that a virus, which replicates at mucosal surfaces, might be associated with an increased risk of psychiatric disorders and cognitive dysfunction. To further explore this interaction we are employing metagenomic sequencing techniques capable of detecting viral sequences at mucosal surfaces. Studies completed to date have been performed using DNA extracted from throat swab samples from individuals with psychiatric disorders and controls. Throat swab samples were initially selected for analysis since they were easy to obtain from the study population in a non-traumatic manner and could be collected on multiple occasions from the same individual. In our initial study, we employed high throughput sequencing to generate more than 100,000,000 sequence reads from samples obtained from 41 individuals with schizophrenia and 33 control individuals without a psychiatric diagnosis (*Yolken* et al., 2015). After matching to available databases, we did not find any known human viruses which distinguished cases from controls or which correlated with cognitive functioning within the groups.

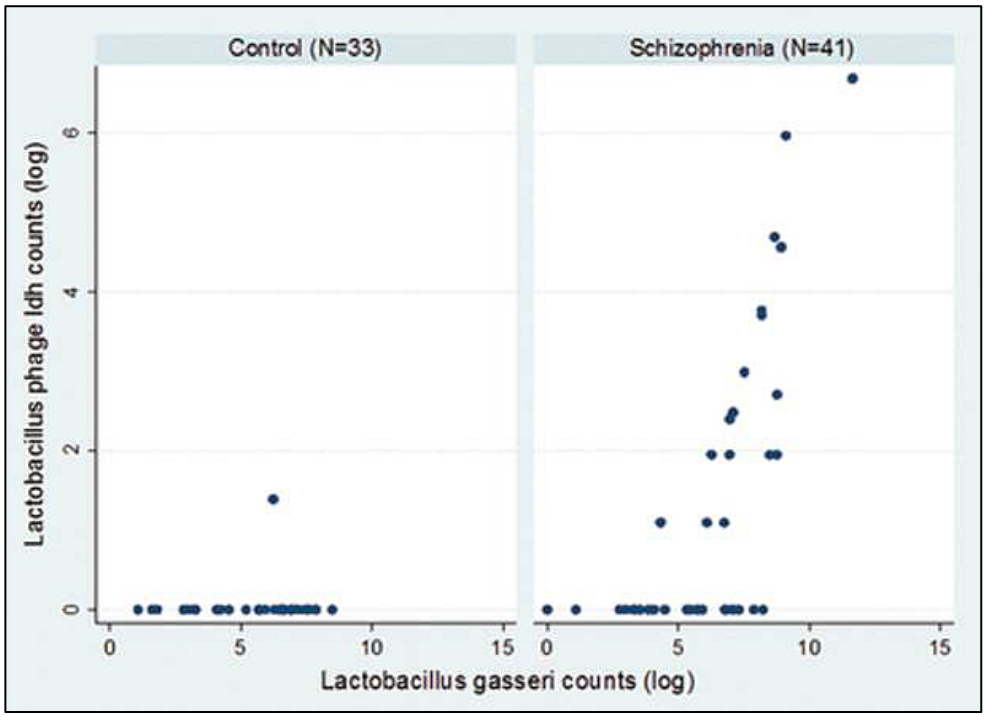

Figure 5: Scatterplot indicating the relationship between number of Lactobacillus phage φadh (NC_000896) and *Lactobacillus gasseri* sequence reads in individuals with schizophrenia (right) and controls (left). Note that schizophrenia diagnosis correlates more strongly with the level of φadh as compared with that of the host bacterial species *Lactobacillus gasseri*. (Figure reproduced from *Yolken* et al. (2015).

A PHAGE ASSOCIATED WITH SCHIZOPHRENIA?

However, we did note some interesting associations with what are usually characterized as "non-human viruses". For example, we identified 79 distinct bacteriophage sequences in the oropharyngeal samples. Of these, one bacteriophage genome, that encoded the lysogenic Lactobacillus phage phi-adh (φadh) **(***Raya* et al., 1989) was significantly different in individuals with schizophrenia ($p<0.00037$, $q<0.03$ adjusted for multiple comparisons) (Figure 4). The different levels of φadh remained significant after controlling for age, gender, race, socioeconomic status, or cigarette smoking ($p<0.006$). Also, the level of φadh correlated better with schizophrenia status than the level of the corresponding host bacteria (Figure 5). Within the group of individuals with schizophrenia, the level of φadh sequences did not correlate with clinical symptoms or demographic variables. However, the level correlated with the prevalence of immunological disorders, particularly type 2 diabetes. The level of sequences homologous to φadh did not correlate with the administration of standard anti-psychotic medications. However, the level did correlate with the administration of the mood stabilizing medication valproic acid. This medication is widely used for the treatment of schizophrenia, particularly in cases of individuals who do not respond to standard medications

