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REVIEW ARTICLE

Viral Metagenomics: A Tool for Virus Discovery and Diversity in Aquaculture

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Abstract Viruses are abundant biological entities on earth and the emergence of viral pathogens has become a serious threat to aquaculture and fisheries worldwide. However, our response to viral pathogens has been largely reactive, in the sense that a new pathogen is usually not discovered until it has already reached epidemic proportions. Current diagnostic methods such as PCR, immunological assays and pan-viral microarrays are limited in their ability to identify novel viruses. In this context, the knowledge on the diversity of viruses in healthy and disease situations becomes important for understanding their role on the health of animals in aquaculture species. Viral metagenomics, which involves viral purification and shotgun sequencing, has proven to be useful for understanding viral diversity and describing novel viruses in new diseases and has been recognized as an important tool for discovering novel viruses in human and veterinary medicine. With the advancements in sequencing technology and development of bioinformatics tools for nucleic acid sequence assembly and annotation, information on novel viruses and diversity of viruses in marine ecosystems has been rapidly expanding through viral metagenomics. Novel circoviruses and RNA viruses in Tampa bay pink shrimp, annelovirus in sea lion, picornavirus in ringed seals and several new viruses of marine animals have been recently described using viral metagenomics and this tool has been also recently used in describing viral diversity in aquaculture ponds. Further, a large amount of information has been generated on the diversity of viruses in the marine environment using viral metagenomics during the last decade. There exists a great potential with viral metagenomics for discovering novel viruses in asymptomatic marine candidate animals of aquaculture/mariculture, some of which may assume pathogenic status under high density culture and stress. Additionally, viral metagenomics can help our understanding of viruses present in aquaculture/mariculture settings and routine pathogen surveillance programmes.

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Introduction

Viruses are the most common biological entities, at least 10 times more abundant than bacteria, with counts of the order of 10^7 and 10^9 in marine waters and sediments, accounting for as much as 94 % of nucleic acid containing particles in the marine environment [18]. Viruses are known to infect from bacteria to mammals in the marine ecosystem [58]. The discovery of viral pathogens has been largely reactive, in a sense that new pathogens are usually discovered only when they are involved in disease or an epizootic. Virus discovery till recently employed methods such as purification of viruses from infected animals by density gradient ultracentrifugation, electron microscopy followed by identification of their nucleic acids and development of molecular diagnostic techniques. However, these methods are time consuming, although successful. Proactive characterization of putative viral pathogens from aquaculture species would be advantageous, especially for disease surveillance and management of aquaculture programmes. During the last decade, with the advancements made in molecular cloning, sequencing and bioinformatic tools, structure of marine biomes, near shore sediments, novel viruses associated with infections in marine animals such as sea turtle and sea lion

have been described using metagenomic approaches [9, 14, 17, 43, 62, 63]. The term 'metagenomics' was coined by Handelsman in [35] 1998 and is defined as sequence based or function based cultivation independent analysis of collective microbial genomes in a given environment. Metagenomic methods can be applied to study viruses in any system including marine, terrestrial, and animal-associated environments. The first of the viral metagenomics was reported a decade ago, which described the uncultured near shore viral communities using shotgun cloning and sequencing [17]. Since then using viral metagenomics a number of viruses associated with cancerous tumors, nasopharyngeal samples, transplanted organs, blood and faeces of humans; terrestrial ecosystems such as deserts, prairie, rain forest soils, paddy soils and plants; extreme environments such as hyper saline environments, deep sea hydrothermal vents and hot springs; and marine and freshwater ecosystems have been described [82]. This tool has not been harnessed in aquaculture sector so far and hence, the present article provides an overview of virus discovery and viral diversity described during the recent years using metagenomic approaches and discusses the application of this technology in aquaculture and fisheries.

Viruses Causing Diseases of Marine Animals Discovered Through Conventional Tools

Viral diseases are widespread in nature and a number of them have been implicated in disease and mortality in marine mammals, finfish, and shellfish. Morbilliviruses, the RNA viruses belonging to the family paramyxoviridae are important pathogens of marine mammals [76], while herpes viruses have been reported to cause neoplastic diseases such as in fibropapillomas in marine turtles [33]. Among the viruses causing disease of finfish in marine environment include iridoviruses, which have been reported in over 140 different fish species worldwide [29]. Infectious salmon anaemia virus (ISAV), an enveloped negative sense single stranded RNA virus of the family orthomyxoviridae [44] in a significant pathogen of salmon mariculture in Norway, Scotland, Canada and the United States. Another important virus of marine fish is the one involved in viral haemorrhagic septicemia (IHN), a rhabdovirus, reported to cause epizootics in wild shoaling fish such as herrings and mackerel, and has been reported from over 50 species of fish [71]. In addition, marine finfish are also reported to be affected by herpesviruses [49] and nodaviruses [57]. Among other marine animals, molluscs are known to be affected by several viral diseases caused by iridoviruses, herpesviruses, birnaviruses and papova-like viruses and have been reported to be responsible for economic losses in Europe and Japan [58]. The most important commercial aquaculture animals, the penaeid shrimp are so far reported to suffer infections due to more than 20 viruses globally [20, 81], among which, white spot syndrome virus (WSSV), placed in a new family nimaviridae [30], yellow head virus (YHV) [86] of the family ronivirdae [30] and Taura syndrome virus (TSV) a picornavirus [37] have been largely responsible for causing serious economic losses to shrimp aquaculture [31]. Other viruses such as parvoviruses, the infectious hypodermal and hematopoietic necrosis virus (IHHNV) [12], hepatopancreatic parvovirus (HPV) [52], lymphoidal parvo-like virus (LPV) [65], baculoviruses such as *Baculovirus penaei,* monodon-type baculovirus, baculoviral midgut gland necrosis type virus [52], iridovirus [53], togaviruses such as lymphoid organ vacuolization virus (LOVV) [13], rhabdoviruses [59] and spawner isolated mortality virus [32] have been also reported to cause infections in shrimp. The number of viral pathogens from different geographical regions has been increasing with the reports of new diseases and syndromes such as infectious myonecrosis [67] and monodon slow growth syndrome [72].

