CHAPTER

2 Metagenomics revealing new virus species in farm and pet animals and aquaculture

Eszter Kaszab[1], Andor Doszpoly[1], Gianvito Lanave[2], Atul Verma[3], Krisztián Bányai[1], Yashpal Singh Malik[3] and Szilvia Marton[1]

[1]*Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary*

[2]*Department of Veterinary Medicine, University of Bari, Valenzano, Bari, Italy*

[3]*Division of Biological Standardization, ICAR-Indian Veterinary Research Institute, Izatnagar, India*

2.1 Introduction

Metagenomics means the genomic analysis of a microbial community from a specific environment. Consequently, viral metagenomics is a culture-independent method that is a useful tool for determining the complexity of viral communities in any type of sample (Bibby, 2013; Handelsman, 2004; Kulski, 2016). Viral nucleic acid suitable for metagenomic analysis could be derived from environmental samples (aquatic or terrestrial environments, surface waters, sediments, agriculture products, human or animal habitations, human and animal vaccines, etc.) or biological specimens (blood, feces, serum, plasma, respiratory, secretion, etc.) (Delwart, 2007; Kulski, 2016; Li et al., 2015a,b).

Over the past decade massively parallel sequencing technologies have become the focus of development. Its potential to gain insight into the structure, function, or regulation of genomes and genes has revolutionized many disciplines of life science. The utilization of these techniques in molecular diagnostics has been exploited in microbiology with the potential to detect novel microorganisms, including viruses in diverse specimens and ecosystems. It can also be used for inspection of viral diversity, evolution and spread, for investigation of pathogenesis of viral agents and for characterization of the viral community (Barzon et al., 2011, 2013; Capobianchi et al., 2013). A popular and widely used approach to describe viral communities without prior virus isolation step is shotgun metagenomics (Capobianchi et al., 2013).

In this chapter we summarize commonly used laboratory methods that had helped to describe viral diversity and structure of viral communities and some

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achievements of this approach related to virus discovery with possible implications in veterinary medicine.

2.2 Technical aspects of viral metagenomics

In the laboratory space viral metagenomics consists of four major steps: (1) viral enrichment to minimize background of prokaryotic and eukaryotic nucleic acids thus increasing the relative proportion of viral nucleic acids, (2) amplification of viral nucleic acids, (3) sequencing with or without cloning of amplified polymerase chain reaction (PCR) fragments, and (4) bioinformatic analysis of the resulting sequence output (Alavandi and Poornima, 2012; Delwart, 2007).

2.3 Virus enrichment and nucleic acid amplification

If necessary, the first step of sample procession is homogenization (physical, e.g., homogenizer, mortar and pestle, freeze-thaw cycles; or enzymatic techniques, e.g., salt solution, detergents, alkaline lysis), centrifugation of the sample and filtration of the supernatant (through 0.22 and 0.45 μm pore membranes) to remove nonviral nucleic acids (i.e., host cellular debris and bacteria). The filtrates can be treated with a mixture of DNases and RNases to further reduce background nucleic acids originating from the host cells and bacteria. The method chosen in this step depends on the physical properties and other characteristics of the sample type (Datta et al., 2015; Mokili et al., 2012; Shah et al., 2014; Vo and Jedlicka, 2014). Following sample homogenization and reduction of the amount of debris and background nucleic acids, viral particles can be concentrated at various efficacies. Commonly used methods include tangential-flow filtration, polyethylene glycol precipitation, and ultracentrifugation. Density gradient ultracentrifugation using cesium chloride gradient provides highly purified virus particles. Concerning the step of viral nucleic acid extraction, the picture is more complex. Viral particles are disrupted by using "lysis buffer," which may contain chaotropic acids (e.g., guanidine hydrochloride), detergents (sodium dodecyl sulfate, Triton X-100), and/or proteases (e.g., proteinase K). During the subsequent separation phase, the nucleic acids could be isolated from other components. This can be done by liquid−liquid extraction or liquid−solid extraction. During the liquid phase extraction different types of alcohol are used (e.g., phenol-chloroform-isoamyl alcohol, isopropanol, etc.). The solid phase extraction may include one of the following procedures: gel filtration, where nucleic acid is separated through gel matrix (e.g., Sephadex), ion exchange chromatography (e.g., anion exchange resin, DEAE-C), and affinity chromatography (silica surface, paramagnetic beads). In general, liquid−solid extraction methods use less hazardous chemicals and provide increased throughput. Various formats have been

marketed providing flexible, fast, and scalable viral nucleic acid extraction (Datta et al., 2015; Thatcher, 2015; Thurber et al., 2009).