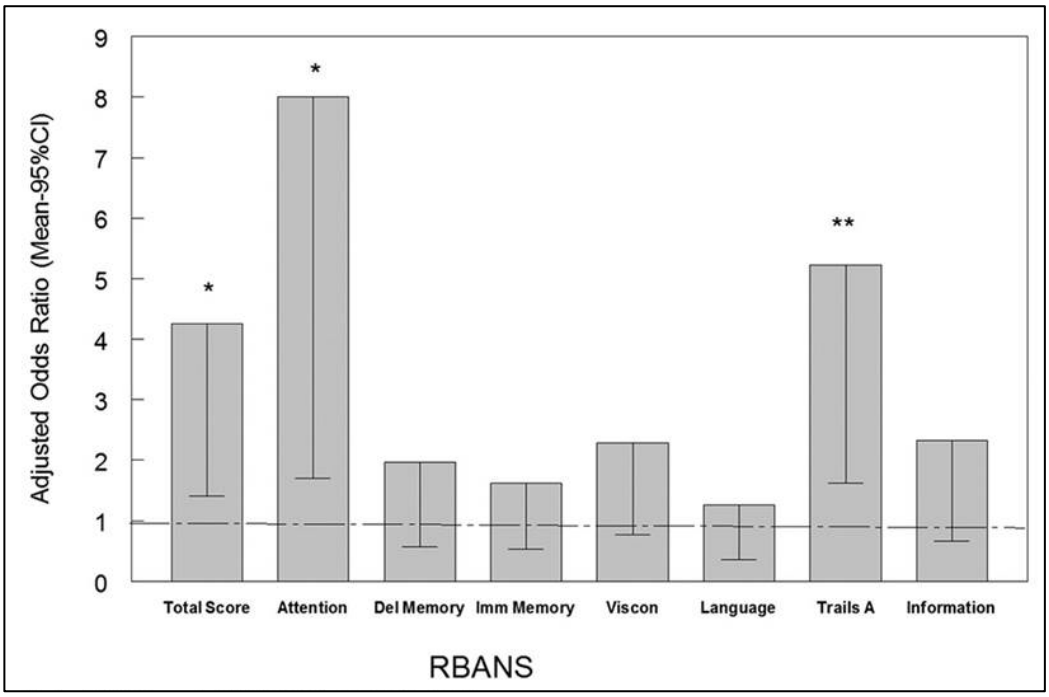

Figure 6: Odds of detecting *Chlorovirus* ATCV-1 in the pharynx by percentile of score on cognitive testing in humans without a psychiatric disorder. Bars represent the mean and 95% confidence interval odds of detecting ATCV-1 DNA in the oropharynx in individuals with the indicated test. The odds ratios are adjusted for the demographic variables of age, sex, race, maternal education, educational status, and place of birth in the United States. Trails A and Information are separate tests and not part of the RBANS. $^{**}p < 0.005$, $^{*}p < 0.01$, adjusted for the same covariates. (Figure reproduced from *Yolken* et al. (2014).

(*Wang* et al., 2016). While it has many biological effects its specific mode of action in schizophrenia is not known with certainty. Previous studies have shown that the administration of valproate alters the microbiome in animal models perhaps related to its homology with fatty acids (*de Theije* et al., 2014). Our finding suggests that valproate may also alter the phage composition of the virome, probably due to alterations in the levels of the bacterial hosts. The possibility that valproate and perhaps other medications employed in schizophrenia exert at least some of their effect through alterations in the microbiome should be the topic of future studies. Further analyses of the role of the immune response to phage in terms of cognitive functioning in individuals with schizophrenia are on-going.

We also measured exposure to φadh by immunoassays that measured the immune response to viral proteins in 620 blood samples obtained from 323 individuals with schizophrenia not of recent onset. We found widespread serological evidence of exposure to φadh as revealed by the prevalence of antibody. Differences in IgG class antibodies did not correlate with available clinical or demographic data. However, the level of IgA class antibodies expressed as normalized scores, were associated with lower levels of cognitive functioning in individuals with schizophrenia as measured by the