Discovery of Novel Viruses of Marine Animals Using Metagenomics

A number of new viruses have been described recently in human and veterinary medicine, plant and marine sciences using viral metagenomics (Table 1). Some such important discoveries include description of a novel picornavirus (SePV1) having only 19.3–30.0 % mean protein sequence identity between the 3D polymerase gene sequence of SePV-1 and those of other picornaviruses in apparently healthy ringed seals (*Phoca hispida*), the most abundant mammal in the Arctic seas, hunted on the shore of Beaufort Sea [43]. Sea turtle tornovirus 1 (STTV1) and California sea lion anellovirus (ZcAV) both sharing only a limited sequence similarity to previously described viral genomes have been recently discovered [62, 63]. STTV1 was discovered from the fibropapilloma of a Florida green sea turtle, having a single-stranded, circular genome of ~1,800 nucleotides in length, and had very low amino acid identity with chicken anemia virus [62]. ZcAV was an anellovirus discovered from the lung of a California sea lion that died in a respiratory-related mortality event, had a single-stranded circular genome of 2,140 nucleotides, with only 35 % amino acid identity to feline anelloviruses in the ORF1 region [63]. Recently, a novel circovirus and two novel noda viruses from asymptomatic wild shrimp (*Penaeus duorum*) from Tarpon Springs, Florida, were discovered using metagenomic sequencing of DNA and RNA viruses purified from these shrimp [61]. The novel shrimp circovirus had a single-stranded DNA genome of 1,955 nucleotides and shared

<50 % amino acid identity to any known viruses in the Genbank database. All previously described circoviruses are reported to infect birds or pigs and represented a phylogenetic branch distinct from the known avian and porcine circoviruses, making this shrimp circovirus the first circovirus described in an invertebrate. Two novel nodaviruses, which shared less than 60 % amino acid identity to known shrimp nodaviruses and likely represent a novel virus genus, were also identified in the shrimp [61]. In aquaculture setting, the only work using such an approach of random shotgun cloning and sequencing has been the description of Laem Singh virus (LSNV), associated with monodon slow growth syndrome of black tiger shrimp by Sritunyalucksana and coworkers [72], and the new virus was reported to be an RNA virus showing significant deduced amino acid sequence similarity to RNA-dependent RNA polymerases (RdRp) of the viruses in the family *Luteoviridae*.

Diversity of Viruses in Marine Ecosystem

Viral metagenomics started with the publication describing viral diversity in seawater in a landmark paper by Breitbart et al. [17]. Extrapolating from about 1,000 cloned sequences they reported between 300 and 7,000 new viral types in seawater, among which, 35 % were phages. They reported that the metagenomic sequences comprised repeat and mobile elements, bacteria, archaea and eukarya, and that a majority of them included major families of dsDNA phages and over 65 % of the viral sequences generated were not significantly similar to known viruses. A similar study of dsDNA viruses in near shore sediments indicated much phylogenetic overlap with seawater bacteriophages and the presence of at least 104 distinct genotypes per kilogram of sediments [14]. Subsequently, the global ocean sampling (GOS) Expedition, by Craig Venter in 2003, was one of the important projects exploring the marine metagenome on a larger scale (Table 1). Angly and coworkers [8] based on metagenomic analyses of 184 viral assemblages collected over a decade and representing 68 sites in four major oceanic regions reported that 60–80 % of the metagenomic sequences were not similar to the viral sequences in the current Genbank databases. Global diversity was very high, presumably several hundred thousand of species, and regional richness varied on a North–South latitudinal gradient. The marine regions had different assemblages of viruses. Cyanophages and a newly discovered clade of single-stranded DNA phages dominated the Sargasso Sea, whereas prophage-like sequences were most common in the Arctic. Most viral species were found to be widespread and the difference between viral assemblages was attributed to variation in the occurrence of the most common viral species and not by exclusion of different viral genomes [8]. They reported that no RNA bacteriophages were detected indicating that most marine bacteriophages have DNA genomes and that most hosts of marine RNA viruses may be eukaryotes. Studies on the RNA viruses in the ocean are relatively scanty. RNA viruses are known to infect marine organisms from bacteria to whales, but RNA virus communities in the sea remain less investigated. Using reverse-transcribed whole-genome shotgun sequencing Culley and his colleagues [23] reported a diverse assemblage of previously unknown RNA viruses including a broad group of marine picorna-like viruses, and distant relatives of viruses infecting arthropods and higher plants. Occurrence of a diverse array of picorna-like viruses in the ocean was reported based on the analysis of conserved sequences of RdRp sequences amplified from marine virus communities [22]. Among the viral types recorded by them, they also reported occurrence of a lytic pathogen of *Heterosigma akashiwo*, a toxic-bloom-forming alga responsible for severe economic losses to the finfish aquaculture industry.

Viral Metagenomics Methods

There is no single gene common to all viruses, analogous to bacterial ribosomal profiling, which posed difficulties, until the advent of improved tools of cloning and sequencing for understanding their diversity and discovering new species [28]. The first of the viral metagenomics was described a decade back, which described the uncultured near shore viral communities using shotgun cloning and sequencing [17]. These earliest DNA viral metagenomes were linker-amplified shotgun libraries (LASLs), created by ligating dsDNA linkers to genomic DNA fragments and cloning them into a vector plasmid for subsequent Sanger sequencing [15, 17, 69]. LASLs were initially limited to dsDNA viruses. However with the advent of strand-displacement amplification using Phi29 polymerases, the technology was extended to ssDNA viruses [16, 45]. The first RNA viral metagenomes were generated using random RT-PCR primers linked to adaptor sequences for cDNA synthesis [5].