The amount of extracted nucleic acids is often too low for sequencing on available next generation sequencing (NGS) platforms; in such a case the amplification of viral nucleic acids is inevitable. Sequence-independent single primer amplification (SISPA) is among the most common amplification methods (Reyes and Kim, 1991). Initially this method was carried out by starting with restriction digestion and followed by ligation of an asymmetric primer or adaptors to both ends of the DNA molecules. By using a primer complementary to the ligated oligonucleotide, the nucleic acid fragments present in the sample could be amplified by PCR. Since then the original method has been modified to achieve more effective enrichment of nucleic acids or identification of viruses, such as DNase-SISPA and Virus discovery based on cDNA-AFLP. Another widely used method is random PCR. It uses a single primer with a known sequence at 5′ end and with a random hexamer or heptamer sequence at 3′ end in the first reaction. After that, the second PCR reaction is executable with a specific primer complementary to the defined 5′ of the first random primer. To amplify circular DNA viral genomes, rolling circle amplification is an alternative option. This method requires short oligonucleotide primers composed of random sequences and polymerase enzyme with strand displacement activity (e.g., bacteriophage ϕ29 DNA polymerase). Another ϕ29 DNA polymerase-based method is the multiple displacement amplification. These techniques are often called whole genome amplification methods (Bexfield and Kellam, 2011; Blomstrom, 2011; Delwart, 2007).

2.4 Sequencing technologies

Owing to efforts in research and innovation (Fig. 2.1), sequencing capacity and speed have dramatically increased over the past decade, while the cost is continuously decreasing. These achievements together led to the capacity to produce billions of nucleotide bases in a single sequencing run, which was unconceivable some time ago (Escobar-Zepeda et al., 2015; Kulski, 2016).

2.4.1 First-generation sequencing

Since 1970s two methods have been developed to determine the precise order of nucleotides (DNA sequencing). In 1975, “plus and minus” method was published by Sanger and Coulson and historically it was the first DNA sequencing method. In 1977, Allan Maxam and Walter Gilbert invented a new protocol (Maxam-Gilbert sequencing or chemical sequencing), which is based on chemical cleavage of the radiolabelled bases and subsequent separation of the resulting DNA fragments by electrophoresis. This method, however, has not become widely adopted. Finally, in 1977, Sanger et al. accomplished the method of DNA sequencing

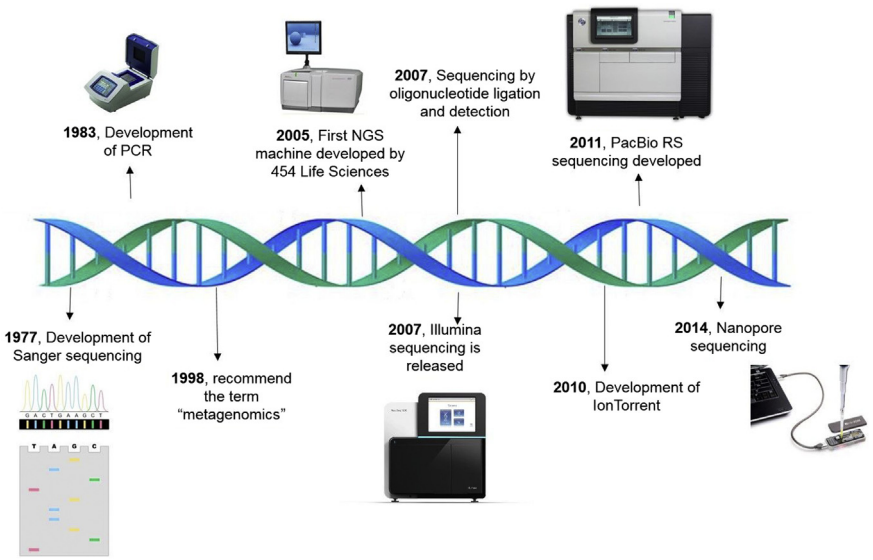

FIGURE 2.1 Metagenomics timeline and milestones.