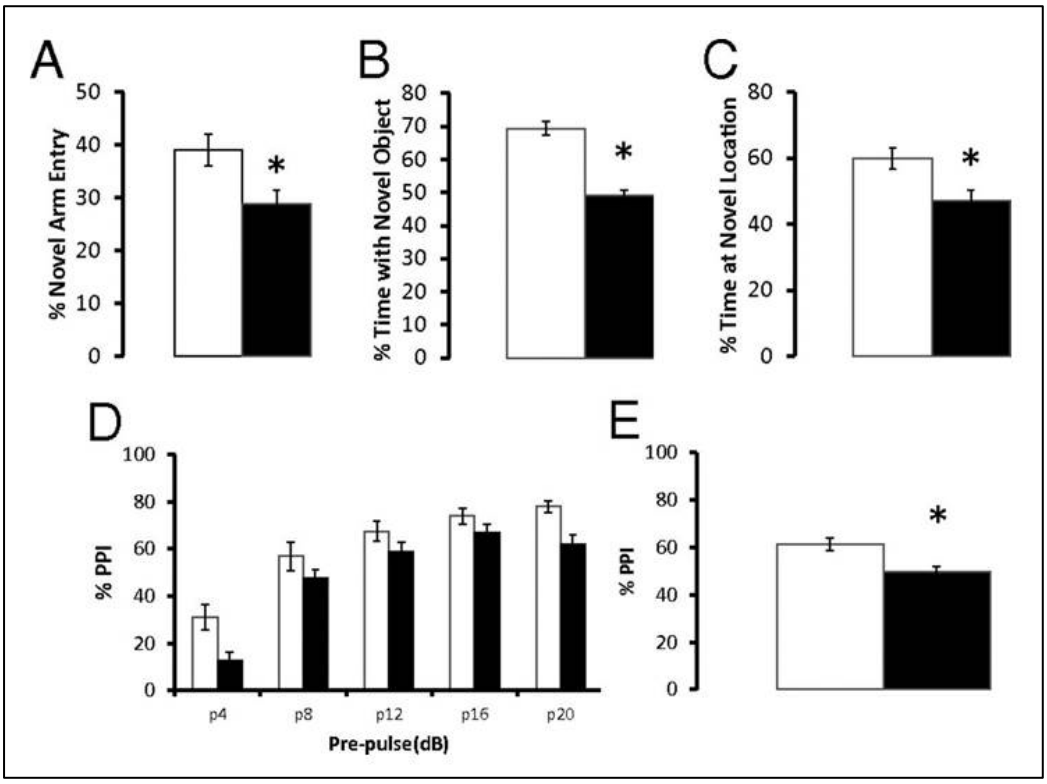

Figure 7: Behavioural effects of oral ATCV-1 exposure. Mice were orally infected with the alga *Chlorella heliozoae* alone (open bars) or with ATCV-1–infected *C. heliozoae* (solid bars) as described in the text. (A) Spatial recognition memory; the y-axis displays the percentage of the previously blocked (i.e., novel) arm entries; *p = 0.015 measured by one-way ANOVA. (B) Novel object recognition; the y axis depicts the percentage of time spent exploring the novel object; *p < 0.001 measured by one-way ANOVA. (C) Place recognition memory recognition; the y axis depicts the percentage of time spent exploring the new location of the familiar object; *p < 0.008 measured by one-way ANOVA. (D) Impaired PPI; mice were exposed to presentation of pulse alone (120 dB) and prepulse–pulse combinations across different prepulse intensities; for example, p4 indicates pairing of the prepulse (4 dB above the background noise of 70 dB) with the pulse alone (120 dB) (see the text for more details); the y axis displays the percentage of PPI. (E) Impaired average PPI; the y axis displays the percentage of PPI; *p < 0.015 measured by post hoc test. (Figure reproduced from *Yolken* et al. (2014).

RBANS total score (regression coefficient -1.21, 95% confidence interval -2.35, -0.06, p = 0.039) and the RBANS visuospatial/constructional score (regression coefficient -1.253208, 95% confidence interval -2.29, -.21, p=0.019) both coefficients adjusted for age, gender, race, level of maternal education and multiple samples per individual. These findings suggest that there is widespread exposure to φadh at mucosal surfaces where IgA antibodies can be generated and that this exposure may be associated with lower levels of cognitive functioning in some individuals with schizophrenia. Analysis of larger sample sizes will be required to explore the relationship between antibodies to phage proteins and cognitive functioning in other populations.