The methods of shotgun sequencing for virus discovery, sequence independent virus discovery and viral diversity have been recently reviewed [6, 74]. The basic steps involved in viral metagenomics include preparation of viral nucleic acid that is free from host and contaminating nucleic acids, sequence independent amplification of viral nucleic acid, sequencing and finally use of bioinformatics tools for analysis of sequences generated. In any given sample, the viral nucleic acid constitutes a very small proportion. Viral isolation and purification protocols use filtration techniques using a 0.22 μm filter to remove bacteria and larger organisms. However, larger viruses

Table 1 Discovery of viruses in humans, terrestrial and marine animals, plants and insects and understanding viral ecology using metagenomics

Objective	Finding	Reference
Discovery of viruses in human diseases		
To develop a method for viral identification in human blood by physical properties dependent selection of viruses combined with sequence-independent amplification and cloning for identification of novel viruses in human blood	Discovery of novel anellovirus sequences in the blood of healthy donors	[16]
Metagenomic analysis of samples from patients with unexplained illness that exhibited reproducible cytopathogenic effects in cell culture and tested negative in standard PCR assays	A range of viruses such as BK polyomavirus, herpes simplex virus, Newcastle disease virus and the recently discovered Saffold viruses (SAFV) detected in the samples	[73]
To identify viruses associated with acute viral infection syndrome using DNase-SISPA (sequence-independent single primer amplification)	Novel parvovirus distinct from known human and animal parvoviruses and of two related TTV-like viruses highly divergent from both the TTV and TTV-like minivirus in human blood identified	[42]
Metagenomic virus screening of human nasopharyngeal aspirates	Identified a novel human KI polyomavirus having very little homology (<30 % amino acid identity) to known polyomaviruses	[3]
Determine the etiologic agent of a hemorrhagic fever outbreak in humans in South Africa characterized by nosocomial transmission and an unprecedented high case fatality rate by pyrosequencing	Identified an RNA virus, Lujo virus (LUJV), a new member of the family *Arenaviridae*	[19]
Identify etiologic agents associated with diarrhea in children by pyrosequencing	Discovery of human klassevirus 1, a member of a novel picornavirus genus, in stool from two infants from Northern California	[34]
Demonstrate the utility of a high-throughput sequencing approach to detect human viral pathogens in nasopharyngeal and fecal samples collected during seasonal influenza virus infections and norovirus outbreaks	Unbiased high-throughput sequencing approach is useful for directly detecting pathogenic viruses without advance genetic information in fecal and nasopharygeal samples	[60]
Identify etiologic agents in stool samples from children with acute flaccid paralysis by pyrosequencing	Novel viruses belonging to *Picornaviridae* and four new viral species (members of the *Dicistroviridae*, *Nodaviridae*, and *Circoviridae* families and the *Bocavirus* genus) discovered	[79]
Identification of viruses in human feces and sewer effluent in samples that failed to detect viruses by cell culture, serological, or nucleic acid tests	Identification of known and highly divergent new RNA viruses belonging to the *Picornaviridae* family showing between 80 and 91 % amino acid identities to known picornaviruses	[80]
Discovery of viruses in terrestrial animals and birds		
Identify viruses using random amplification of particle-associated nucleic acids in Poult enteritis mortality syndrome (PEMS) of turkeys and runting-stunting syndrome (RSS) of chickens	Discovery of parvovirus DNA sequences in intestinal homogenates of affected birds. Sequence analysis of complete non-structural gene, revealed that the chicken and turkey parvoviruses were closely related to each other and were representative of a novel member of the Parvovirus family	[88]
To understand the range of viruses carried by common bats in the United States	Full and partial descriptions of viral genomes identified using metagenomics in the guano of bats from California and Texas. A total of 34–58 % of viral sequences generated were related to eukaryotic viruses, and largest proportion of which infect insects, reflecting the diet of these insectivorous bats, including members of the viral families *Dicistroviridae*, *Iflaviridae*, *Tetraviridae*, and *Nodaviridae* and the subfamily *Densovirinae*	[51]
Analysis for Viral particles in stool samples from wild-living chimpanzees using random PCR amplification and sequencing	Sequences encoding proteins distantly related to the replicase protein of single-stranded circular DNA viruses were identified. The viral genomes were related in size and genome organization to vertebrate circoviruses and plant geminiviruses, named them chimpanzee stool-associated circular viruses (ChiSCV)	[10]

Table 1 continued

Objective	Finding	Reference
To investigate the virus associated with stillborn foetuses and deaths of 3–4-week-old piglets using sequence independent single primer amplification (SISPA)	Identified a novel virus associated with stillborn foetuses and deaths of 3–4-week-old piglets. Conserved 5′ UTR motifs, the presence of a putative N^{pro} coding region and limited antigenic cross-reactivity with other members of the *Pestivirus* genus. Phylogenetic analysis of the 5′UTR, N^{pro} and E2 coding regions showed this virus to be the most divergent pestivirus identified to date	[46]
Investigate the cause of neurological disorder of unknown etiology (Shaking Mink Syndrome) of farmed mink kits in Denmark	Detected an astrovirus in the brain tissue of naturally and experimentally infected mink kits using random amplification and large-scale sequencing. The complete coding region of this astrovirus showed 80.4 and 52.3 % identity with sequences of mink astrovirus associated with preweaning diarrhea and to a recently discovered human astrovirus associated with a case of encephalitis in a boy with x-linked agammaglobulinemia respectively	[11]
Genetic characterization of two novel, divergent simian hemorrhagic fever virus (SHFV) variants co-infecting male red colobus monkey from Kibale National Park, Uganda, by direct pyrosequencing	Two new SHFV variants, SHFV-krc1 and SHFV-krc2 highly divergent from each other (51.9 % nucleotide sequence identity) and from the SHFV type strain LVR 42-0/M6941 (52.0–51.8 % nucleotide sequence identity, respectively) with greater phylogenetic diversity within SHFV discovered	[50]
Identify etiologic agents in parrots with proventricular dilation disease by pyrosequencing	Two strains of a novel Borna viruses in psittacine birds discovered	[39]
Discovery of viruses in plants		
Develop a diagnostic tool to detect viral Pathogens in plants such as *Gomphrena globosa* using pyrosequencing	Discovery of a new cucumovirus, named as ‘Gayfeather mild mottle virus’, and sequencing of full viral genome	[1]
In a search for viruses associated with decline symptoms of Syrah grapevines, by analysis of total plant RNA sequences using Life Sciences 454 high-throughput sequencing	Detection of mixed viral infection that included seven different RNA genomes associated with Syrah grapevine decline symptoms	[2]
Discovery of viruses in insects		
To survey microflora and identify candidate pathogens in *Apis mellifera* honeybee colony collapse disorder (CCD) hives, normal hives, and imported royal jelly by metagenomic approach	Identified seven positive-sense single-stranded RNA viruses previously associated with disease in honey bees, including members of the family *Dicistroviridae* and the genus Iflavirus Israeli acute paralysis virus. Two iflaviruses, Sacbrood virus (SBV) and Deformed wing virus (DWV), as well as two dicistroviruses, Black queen cell virus (BQCV) and Acute bee paralysis virus (ABPV), were found in both CCD and non-CCD operations	[21]
Characterization of viruses of *Culex*, *Culiseta*, and *Ochlerotatus* mosquitoes from Spain	Identification of three novel flaviviruses Marisma Mosquito virus, *Ochlerotatus flavivirus* and Spanish *Culex flavivirus* followed by development of a sensitive RT-nested polymerase chain reaction for surveillance of flavivirus directly in clinical or environmental samples	[78]
Discovery of viruses in marine animals		
To identify the causative agent of filtrates showing CPE in Vero cells recovered from ringed seals hunted in 2000–2002 on the shore of the Beaufort Sea using sequence independent amplification	Discovery of a novel picornvirus (SePV-1) in ringed seals (*Phoca hispida*), deciphering full genome sequence of a novel picornavirus	[43]
To investigate viral pathogens potentially involved in a mortality event of 21 Pacific harbor seals (*Phoca vitulina richardsii*) in California in 2000 using viral metagenomics	Discovery of a novel seal anellovirus (SealAV), which clustered phylogenetically with anelloviruses from California sea lions and domestic cats.	[64]
Identify viruses associated with fibropapillomas in sea turtles, a debilitating neoplastic disease affecting sea turtles	Discovery of novel single-stranded DNA virus, STTV1 having circular genome of $\sim$1,800 nucleotides	[62]