Timeline showing advances in sequencing technologies from Sanger sequencing to Nanopore sequencing.

which ultimately led to the development of the "dideoxy method" or chain-termination sequencing technique. This process—which definitively amended the progress of DNA sequencing technology—depends on the application of dideoxynucleotides (ddNTPs) to cause base-specific termination of primed DNA synthesis. Initially, the radio-labeled fragments were separated by denaturating polyacrylamide-urea gel electrophoresis. These days the technique is coupled with fluorescence and capillary electrophoresis-based sequencers.

Sequencing methods have benefited from the development of PCR, recombinant DNA technologies, discovery and molecular cloning of thermostable sequencing polymerases (e.g., Taq polymerase), and reverse transcriptase, as well as the development of fluorescent dye-labeled ddNTPs and process parallelization, which facilitated the birth of genome projects and the field of metagenomics (Heather and Chain, 2016; Hutchison, 2007; Kulski, 2016; Mardis, 2013; Schuster, 2008). Initially, laboratory methods of viral metagenomics relied on molecular cloning of amplified genomic fragments coupled with Sanger sequencing of hundreds to thousands of individual clones (Allander et al., 2005; Finkbeiner et al., 2008). Collectively, the major advantages of this technology are the long read lengths and the excellent resolution of sequence repeats and homopolymer regions. The drawbacks include the limited throughput and the relatively high per base sequencing cost (Hutchison, 2007; Schuster, 2008).

2.4.2 Second-generation sequencing

These methods can handle the parallel sequencing of several samples by molecular barcoding at very high-throughput and at reduced per nucleotide costs due to the miniaturization of the sequencing reactions. For metagenomic applications an added advantage was the lack of need for prior cloning of amplified target genomic DNA/RNA. With the initiation of NGS methods, the efficiency of DNA and RNA-base examinations explosively increased and the relative costs of sequencing have dramatically decreased (Escobar-Zepeda et al., 2015; Kulski, 2016; Metzker, 2010).

2.4.2.1 Pyrosequencing

The first commercially available NGS technology was the "pyrosequencing technique" developed by 454 Life Sciences in 2005 and acquired by Roche in 2007, which is a sequencing-by-synthesis method (Barzon et al., 2011; Hutchison, 2007; Kulski, 2016; Schuster, 2008). During the process, pyrophosphate synthesis is measured with the utilization of chemiluminescent method. In brief, when a nucleotide is incorporated into the DNA chain, pyrophosphate is released (Fig. 2.2). The Adenosine triphosphate sulfurylase converts pyrophosphate into ATP, which is used as a substrate for luciferase, hence visible light is detected and measured (chemiluminescence) with charge-coupled device (CCD) camera. The process is reinitiated by adding the next complementary dNTP in the next dispensing cycle (Heather and Chain, 2016; Hutchison, 2007; Kulski, 2016). The method has disadvantages, including the high error rates in homopolymer repeats, the appearance of indels (insertions, deletions), as well as the time consuming sample preparation protocols. In contrast, the number of sequencing reactions and reads dramatically extended, the quality of data improved by reducing cross-talk between reaction wells, and the system was miniaturized (PicoTiterPlates) enabling the reduction of reaction volume. Furthermore, this platform provides long read lengths (400 bp to 1 kb) (Heather and Chain, 2016; Kulski, 2016; Metzker, 2010; Schuster, 2008).

2.4.2.2 Illumina/solexa sequencing

Solexa developed the Genome Analyzer in 2006 that was acquired by Illumina in 2007. Currently, it is the most widespread sequencing system with the technology of sequencing-by-synthesis (Fig. 2.2). It differs from the Roche 454 sequencer in that it uses reversible chain-terminating nucleotides, which are fluorescently labeled. The fluorescent tags block the 3′-OH of the new nucleotide and so the next base can only be added when the tag is removed. When a nucleotide is incorporated into DNA template strands, the photon emission from each cluster is recorded by a CCD camera. Illumina provides currently benchtop sequencers (iSeq 100, MiniSeq, MiSeq/MiSeq Dx/MiSeq FGx, and NextSeq 550/NextSeq 550 Dx) and production-scale sequencers (HiSeq 2500/HiSeq 3000/HiSeq 4000, HiSeq X series Five or Ten, and NovaSeq 6000).

