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Coupling cell culture and next-generation sequencing to study aquaculture viral diseases: a review

Abstract

In this short review, we highlight the importance of combining cell culture and next-generation sequencing for the study of viruses in aquaculture. Moreover, we summarize some key examples of previously published studies that have implemented this approach and discuss the advantages of nanopore sequencing and other long-read sequencing technologies. With the rapid advances of genomic research, selection of the best tool to carry out analyses, it's a computational challenge, but the potential for their applications is enormous. Therefore, this mini-review highlights important NGS bioinformatic tools and recent studies in cell culture for the study of aquatic virology.

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Introduction

Aquaculture is among the fastest-growing industries worldwide, with seafood accounting for approximately 20% of global animal protein consumption. However, viral diseases have had devastating economic effects in the aquaculture sector. This review introduces the most recent advances of sequencing technologies, to promote study of aquaculture viral diseases development. The impact of genomics in our understanding of viral genomes evolution and its potential implications in the analysis of viral mutations *in vitro*.

The field of aquaculture genomics was established in the 1990s. Since then, sequencing capacity and speed have increased considerably, particularly over the past decade, and sequencing costs have also steadily decreased.[1,2] Notably, next-generation sequencing (NGS; i.e., a method that allows for the massively parallel sequencing of entire genomes) has revolutionized the study of viral dynamics. Therefore, it is currently much easier and cheaper to sequence the entire genomes of a wide variety of species than it was only a few years ago.[3] In addition to genome sequencing, NGS can be applied to characterize transcriptomes and non-coding genes, as well as to interpret proteomics data. Consequently, a plethora of increasingly powerful bioinformatic tools have been developed in recent years to analyze and interpret biological data.[3]

Viruses are among the main threats to aquaculture development. These entities are obligate intracellular parasites, and therefore viral replication in cell cultures is essential for diagnosis, vaccine design, and *in vitro* characterization of novel viruses.[1-7] However stable cell lines for the study of aquatic virology remain scarce.[2] *In vitro* cell culture systems are an exceptionally important tool in aquaculture viral disease research. Nonetheless, the establishment of cell lines from aquatic organisms has faced several obstacles, as it is not possible to use a common methodology for all species due to inherent differences in cell physiology, biochemistry, and biology.[3] Therefore, there is an urgent need to establish aquatic organism cell culture systems to allow for the replication of enough viral particles for genome purification and NGS analysis with sufficient sequence coverage.[4]

Next-generation sequencing applications in viral disease research

NGS technology has been proven to be very useful in aquatic virology, as it provides an effective means to discover new viruses and the role of viral genes in disease onset, as well as for the study of molecular epidemiology, molecular evolution, and transcriptome analysis in combination with RNA-Seq techniques.[2,3,7-18] Most of the aquaculture species genomes have been sequenced using the illumina technology, and in some cases supplemented with third generation sequencing such as PacBio and MinION sequencing technologies. In recent years, fourth generation sequencing such as MinION based on nanopore, allows sequencing long read lengths (up to 30 Gb of DNA), as single DNA molecule,[17] however these nanopore technologies are less frequently used in aquaculture. For instance, the entire cyprinid herpesvirus 3 (CyHV-3) genome was sequenced from infected *Cyprinus carpio L.* tissues, after which sequence analyses indicated the occurrence of genetically distinct CyHV-3 strains (i.e., mixed infection) in infected carps.[5,6] A CyHV-3 isolate (KHV-T) was serially passaged 99 times in common carp (*Cyprinus carpio*) brain cells (CCB), and virus virulence was assessed at different passaging levels. A comparative analysis was then conducted in selected passaged (P) isolates [P0 (wild-type), P78, and P99]. P78 exhibited the largest deletion (1,363 bp) in ORF150, which was absent in P99. Interestingly, the passage 78 isolate was less virulent than P0 or P99, suggesting that the *in vitro* evolution of CyHV-3 resulted in a haplotype mixture.[7] Therefore, bioinformatic tools could be employed to characterize the phylogenetic and molecular evolution of viral taxa. For example, understanding viral quasispecies variations would allow for the identification of infectious disease routes and transmission in aquatic organisms.[20] Additionally, the complete genome of the white