Table 1 continued

Objective	Finding	Reference
To investigate putative viral pathogens associated with a mortality event of three captive California sea lions (*Zalophus californianus*)	Identified a novel California sea lion anellovirus (ZcAV), with 35 % amino acid identity in the ORF1 region to feline anelloviruses associated with the death of these animals	[63]
Identify viruses in apparently normal shrimp (*Penaeus duorarum*) caught from Tampa Bay (Western Florida)	Discovery of a novel circovirus having a single-stranded DNA genome of 1,955 nucleotides and shared <50 % amino acid identity to any known viruses in the Genbank and two novel noda viruses which shared <60 % amino acid identity to known shrimp nodaviruses	[61]
Assess differences between viral communities associated with healthy and diseased corals Diploria strigosa by metagenomic analyses	Herpes-like sequences accounted for 4–8 % of the total sequences in each metagenome. Viruses similar to those that infect algae and plants were also present in the coral viral assemblage. Cyanophages were abundant in both healthy and bleaching corals along with vibriophages	[55]
Viral ecology in coastal and marine ecosystems		
Comparative metagenomic analyses of as many as 15 million microbial and viral metagenomic sequences to elucidate the functional potential of subterranean (mine samples), hypersaline ponds from solar salterns, marine, freshwater, coral-associated, microbialites (including stromatolites and thrombolites), *aquaculture-fish-associated*, terrestrial-animal-associated and mosquito-associated biomes	Differences between metagenomes indicated strong discriminatory metabolic profiles across environments and the biogeochemical conditions of each environment. The magnitude of the microbial metabolic capabilities encoded by the viromes was extensive and influenced global evolutionary and metabolic processes Identified overrepresented sulphur metabolisms (alkanesulphonate and taurine metabolism) in fish-associated metagenomes in aquaculture ponds	[27]
Characterize the diversity of uncultured marine viral communities	Majority of the viruses belonged to major families of dsDNA tailed phages, and some algal viruses. Over 65 % of the viral sequences were not significantly similar to previously reported sequences in offshore waters	[17]
Metagenomic analysis of uncultured near-shore marine-sediment viral communities	Majority of the viruses in marine sediments belonged to double-stranded DNA phage, predominated by lysogenic forms	[14]
Characterize viral distribution and diversity in four oceanic regions by pyrosequencing	Domination of cyanophages and a newly discovered clade of single-stranded DNA phages in the Sargasso Sea; prophage-like sequences in the Arctic	[8]
Characterize the diversity of marine RNA viruses from off coast of Vancouver, British Columbia	A diverse assemblage of RNA viruses, including picorna-like viruses, and distant relatives of viruses infecting arthropods and higher plants	[23]
Distribution of phage communities by comparative metagenomic analysis of viral communities associated with marine and freshwater microbialites	Occurrence of genetically unique phages in microbialites: Highborne Cay metagenome were dominated by single-stranded DNA microphages that were not detected in any other samples examined, including sea water, fresh water, sediment, terrestrial, extreme, metazoan-associated and marine microbial mats Detection of marine signature in the phage community of the Pozas Azules II microbialites, which has not been in contact with the ocean for tens of millions of years	[26]

such as giruses [77] may be eliminated in this step. Cesium chloride gradients have been commonly used to separate viral particles from free DNA and cellular material based on buoyant density [74]. While refined methods enrich and selection, at each step viruses can be lost decreasing the overall viral diversity detected [74]. Hence, viral metagenomes have been generated using filtration techniques to remove larger non-viral particles, without subsequent density centrifugation step. Metagenomic protocols also include steps to remove host genomic DNA/RNA. Viral preparations derived from plants or animals can be contaminated with DNA from host organisms, microbial flora, and microbial DNA from reagents [4]. DNAse treatment is commonly used to degrade unwanted free DNA prior to extraction and amplification of viral nucleic acid [4, 74]. While DNAse treatment does reduce the amount of contaminating DNA in viral samples, it does not completely remove it, and the use of bioinformatic filters may be necessary after sequencing [4]. The purity of viral nucleic acid preparations can be examined especially for contaminating bacterial and host genomic nucleic acids by PCR.

The amount of total nucleic acids isolated from viral particles is often too low for sequencing, and may require amplifying viral nucleic acid depending on the sequencing technology used. The viral genomes present in the nucleic acid sample are simultaneously amplified independent of their sequences and these methods have been reviewed recently [6, 25]. Sequence-independent of amplification of nucleic acids can be achieved by methods such as sequence-independent single-primer amplification (SISPA), random PCR or displacement amplification. In SISPA, adapters are ligated to DNA or cDNA to enable sequence-independent amplification [68]. The random PCR method involves use of a primer consisting of known adapter sequence at 5′ end and a degenerate hexa or heptamer at 3′ end during the cDNA synthesis step, when the cDNA is labeled with adapter sequences at both ends. A similar treatment can be done with DNA sample, enabling primer targeted sequencing. Multiple displacement amplification uses random primers in combination with high fidelity displacement polymerase such as Phi29 polymerase, enabling rolling circle amplification of nanogram to microgram quantities of nucleic acid of total viral DNA or cDNA, and generate adequate template for sequencing [24, 41, 75]. Viral RNA may also be amplified using whole transcriptome amplification methods [74, 75].