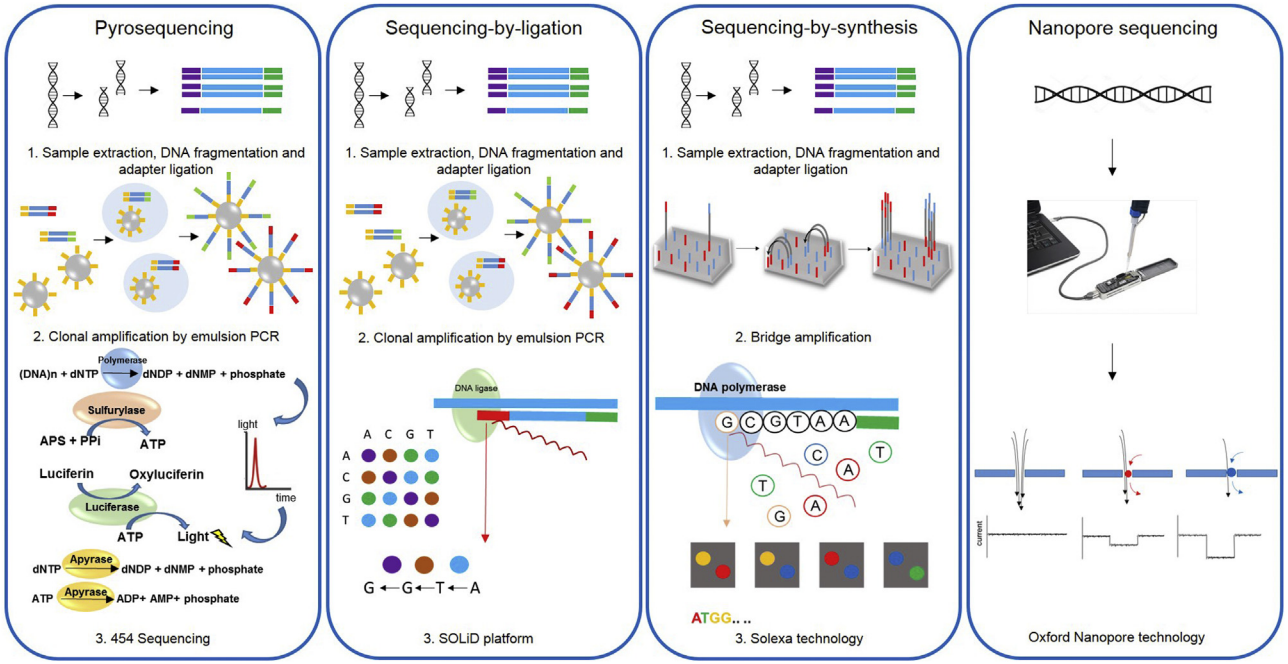

FIGURE 2.2 Principle of next generation sequencing platforms currently available.

Roche 454, specific adaptor containing DNAs are denatured into single strands and captured by amplification beads followed by their emulsion PCR then pyrosequencing where oxyluciferin release is monitored. *SOLiD*, this is a ligation based method that uses DNA ligase enzyme to identify the presence of nucleotide at a given position in a DNA sequence. *Illumina*, selected DNA fragments are ligated with adaptors, and primers designed against them are used for the synthesis. Bridge amplification is carried by PCR and polony generations take place. *Oxford Nanopore*, This is actually based on the tunneling of polymer molecules passing through a protein nanopore, separating two compartments. Movement of DNA through this pore causes disruption in the current set against the voltage across the pore. Change in the current helps to identify the specific molecule and real time advances the quick data analysis.

With the first sequencer (Genome Analyzer) it was possible to sequence 1 gigabase (Gb) of data in a single run. Nowadays, the output reaches 6000 Gb when using NovaSeq6000. The technology allows advantages and drawbacks too. It provides the lowest per base cost, huge amount of data, better performance along homopolymeric regions, and low error rate. The read length limitation (maximum 2×150 bp or 2×300 bp) remains an unsolved problem [Ari and Arikan, 2016; Heather and Chain, 2016; Kulski, 2016; Liu et al., 2012; Mardis, 2013; <https://www.illumina.com/systems/sequencing-platforms.html> (accessed: 2018.09.22)].