spot syndrome virus (WSSV) was assembled from shrimp tissues via NGS. The WSSV genome was 305,094 bp, from which 38 single nucleotide polymorphisms (SNPs) were detected.[8] Improvements in NGS and bioinformatics methods provide an opportunity to investigate virus epidemiology and evolution. In turn, optimized whole-genome sequence analysis tools would facilitate the detection and characterization of a wider range of novel WSSV subtypes and intra-host genetic variations.[23,8,7]

In mollusks, the genome sequences obtained from ostreid herpesvirus 1 (OsHV-1) variants using two different sequencing methods (Illumina HiSeq and PacBio RS II) exhibited high genetic diversity and the results of phylogenetic analyses were indicative of spatial segregation.[9] Among these variants, the OsHV-1µVar[10] genome has been identified and characterized in five biparental families of oysters (*Crassostrea gigas*) in the Atlantic and Mediterranean French coasts. Viral diversity analyses identified 268 single nucleotide polymorphisms (SNPs) in the Atlantic and 59 SNPs in the Mediterranean, of which 109 SNPs were common in both sites. SNP frequency was higher in the Atlantic environment, suggesting a more diversified viral population in this site and that different oyster families were infected by different viral haplotypes.[11]

The capacity of some viruses to adapt to new hosts and environments depends on their ability to quickly respond to specific selective pressures.[21] Viral quasispecies are non-identical but related genomes that are subject to a continuous process of genetic variation, competition, and selection. Cell culture experiments have demonstrated that new viral particles compete with existing ones for the invasion of neighboring cells that originated from different replicative units.[22,23] Therefore, understanding viral quasispecies variations facilitates the characterization of infectious disease transmission mechanisms.[19,23]

Cell culture and next-generation sequencing-assisted virus vaccine development

Viruses can evolve *in vitro* through mutations after serial passages in cell cultures or infectious bioassays, which can lead to virulence loss, thus rendering attenuated viral strains.[7] Although attenuated viruses are not virulent, they are known to still elicit an immune response in vertebrates and can spread through large populations over extended periods.[12,13] Vaccines can also stimulate the innate immune responses of invertebrates, thereby attenuating viral infections.[24]

For example, carp nephritis and gill necrosis virus (CNGV) was attenuated via non-permissive temperature (30°C) and serial *in vitro* passages in koi carp cell line cultured 40–60 times. In this study, the authors isolated attenuated non-pathogenic viruses that could be used as live vaccines to control lethal diseases in *Cyprinus carpio*.[12] CyHV-3 from *Cyprinus carpio L*. was serially passaged (100 times) in CCB cells and the 78 passage exhibited a 1,363 bp deletion in ORF150, resulting in symptom loss. These kinds of haplotypes could be used as live attenuated vaccines. Therefore, although little is known about the role of ORF150 in virulence, the findings of therefore mentioned study suggest that this mutation could contribute to virus attenuation.[7] Specific studies are necessary to understand the molecular mechanisms (genomic and transcriptomic) of virus attenuation. Primary cell culture from *Marsupenaeus japonicus* testicular tissue and RNA sequencing (RNA-Seq) were implemented to assess caspase 3 gene expression levels to determine cell proliferation and identify regulator genes that control cell cycle arrest. Genes associated with mitosis initiation (LRWD1, TMEM127, CDCA3, PPP2R1A, and GOLGA2) were down regulated, whereas ARAF, a gene that activates cell cycle arrest, was upregulated.[14] Therefore, coupling transcriptome and viral genome NGS analysis with *in vitro* methods using aquatic animal cell lines will likely impact all areas of aquaculture virology, diagnosis, pathogenesis, viral evolution, ecology, and vaccine design.[12,14,25,26]