Laboratory methods such as pulsed field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD-PCR) assays have been used to quantify viral richness [38, 84, 85]. However, these methods do not always provide an accurate assessment of diversity, e.g. one band can represent multiple genomes in PFGE [84].

Metagenomic Sequencing

Metagenomic sequencing technologies differ in library preparation methods and the length of reads produced. An important approach is to construct viral shotgun libraries for sequencing by Sanger's method as proposed by Breitbart and Rohwer [16]. Sanger's sequencing method yields high quality sequence data with read lengths about 400 base pairs. The next generation high-throughput pyrosequencing by 454 Life Sciences provide greater amounts of sequence data compared to Sanger's sequencing method, enabling detection of even low number of viruses present in the sample [56] with no need of cloning [54]. The recent 454 Life Sciences GSFLX system provides read lengths ranging from 250 to 400 base pairs and is widely used. Even higher throughput technologies such as Solexa/Illumina and SOLiD systems are now available, which can provide data of as much as 3–6 Gb per run. However, the average read length obtained with these systems is about 50–100 bp [66].

Bioinformatics for viral metagenomics

Bioinformatic analyses of viral metagenomes attempts to answer three questions: how many viruses are there (diversity), 'what are they (taxonomy), and what are they doing (function)? [83]. The sequence data generated need to be first assembled. Bioinformatic screens to identify and filter out host and other non-viral sequences from metagenomes should also be included. Several software such as MEGAN [40], PathSeq [47] and CAMERA [70] etc. are available for analysis of metagenomic sequences. Several software Packages are available to rapidly parse and visualize BLAST results, and also to assist in taxonomic assignment. Bioinformatic tools compare the viral sequences generated with known sequences maintained in an annotated database such as NCBI using the programmes such as Blastn and Blastx to assign taxonomy (by comparing with known viral sequences and indicate divergent ones) and function to metagenomic sequences. MEGAN (http://ab.inf.uni-tuebingen.de/software/megan/) assigns metagenomic sequences to NCBI taxonomic classes based on significant BLAST similarities, and assigns taxonomy at the lowest (i.e. most specific) level possible using a least common ancestor algorithm [40]. The genome relative abundance and average size (GAAS) (http://www.sourceforge.net/GAAS) metagenomic tool also uses BLAST similarities to assign taxonomy. GAAS provides a set of viral community relative abundances based on all BLAST similarities for all sequences [9]. GAAS also normalizes for the length of the target genome in the database, which provides more accurate estimates of community composition [9]. The Community Cyber infrastructure for

Advanced Marine Microbial Ecology Research and Analysis (CAMERA) project (http://camera.calit2.net.) was established for attempting to bridge gaps and in developing global methods for monitoring microbial communities in the ocean. CAMERA's database includes environmental metagenomic and genomic sequence data, associated environmental parameters ("metadata"), pre-computed search results, and software tools to support powerful cross-analysis of environmental samples. However, these tools do not help detection of completely novel viruses since viral metagenomes often contain a large number of sequences with no similarity to known sequences, as reported in microbialite viromes, where, unknown sequences accounted for 99 % [26]. Characterizing Short Read Metagenomes (CARMA) (http://www.cebitec.uni-bielefeld.de/brf/carma/carma.html) is a software for characterizing the taxonomic composition and genetic diversity of short-read metagenomes and was originally designed for the analysis of environmental metagenomes obtained by the ultra-fast 454 pyrosequencing system [48]. Functional annotation for viral metagenomes may be assigned by BLAST analyses to a small percentage of viral sequences derived. There are other methods available for functional annotation, including profile Hidden Markov Model approaches [87] and gene neighborhood analysis [36]. Viral diversity and community structure cannot usually be determined from BLAST comparisons, since many metagenomic sequences have no significant similarities to known organisms. PHAge Communities from Contig Spectrum (PHACCS) implements mathematical models to determine viral community structure and calculate alpha diversity measures from contig spectra [7, 17]. When metagenomic sequences are assembled, overlapping sequences are grouped together to form contigs, or contiguous sequences. PHACCs can be used from a web interface (http://biome.sdsu.edu/phaccs/). The programme takes four inputs: the calculated contig spectrum, the average fragment size in the metagenomic library, the minimum overlap length, and the average genome size [7, 9]. PHACCs tests several viral community structure models, and outputs the best fit model, along with estimated species richness, evenness, and the Shannon diversity index [7].

Concluding Remarks

Viral metagenomics has been recognised as an important tool in human, veterinary medicine and has contributed significantly in discovering novel viruses associated with disease in humans and animals. A great deal of information has been generated on the diversity of viruses in marine ecosystems and a number of new viruses associated with disease in marine animals have been discovered using this tool. So far, to our knowledge, description of LSNV associated with monodon slow growth syndrome in farmed shrimp in Thailand is the only one study that has used shotgun cloning and sequencing in aquaculture [72]. Recently two novel circoviruses and a nodavirus have been identified in apparently healthy shrimp from western Florida using viral metagenomics [61]. Such proactive characterization of putative viral pathogens from aquaculture species before epidemics occur will have tremendous advantages, benefitting both disease monitoring efforts and aquaculture management. It is time that this tool is utilised in understanding the viral diversity and putative viral pathogens in aquaculture settings. Such information can have implications on the biosecurity in aquaculture. Further, understanding viral ecology in aquaculture ecosystem can throw light on the role of viruses in the food web dynamics in the aquaculture ecosystems.