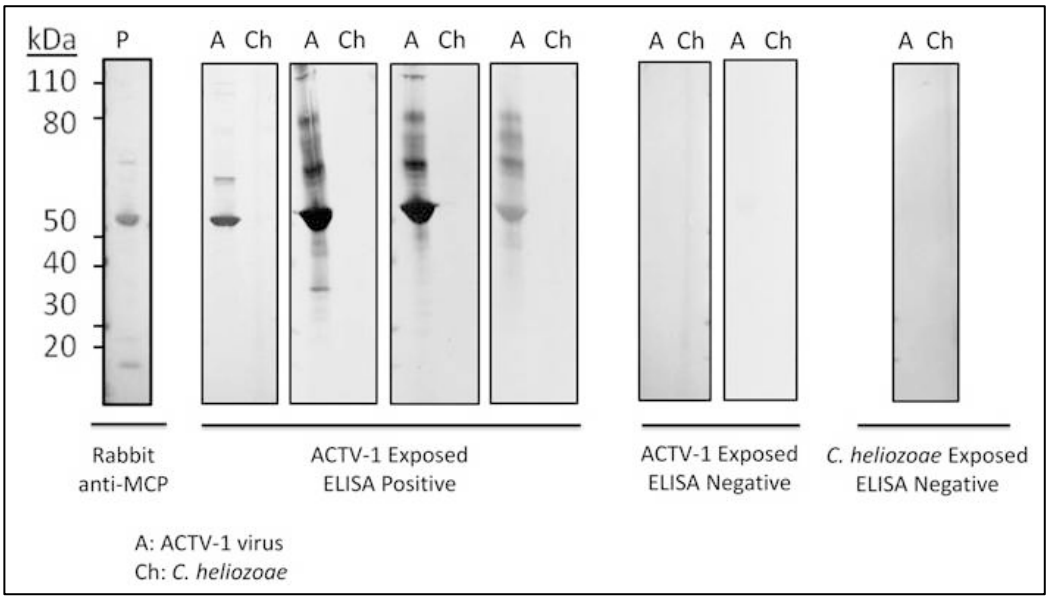

Figure 8: Western Blot assays performed with antigens derived from purified ATCV-1 (A) and *C. heliozoae* (Ch). The first lane (labelled P) is from rabbit antibody prepared against the major capsid protein (A430L) of chlorovirus PBCV-1 as a reference. ATCV-1 exposed ELISA positive, reactivity of sera from mice exposed to ATCV-1 and reactive to ATCV-1 antigens by ELISA. ATCV-1 exposed ELISA negative, reactivity of sera from mice exposed to ATCV-1 and not reactive to ATCV-1 antigens by ELISA. *C. heliozoae* exposed ELISA negative, reactivity of serum from a mouse exposed to *C. heliozoae* in the absence of ATCV-1. All mice with this exposure were nonreactive by ELISA. (Figure reproduced from *Yolken* et al. (2014).

A CHLOROVIRUS ASSOCIATED WITH COGNITIVE BEHAVIOUR?

The above studies indicate that a virus not generally considered important to human health is associated with increased risk of a psychiatric disorder and a decreased level of cognitive functioning. Metagenomic sequencing performed on throat swab samples obtained from 92 individuals without a psychiatric diagnosis revealed other viruses, which are generally not considered to infect humans or animals. Of particular interest in this regard are a group of viruses called Chloroviruses, the natural hosts of which are eukaryotic green algae (*Van Etten* and *Dunigan*, 2012). One of these chloroviruses, called Acanthocystis turfacea chlorella virus 1 (ATCV-1) was associated with lower scores on some cognitive tests in individuals without a psychiatric diagnosis. The most affected tests were ones that measured visual processing and visual motor speed (Figure 6). In order to verify an association between ATCV-1 and lower cognitive functioning we exposed mice to ATCV-1 by gavage and noted alterations in several cognitive domains, including ones involving recognition memory and sensory gating (Figure 7) (*Yolken* et al., 2014). In a subsequent experiment intracranial inoculation of ATCV-1 into mice resulted in impaired memory as well as altered levels of immune markers (*Petro* et al., 2016). Additional studies indicated that ATCV-1 achieves partial replication in mouse macrophages resulting in the expression of