In vitro virology as an alternative for successful NGS performance

In vitro models allow for more controlled experimental conditions, thereby reducing variability. *In vitro* assays have been used to investigate pathogenicity mechanisms throughout different disease stages.[24,26] The most important challenge when sequencing viral genomes via NSG technology is the difficulty to obtain sufficient viral DNA with high purity, as it is extremely difficult to detect viral genomes at low concentrations.[2,15] These limitations were demonstrated during OsHV-1 propagation, where the unavailability of suitable cell lines made it especially challenging to obtain high-purity OsHV-1 DNA for NGS analysis.[10,16] Furthermore, the major challenge affecting aquatic metagenomics is that the samples are often highly contaminated.[2] Therefore, for better identification of viruses at the genus or species levels, identification procedures should ideally include both NGS and cell culture techniques. Viral replication in cellular culture is thus still considered the most important technique for the diagnosis and discovery of novel viruses.[1] However, future efforts must focus on filling the gaps in the availability of stable aquatic organism cell lines (Table 1)[2,16,10]

Nanopore sequencing (MinION) as a useful tool in aquatic virology

MinION is a revolutionary fourth-generation DNA sequencing technology based on engineered protein nanopores with small channels, which allow small molecules to pass through. The extreme miniaturization of the fundamental mechanisms on which this technology is based has allowed for the creation of DNA sequencing devices that are approximately the size of a USB memory stick.[17] The MinION device is capable of analyzing DNA, RNA, peptides, proteins, polymers, and other molecules.[17] Long-read nanopore sequencing technology can process up to 30 Gb of DNA sequences or 7–12 million reads and can also characterize ultra-long RNA reads.[17,18] This allows for less ambiguous genome assembly, thereby facilitating the characterization of gene regulation and function, gene expression, and isoform diversity.[18] Despite being a low-cost and highly portable sequencing technology, MinION run times can be rather lengthy (i.e., several days) depending on the amount of data required for a given experiment[17,19] and very few studies have implemented this technology to study aquatic viral diseases (Table 1). Among them, one study reported the rapid sequencing of two viruses that affects Salmonid culture, salmonid alphavirus (SAV) and infectious salmon anemia virus (ISAV), using MinION technology.

MinION may be used to complement the results of Sanger sequencing to retrieve genomic information. For instance, the sequencing required for the phylogenetic reconstruction of the SAV subtype 6 genome was achieved in three hours using MinION technology, producing over 400 Mb of data and almost full-genome coverage.[19] MinION technology offers important cost- and time-saving advantages over Sanger and Illumina systems, and thus could become instrumental for aquatic viral genotype and subtype characterization and monitoring in the future.[17,18,19,27-40]

In recent years, the application of viroinformatics (i.e., an amalgamation of virology with bioinformatics) has provided new

ways to formulate scientific questions, thereby strengthening the progress of viral pathogenesis research. NGS applications involve the management of large datasets, requiring highly-specialized bioinformatics software and pipelines to process and analyze sequencing data using parallel computing (i.e., supercomputers). The development and optimization of tools for the analysis of large datasets poses an important computational challenge of large, and previous reviews have addressed the current state of viroinformatics resources in depth.[41] Therefore, this mini-review focused exclusively on recent studies in which cell culture and cutting-edge NGS bioinformatic tools were jointly implemented for the study of aquatic virology (Table 1). As discussed above, very few studies have applied this combined approach and it appears that the relevance of cell culture has not been fully exploited in this context, which may due to a lack of interest and/or budget allocation for virology research in aquatic animal cell lines.

Conclusion

Combining cell culture with NGS, nanopore, or PacBio sequencing technologies (the latter being currently the technology with the longest read sequencing capacity) would allow for the efficient exploration of viral infection properties and dynamics in aquaculture systems. This is especially important in the context of highly dynamic system and organisms with relatively short generation times and wide geographic ranges due to migratory behaviors, with some important exceptions such as abalone or geo duck clams. Although very few studies have implemented this combined approach, these seminal works have already provided important insights, thus highlighting the potential applicability of this method for future viral research.

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Conflicts of interest

The author declares that there is no conflicts of interest.

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