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References

1. Adams IP, Glover RH, Monger WA, Mumford R, Jackeviciene E, Navalinskiene M, Samuitiene M, Boonham N. Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Mol Plant Pathol. 2009;10:537–45.
2. Al Rwahnih M, Daubert S, Golino D, Rowhani A. Deep sequencing analysis of RNAs from a grapevine showing Syrah decline symptoms reveals a multiple virus infection that includes a novel virus. Virology. 2009;387:395–401.
3. Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B. Identification of a third human polyomavirus. J Virol. 2007;81:4130–6.
4. Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. Proc Natl Acad Sci USA. 2001;98:11609–14.
5. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Anderson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci USA. 2005;102:12891–6.
6. Ambrose HE, Clewley JP. Virus discovery by sequence independent genome amplification. Rev Med Virol. 2006;16:365–83.
7. Angly F, Rodiguez-Brito B, Bangor D, McNairnie P, Breitbart M, Salamon P, Felts B, Nulton J, Mahaffy J, Rohwer F. PHACCS, an online tool for estimating the structure and diversity of uncultured viral communities using metagenomic information. BMC Bioinformatics. 2005;6:41.
8. Angly FE, Felts B, Breitbart M, Salamon P, Edwards RA, Carlson C, Chan AM, Haynes M, Kelley S, Liu H, Mahaffy J, Mueller JE, Nulton J, Olson R, Parsons R, Rayhawk S, Suttle CA, Rohwer F. The marine viromes of four oceanic regions. PLoS Biol. 2006;4:e368.
9. Angly FE, Willner D, Prieto-Davó A, Edwards RA, Schmieder R, Vega-Thurber R, Antonopoulos DA, Barott K, Cottrell MT, Desnues C, Dinsdale EA, Furlan M, Haynes M, Henn MR, Hu Y, Kirchman DL, McDole T, McPherson JD, Meyer F, Miller RM, Mundt E, Naviaux RK, Rodriguez-Mueller B, Stevens R, Wegley L, Zhang L, Zhu B, Rohwer F. The GAAS metagenomic tool and

its estimations of viral and microbial average genome size in four major biomes. PLoS Comput Biol. 2009;5:e1000593.
10. Blinkova O, Victoria J, Li Y, Keele BF, Sanz C, Ndjango JBN, Peeters M, Travis D, Lonsdorf EV, Wilson ML, Pusey AE, Hahn BH, Delwart EL. Novel circular DNA viruses in stool samples of wild-living chimpanzees. J Gen Virol. 2010;91:74–86.
11. Blomstrom AL, Widen F, Hammer AS, Belak S, Berg M. Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. J Clin Microbiol. 2010;48:4392–6.
12. Bonami RJ, Trumper B, Mari J, Brehelin M, Lightner DV. Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. J Gen Virol. 1990;71:2657–64.
13. Bonami JR, Lightner DV, Redman RM, Poulos BT. Partial characterization of a togavirus (LOVV) associated with histopathological changes of the lymphoid organ of penaeid shrimps. Dis Aquat Organ. 1992;14:145–52.
14. Breitbart M, Felts B, Kelley S, Mahaffy JM, Nulton J, Salamon P, Rohwer F. Diversity and population structure of a near-shore marine-sediment viral community. Proc Royal Soc London B. 2004;271:565–74.
15. Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P, Rohwer F. Metagenomic analyses of an uncultured viral community from human feces. J Bacteriol. 2003;185:6220–3.
16. Breitbart M, Rohwer F. Method for discovering novel DNA viruses in blood using viral particle selection and shotgun sequencing. Biotechniques. 2005;39:729–36.
17. Breitbart M, Salamon P, Andresen B, Mahaffy JM, Segall AM, Mead D, Azam F, Rohwer F. Genomic analysis of uncultured marine viral communities. Proc Natl Acad Sci USA. 2002;99:14250–5.
18. Breitbart M. Marine Viruses: Truth or Dare. Annu Rev Mar Sci. 2011; doi:10.1146/annurev-marine-120709-142805.
19. Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, Khristova ML, Weyer J, Swanepoel R, Egholm M, Nichol ST, Lipkin WL. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated Arenavirus from Southern Africa. PLoS Pathog. 2009;5(5):e1000455.
20. Briggs M, Funge-Smith S, Subasinghe, RP, Phillips M. Introductions and movement of two penaeid shrimp species in Asia and the Pacific. FAO Fish Tech Paper 476, FAO Rome, 2005; ftp://ftp.fao.org/docrep/fao/008/a0086e/a0086e00.pdf.
21. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan P, Briese T, Hornig M, Geiser DM, Martinson V, van Engelsdorp D, Kalkstein AL, Drysdale A, Hui J, Zhai J, Cui L, Hutchison SK, Simons JF, Egholm M, Pettis JS, Lipkin WI. A metagenomic survey of microbes in honey bee colony collapse disorder. Science. 2007;318:283–7.
22. Culley AI, Lang AS, Suttle CA. High diversity of unknown picorna-like viruses in the sea. Nature. 2003;424:1054–7.
23. Culley AI, Lang AS, Suttle CA. Metagenomic analysis of coastal RNA virus communities. Science. 2006;312:1795–8.
24. Dean FB, Nelson JR, Giesler TL, Lasken RS. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res. 2001;1:1095–9.
25. Delwart EL. Viral metagenomics. Rev Med Virol. 2007;17:115–31.
26. Desnues C, Rodriguez-Brito B, Rayhawk S, Kellev S, Trati T, Havnes M, Liu H, Furlan M, Weglev L, Chau B, Ruan Y, Hall D, Anglv FE, Edwards RA, Li L, Thurber RV, Reid RP, Siefert J, Souza V, Valentine DL, Swan BK, Bieitbart M, Rohwer F. Biodiversity and biogeography of phages in modern stromatolites and thromatolites. Nature. 2008;452:340–3.
27. Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, Brulc JM, Furlan M, Desnues C, Haynes M, Li L, McDaniel L, Moran MA, Nelson KE, Nilsson C, Olson R, Paul J, Brito BR, Ruan Y, Swan BK, Stevens R, Valentine DL, Thurber RV, Wegley L, White BA, Rohwer F. Functional metagenomic profiling of nine biomes. Nature. 2008;452:629–32.
28. Edwards RA, Rohwer F. Viral metagenomics. Nat Rev Microbiol. 2005;3:504–10.
29. Essbauer S, Ahne W. Viruses of lower vertebrates. J Vet Med (B) Infect Dis Vet Public Health. 2001;48:403–75.
30. Fauquet CM, Mayo MA, Maniloj J, Desselberger U, Ball LA. Virus Taxonomy, VIII Report of the ICTV. London: Elsevier/Academic Press, 2005.
31. Flegel TW. Special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microbiol Biotechnol. 1997;13:433–42.
32. Fraser CA, Owens L. Spawner-isolated mortality virus from Australian *Penaeus monodon*. Dis Aquat Organ. 1996;27:141–8.
33. Greenblatt RJ, Sutton CA, Casey RN, Casey JW, Work TM, Balazs GH. The Ozobranchus leech is a candidate mechanical vector for the fibropapilloma-associated turtle herpesvirus found latently infecting skin tumors on Hawaiian green turtles (*Chelonia mydas*). Virology. 2004;321:101–10.
34. Greninger AL, Runckel C, Chiu CY, Haggerty T, Parsonnet J, Ganem D, DeRis JL. The complete genome of klassevirus—a novel picornavirus in pediatric stool. Virol J. 2009;6:82.
35. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol. 1998;5:245–9.
36. Harrington ED, Singh AH, Doerks T, Letunic I, von Mering C, Jensen U, Raes J, Bork P. Quantitative assessment of protein function prediction from metagenomics shotgun sequences. Proc Natl Acad Sci USA. 2007;104:13913–8.
37. Hasson KW, Lightner DV, Poulos BT, Redman RM, White BL, Brock JA, Bonami JR. Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology. Dis Aquat Organ. 1995;23:115–26.
38. Helton RR, Wommack KE. Seasonal dynamics and metagenomic characterization of estuarine viriobenthos assemblages by randomly amplified polymorphic DNA PCR. Appl Environ Microbiol. 2009;75:2259–65.
39. Honkavuori KS, Shivaprasad HL, Williams BL, Quan PL, Hornig M, Street C, Palacios G, Hutchison SK, Franca M, Egholm M, Briese T, Lipkin WI. Novel borna virus in psittacine birds with proventricular dilatation disease. Emerg Infect Dis. 2008;14:1883–6.
40. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007;17:377–86.
41. Johne R, Muller H, Rector A, van Ranst M, Stevens H. Rolling-circle amplification of viral DNA genomes using phi29 polymerase. Trends Microbiol. 2009;17:205–11.
42. Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E. New DNA viruses identified in patients with acute viral infection syndrome. J Virol. 2005;79:8230–6.
43. Kapoor A, Victoria J, Simmonds P, Wang C, Shafer RW, Nims R, Nielsen O, Delwart E. A highly divergent picornavirus in a marine mammal. J Virol. 2008;82:311–20.
44. Kibenge FS, Munir K, Kibenge MJ, Joseph T, Moneke E. Infectious salmon anemia virus: causative agent, pathogenesis and immunity. Anim Health Res Rev. 2004;5:65–78.
45. Kim K, Chang H, Nam Y, Rob SW, Kim M, Sting Y, Jeon CO, Oh H, Bae J. Amplification of uncultured single-stranded DNA viruses from rice paddy soil. Appl Environ Microbiol. 2008;74:5975–85.