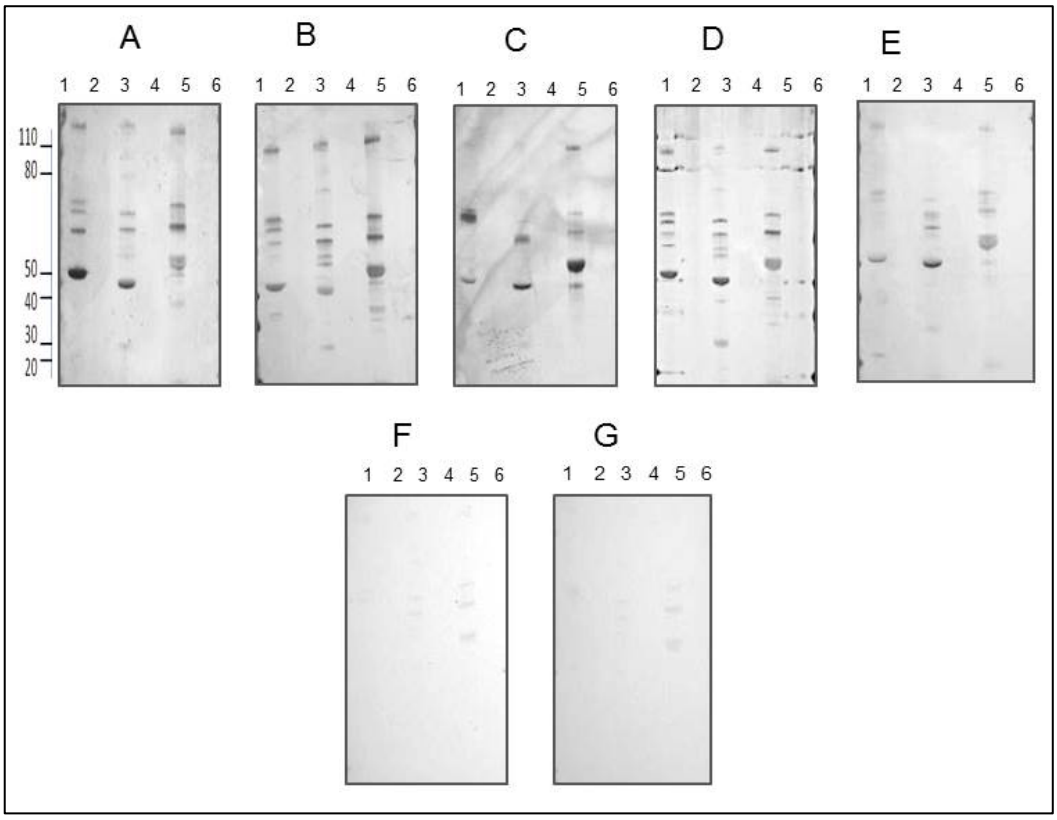

Figure 9: Western Blot reactivity of human sera to proteins derived from ATCV-1 as well as 2 additional chloroviruses, Paramecium bursaria chlorella virus 1 (PBCV-1) and Paramecium bursaria chlorella virus CVM-1 (CVM-1) as well as their host algae.
Antigens contained in the lanes are as follows: 1. ATCV-1 chlorella virus; 2. *C. heliozoae* (SAG-3) host for ATCV-1 3; CVM-1 chlorella virus; 4. *Micractinium conductrix* Pbi host for CVM-1; 5. PBCV-1 chlorella virus; 6. *Chlorella variabilis* NC64A host for PBCV-1.
The samples tested were: A: 46 year-old female with Bipolar Disorder born in Maryland. B: 20 year old male with recent onset psychosis born in Maryland. C: 31-year-old male with recent onset psychosis born in New York. D: 40-year-old female with schizophrenia born in New York. E: 26-year-old woman with mania born in Maryland. F: 29-year-old female with schizophrenia born in Pennsylvania. Panel G depicts the reactivity to labelled anti-human IgG in the absence of added serum.

immune mediators (*Petro* et al., 2015). These studies suggest that ATCV-1 may be exerting its effect on cognition through immune activation, a process that has previously been found to link other infectious agents with altered cognitive functioning in humans (*Rempel* et al., 2013).

We have further investigated the immune response to ATCV-1 in humans. Initial studies were performed using Western Blotting. Antibodies to multiple proteins were detected in mice exposed to ATCV-1 (Figure 8). Furthermore, antibodies to several ATCV-1 proteins and antigenically related chloroviruses were detected in individuals with a psychiatric disorder (Figure 9) as well as in individuals without a psychiatric disorder. Mice and human sera also reacted with two recombinant, hypothetical ATCV-1 proteins, Z227L