46. Kirkland PD, Frost MJ, Finlaison DS, King KR, Ridpath JF, Gu X. Identification of a novel virus in pigs-Bungowannah virus: a possible new species of pestivirus. Virus Res. 2007;129:26–34.
47. Kostic AD, Ojesina AI, Pedamallu CS, Jung J, Verhaak RG, Getz G, Meyerson M. PathSeq: software to identify or discover microbes by deep sequencing of human tissue. Nat Biotechnol. 2011;29:393–6.
48. Krause L, Diaz NN, Goesmann A, Kelley S, Nattkemper TW, Rohwer F, Edwards PA, Stoye J. Phylogenetic classification of short environmental DNA fragments. Nucleic Acids Res. 2008; 36:2230–9.
49. Kumagai A, Takahashi K, Fukuda H. Infection source of herpesvirus disease in coho salmon culture and its control. Fish Pathol. 1997;32:103–8.
50. Lauck M, Hyeroba D, Tumukunde A, Weny G, Lank SM, Chapman CA, O'Connor DH, Friedrich TC, Goldberg TL. Novel, divergent simian hemorrhagic fever viruses in a wild ugandan red colobus monkey discovered using direct pyrosequencing. PLoS ONE. 2011;6:e19056. doi:10.1371/journal.pone.0019056.
51. Li L, Victoria JG, Wang C, Jones M, Fellers GM, Kunz TH, Delwart E. Bat Guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. J Virol. 2010;84:6955–65.
52. Lightner DV. A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. Baton Rouge: World Aquaculture Society; 1996.
53. Lightner DV, Redman RM. A putative iridovirus from the penaeid shrimp *Protrachypene precipua* Burkenroad (Crustacea: Decapoda). J Invertebr Pathol. 1993;62:107–9.
54. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen Y, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzvk GP, Jando SC, Alenquer MLI, Jarvie TP, Jirage KB, Kim J, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. Genome sequencing in microfabricated high-density picolitre reactors. Nature. 2005;437:376–80.
55. Marhaver KL, Edwards RA, Rohwer F. Viral communities associated with healthy and bleaching corals. Environ Microbiol. 2008;10:2277–86.
56. Metzker ML. Sequencing technologies—the next generation. Nat Rev Genet. 2010;11:31–46.
57. Munday BL, Kwang J, Moody N. Betanodavirus infections of teleost fish: a review. J Fish Dis. 2002;25:127–42.
58. Munn CB. Viruses as pathogens of marine organisms-from bacteria to whales. J Mar Biological Assn UK. 2006;86:453–67.
59. Nadala ECB Jr, Lu Y, Loh PC, Brock JA. Infection of *Penaeus stylirostris* (Boone) with a rhabdovirus isolated from *Penaeus* spp. Fish Pathol. 1992;27:143–7.
60. Nakamura S, Yang C-S, Sakon N, Ueda M, Tougan T, Yamashita A, Goto N, Takahashi K, Yasunaga T, Ikuta K, Mizutani T, Okamoto Y, Tagami M, Morita R, Maeda N, Kawai J, Hayashizaki Y, Nagai Y, Horii T, Iida T, Nakaya T. Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. PLoS ONE. 2009;4(1):e4219.
61. Ng TF, Alavandi SV, Breitbart M. Metagenomic studies reveal novel viruses in shrimp. Sixth International symposium on 'Aquatic animal health, Global strategies for a changing Environment' Sep 5–9, 2010 Tampa.
62. Ng TF, Manire C, Borrowman K, Langer T, Ehrhart L, Breitbart M. Discovery of a novel single-stranded DNA virus from a sea turtle fibropapilloma using viral metagenomics. J Virol. 2009;83: 2500–9.
63. Ng TF, Suedmeyer WK, Wheeler E, Gulland F, Breitbart M. Novel anellovirus discovered from a mortality event of captive California sea lions. J Gen Virol. 2009;90:1256–61.
64. Ng TF, Wheeler E, Greig D, Waltzek TB, Gulland F, Breitbart M. Metagenomic identification of a novel anellovirus in Pacific harbor seal (*Phoca vitulina richardsii*) lung samples and its detection in samples from multiple years. J Gen Virol. 2011;92:1318–23.
65. Owens L, De Beer S, Smith J. Lymphoidal parvovirus-like particles in Australian penaeid prawns. Dis Aquat Organ. 1991;11: 129–34.
66. Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. J Appl Genet. 2011;52:413–35.
67. Poulos BT, Tang KFJ, Pantoja CR, Bonami JR, Lightner DV. Purification and characterization of infectious myonecrosis virus of penaeid shrimp. J Gen Virol. 2006;87:987–96.
68. Reyes GR, Kim JP. Sequence-independent, single primer amplification (SISPA) of complex DNA populations. Mol Cell Probes. 1991;5:473–81.
69. Rohwer F, Seguritan V, Choi DH, Segall AM, Azam F. Production of shotgun libraries using random amplification. Biotechniques 2001;31:108–12, 114–16, 118.
70. Seshadri R, Kravitz SA, Smarr L, Gilna P, Frazier M. CAMERA: a community resource for metagenomics. PLoS Biol. 2007;5:e75.
71. Skall HF, Olesen NJ, Mellergaard S. Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming-a review. J Fish Dis. 2005;28:509–29.
72. Sritunyalucksana K, Apisawetakan S, Boon-nat A, Withyachumnarnkul B, Flegel TW. A new RNA virus found in black tiger shrimp *Penaeus monodon* from Thailand. Virus Res. 2006; 118:31–8.
73. Svraka S, Rosario K, Duizer E, van der Avoort H, Breitbart M, Koopmans M. Metagenomic sequencing for virus identification in a public-health setting. J Gen Virol. 2010;91:2846–56.
74. Thurber RV, Haynes M, Breitbart M, Wegley L, Rohwer F. Laboratory procedures to generate viral metagenomes. Nat Protocols. 2009;4:470–83.
75. Tomlins SA, Mehia R, Rhodes DR, Shah RB, Rubin MA, Bruening E, Makarov V, Chinnaiyan AM. Whole transcriptome amplification for gene expression profiling and development of molecular archives. Neoplasia. 2006;8:153–62.
76. Van Bressem MF, Van Waerebeek K, Raga JA. A review of virus infections of cetaceans and the potential impact of morbilliviruses, poxviruses and papillomaviruses on host population dynamics. Dis Aquat Organ. 1999;38:53–65.
77. Van Etten JL, Lane LC, Dunigan DD. DNA viruses: the really big ones (giruses). Annu Rev Microbiol. 2010;64:83–99.
78. Vázquez A, Sánchez-Seco MP, Palacios G, Molero F, Reyes N, Ruiz S, Aranda C, Marqués E, Escosa R, Moreno J, Figuerola J, Tenorio A. Novel Flaviviruses detected in different species of mosquitoes in spain. Vector Borne Zoonotic Dis. 2011; doi: 10.1089/vbz.2011.0687.
79. Victoria JG, Kapoor A, Li L, Blinkova O, Slikas B, Wang C, Naeem A, Zaidi S, Delwart E. Metagenomic analyses of viruses in stool samples from children with acute flaccid paralysis. J Virol. 2009;83:4642–51.
80. Victoria JG, Kapoor A, Dupuis K, Schnurr DP, Delwart EL. Rapid identification of known and new RNA viruses from animal tissues. PLoS Pathog. 2008;4:el000163.
81. Walker PJ, Mohan CV. Viral disease emergence in shrimp aquaculture: origins, impact and the effectiveness of health management strategies. Rev Aquacult. 2009;1:125–54.
82. Williamson SJ. Viral Metagenomics. In: de Bruijn, editor, Handbook of molecular microbial ecology II: metagenomics in different habitats. Wiley-Blackwell. 2011. p. 4–12.