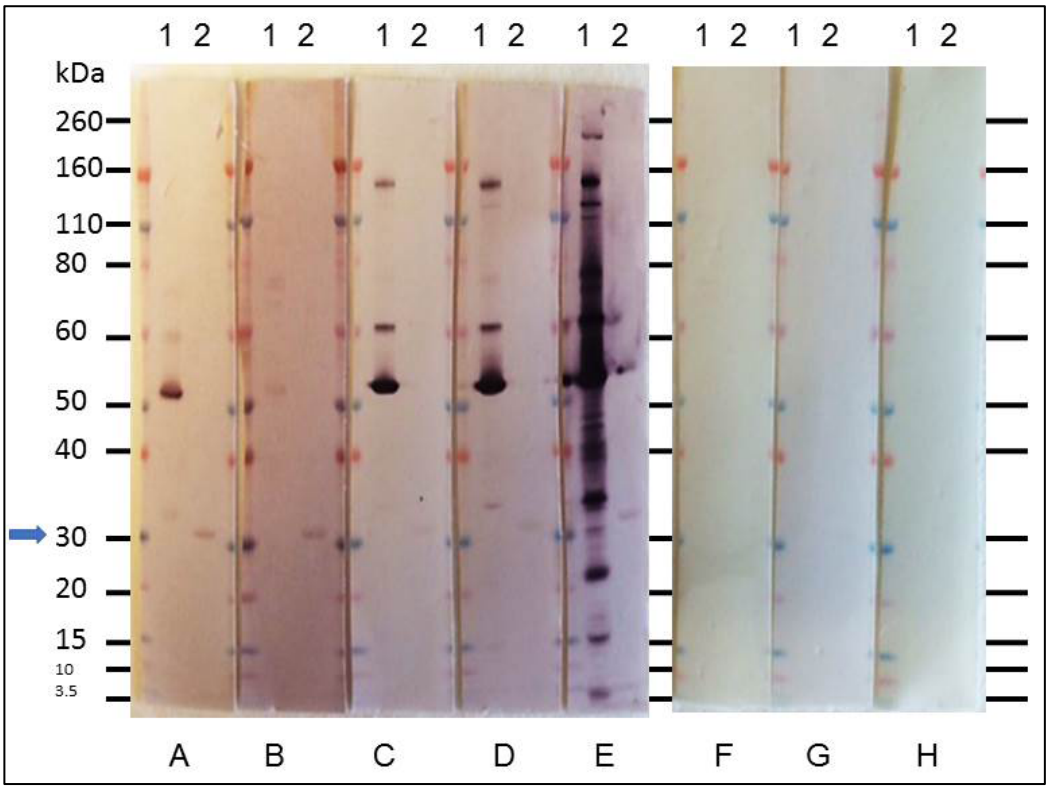

Figure 10: Reactivity of human and mouse sera to proteins derived from ATCV-1 virion- (lanes marked "1") and recombinant protein ATCV-1_Z227L (lanes marked "2"). The expected position of the cloned recombinant protein ATCV1_Z227L is shown by the arrow.
The samples tested were: A. 58 year old female without a psychiatric disorder born in Maryland; B. 49 year old female with schizophrenia born in Maryland; C. Mouse exposed to ATCV-1 through the gastrointestinal tract; D. Mouse exposed to ATCV-1 through the gastrointestinal tract. E. Rabbit immunized with ATCV-1. F-H. Samples without added serum processed with secondary antibody directed at human, mouse and rabbit immunoglobulins, respectively.

(GenBank: ABT16361.1) and Z223L (NCBI Reference Sequence: YP_001426704.1). These antigenically related proteins were originally selected because they have no homologs in GenBank and they are not part of the virus proteome. Therefore, the host immune system would presumably only be exposed to the proteins during viral replication (*Van Etten* and *Dunigan*, 2012) We found that human and mouse sera with antibodies to other ATCV-1 proteins react to hypothetical ATCV-1 protein Z227L cloned into a baculovirus vector (Figure 10). We also discovered that some human sera recognize epitopes in these proteins as measured by reactivity to overlapping synthetic peptides derived from hypothetical ATCV-1 protein Z223L (Figure 11). These findings indicate that for some humans there is a clear interaction between the systemic immune system and proteins antigenically related to ATCV-1 encoded proteins. Additional studies using Western Blot assays and synthetic peptide techniques are on-going.

We have performed additional studies in mice in an attempt to identify the pathological consequences of ATCV-1 exposure. We found that mice infected with ATCV-1 administered by the

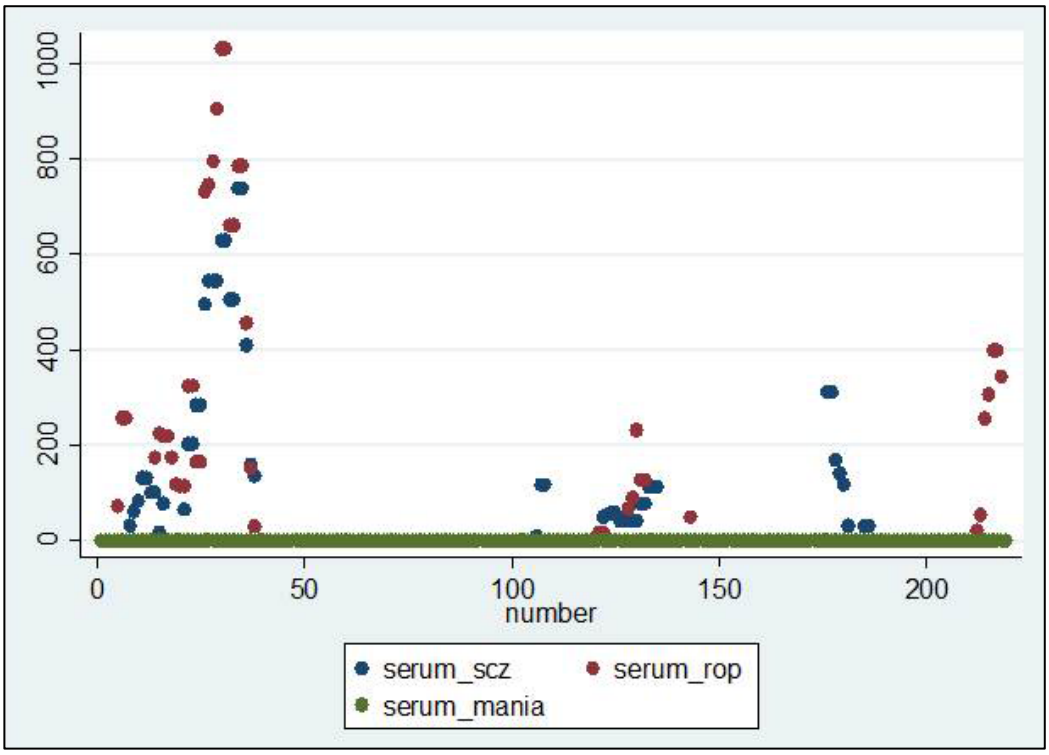

Figure 11: Reactivity of 3 human sera to overlapping peptides spanning the entire length of hypothetical protein ATCV-1_Z223L. The numbers on the x-axis indicate the peptide number based on the target sequence (NCBI Reference Sequence: YP_001426704). The numbers on the y-axis indicate the relative activity in arbitrary units. Each dot represents the reactivity to a single peptide starting at the indicated position. "Serum_scz" indicates sample from an individual with established schizophrenia, "serum_rop" indicates a sample from an individual with recent onset psychosis and "serum_mania" indicates a sample from an individual with mania.