83. Willner DL. Viral metagenomics in host-associated systems. San Diego: San Diego state University; 2010.
84. Winget DM, Wommack KE. Randomly amplified polymorphic DNA PCR as a tool for assessment of marine viral richness. Appl Environ Microbiol. 2008;74:2612–8.
85. Wommack KE, Ravel J, Hill RT, Chun J, Colwell RR. Population dynamics of Chesapeake Bay virioplankton: total-community analysis by pulsed-field gel electrophoresis. Appl Environ Microbiol. 1999;65:231–40.
86. Wongteerasupaya C, Sriurairatana S, Vickers JE, Anutara A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW. Yellow-head virus of *Penaeus monodon* is an RNA virus. Dis Aquat Organ. 1995;22:45–50.
87. Yooseph S, Sutton G, Rusch DB, Halpern AL, Williamson SJ, Remington K, Eisen JA, Heidelberg KB, Manning G, Li W, Jaroszewski L, Cieplak P, Miller CS, Li H, Mashiyama ST, Joachimiak MP, van Belle C, Chandonia J, Soergel DA, Zhai Y, Natarajan K, Lee S, Raphael BJ, Bafna V, Friedman R, Brenner SE, Godzik A, Eisenberg D, Dixon JE, Taylor SS, Strausberg RL, Frazier M, Venter JC. The Sorcerer II Global Ocean sampling expedition: expanding the universe of protein families. PLoS Biol. 2007;5:e16.
88. Zsak L, Strother KO, Kisary J. Partial genome sequence analysis of parvoviruses associated with enteric disease in poultry. Avian Pathol. 2008;37:435–41.