intraperitoneal route are associated with periportal inflammation in the liver (Figure 12). We also identified ATCV-1 antigens in the livers of mice infected by this route, most likely in cells with phagocytic activity (Figure 13). The testing of samples from humans with liver diseases is on-going.

We have also examined other body sites for the presence of DNA sequences related to ATCV-1. We did not find any sequences in faecal samples obtained from a small cohort of individuals with a psychiatric disease or controls (*Schwarz* et al., 2017). We also examined plasma samples for the presence of ATCV-1 DNA using a previously described quantitative PCR method (*Yolken* et al., 2014). Testing of more than 200 samples from individuals with psychiatric disorders and controls resulted in the identification of one sample containing detectable ATCV-1 DNA. This sample was obtained from a 51 year old man from Maryland with schizophrenia and lung cancer. Metagenomic sequencing of the sample identified 304 sequence reads, which had high identity to ATCV-1 (Figure 14). A skin swab sample taken from this individual at the same time did not have any detectable DNA sequences homologous to ATCV-1 indicating that the detection in the plasma

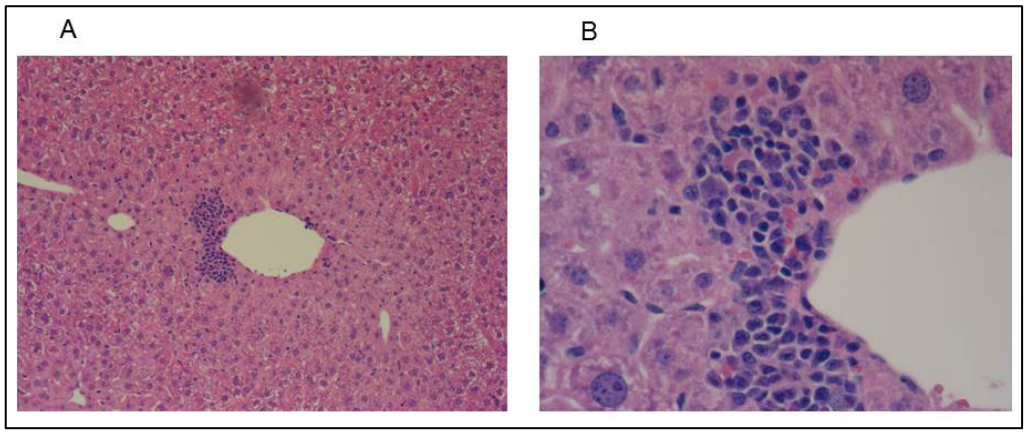

Figure 12: Haematoxylin and eosin stained sections of liver from mice exposed to ATCV-1 by the intraperitoneal injection route displaying cellular infiltration around the portal tract. The magnifications are 10x (A) and 40x (B). Significant infiltration adjacent to the portal tract was present on approximately 50% of hepatic portal veins in all ATCV-1 exposed animals and was not observed at all in ATCV-1 naive animals.

was not simply due to skin contamination. These findings indicate that ATCV-1 may in some cases result in systemic infection.

CONCLUSIONS

Our studies indicate that exposure to infectious agents contribute to the pathogenesis of human psychiatric disorders. Similarly, exposure to microbial agents is also associated with lower levels of cognitive functioning in individuals with psychiatric disorders as well as individuals without a psychiatric disorder. Some of the microbial agents are well-recognized human pathogens. However, other viral agents identified by metagenomic sequencing and associated with these states are not normally considered to be "human" viruses or even animal viruses because their known primary hosts are either bacteria or algae. The surprising finding that "non-human" viruses can produce an immune response in humans and alter behaviour in animal models suggests that even though the viruses are considered to be "non-human" they need to be considered as factors affecting human health. The mechanism(s) by which these "non-human" viruses affect humans is not known with certainty but they are likely to exert their affect through changes in the immune system or alterations in the microbiome. The exact mechanisms by which these "non-human" viruses can affect human health remain an exciting area for future research.

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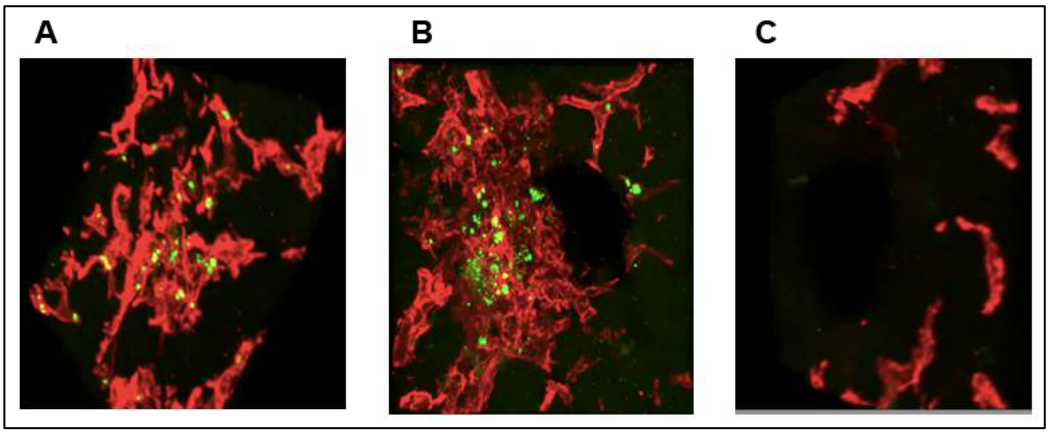

Figure 13: Reactivity of fluorescent-labelled rabbit antibody to ATCV-1 (green) and rat antibody to mouse IBA-1 (red). (A) IP injected ATCV-1 exposed mouse, section of mid liver. (B) ATCV-1 exposed mouse, section of the peri-portal region of the liver. (C) Peri-portal region of an unexposed mouse. Magnifications are at 100x. Three-dimensional analysis confirms the presence of foci of ATCV-1 antigen clustered inside IBA-1 positive cells, indicating that the viral antigen is found intracellularly almost exclusively in phagocytic cells. These cells may be infiltrative phagocytic cells, or consistent with their location within the liver tissue, are likely to include hepatic resident macrophages.

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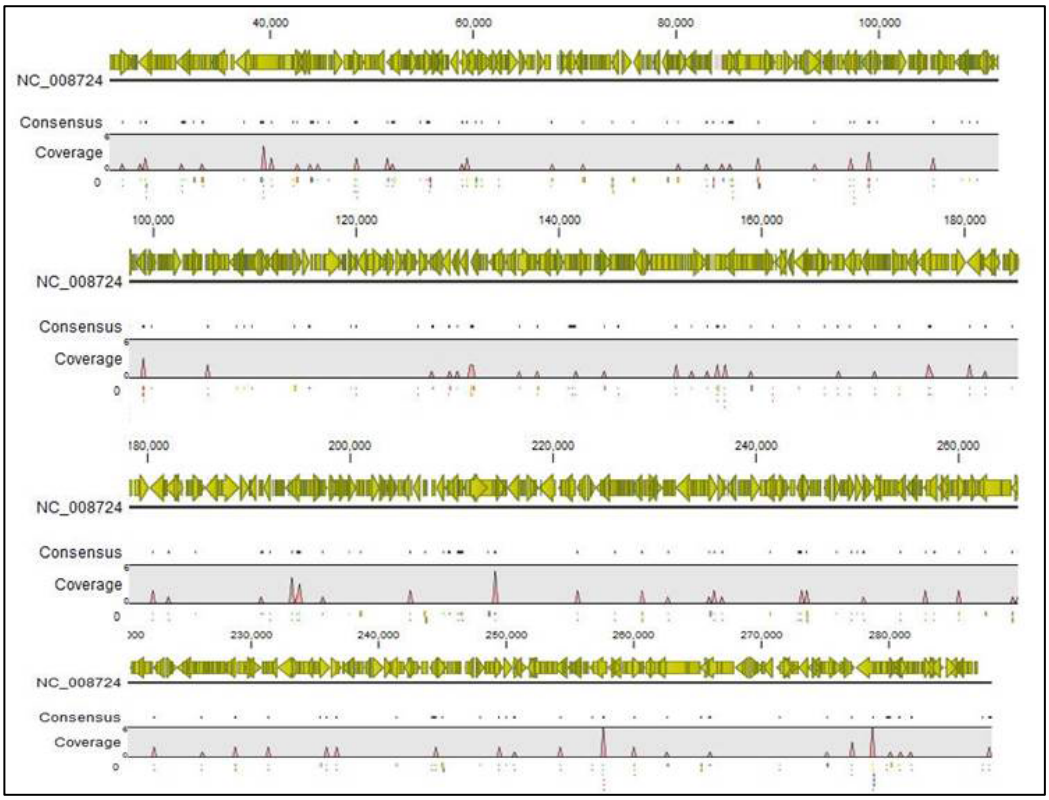

Figure 14: Sequence matches to ATCV-1 found in the blood of a 51 year old man with schizophrenia and lung cancer. The methods used were similar to those employed for ATCV-1 sequences derived from throat swab samples as described in *Yolken* et al. (2014).

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