2.4.2.3 Sequencing by oligonucleotide ligation and detection

Another commercially available NGS platform, the Sequencing by oligonucleotide ligation and detection (SOLiD) technology, also known as polony sequencing, was developed by George Church. The technology was purchased and improved by Applied Biosystems in 2007. The sequencer adopts the technology of two-base sequencing based on ligation sequencing. These probes are fluorescently labeled and compete for ligation to the sequencing primer by DNA ligase. The fluorescent signal is captured when the probe complement to the template is ligated. The cycle can be repeated by using cleavable probes to remove the fluorescent dye or by removing and hybridizing a new primer to the template.

Due to the di-base encoding system and the exact call chemistry (ECC), the accuracy is greater than 99.94% (5500 Series SOLiD System's accuracy is 99.99%). It provides inherent error correction, because the probes interrogate two base per reaction. In addition, sequencing of homopolymeric regions by the SOLiD technology is not a major issue. Besides more efficient sequencing, the throughput is increased on 5500 series (up to 10−15 Gb on 5500, up to 120 Gb per run on 5500xl W). Nonetheless, the major drawback of the method is the very short read lengths (75 bp (fragment), 75 bp × 35 bp (paired-end), up to 60 bp × 60 bp (mate-paired)) and the need for advanced computational infrastructure [Ari and Arikan, 2016; Heather and Chain, 2016; Hutchison, 2007; Kulski, 2016; Liu et al., 2012; Metzker, 2010; <http://www.appliedbiosystems.com/absite/us/en/home.html> (accessed: 2018.09.22)].

2.4.2.4 Semiconductor sequencing

With Ion Torrent semiconductor sequencing a new paradigm in next generation sequencing was established in 2010. The sequencing technology exploits the fact that addition of a dNTP to a DNA polymer releases an $H+$ ion and changes the pH of the solution that is proportional to the numbers of incorporated nucleotides. If there are two identical bases on the DNA strand, the sequencing chip will record two identical bases. If the next nucleotide is not a match, no base will be recorded. This process can be detected by a sequencing chip, which functions like a small solid-state pH meter. Overall, the method provides shorter workflow and simpler, faster, and more affordable sequencing to users. Life Technologies offers several next generation sequencing systems and sequencing chips: Ion Proton

with Ion PI Chip, Ion S5 System with Ion 520, 530 540 Chip Kit, Ion Personal Genome Machine (PGM) with the Ion 314, 316, 318 Chip. The read lengths (200−400 bp), the accuracy (99%) and the run time (2−7 hours) are similar, but there are differences in the output (reads per run ranges from 0−5 to 60−80 million reads/run; with up to 2−10 Gb per run). The major disadvantages of this technology are biases in detection of insertions, deletions, and homopolymer sequences. However, there are numerous advantages like stable quality of sequencing, better GC depth distribution, higher map rate, relatively longer read lengths, and fastness [Kulski, 2016; Liu et al., 2012; Mardis, 2013; <https://www.thermofisher.com/hu/en/home/life-science/sequencing/next-generation-sequencing.html> (accessed: 2018.09.22)].

In conclusion, of the four NGS systems described above, the Illumina HiSeq2500−4000 and NovaSeq6000 feature the biggest output and lowest per base reagent cost, the Ion Torrent has the fastest run time, the SOLiD has the highest accuracy, while the Roche 454 system has the longest read length.

2.4.3 Third-generation sequencing

To overcome some inconveniences related to second-generation sequencing technologies (e.g., the lengthy procedure of library preparation step, difficult genome assembly due to the relatively short read lengths, biases in identification of indels and homopolymer regions), the third-generation sequencing methods has been developed. At present two approaches are available in the market.

2.4.3.1 Single molecule real-time sequencing

Pacific Biosciences developed the single molecule real-time (SMRT) sequencing. SMRT is based on DNA replication via DNA polymerase. When a fluorescent nucleotide is incorporated into the growing DNA chain, the enzyme cleaves off the fluorescent dye. The emitted signals are detectable in real time by a CCD camera placed inside the machine. The other key point of the method is the usage of zero-mode waveguides (ZMW), which are fine wells in a metallic film covering a chip. Each ZMW contains single DNA polymerase attached to their bottom and single DNA target. This allows the observation of a fluorescent labeled single molecule in real time, kept apart from the signal noise background (other fluorescent labeled molecules). With this new approach the read length has dramatically increased with maximum read lengths up to >100 kbp. A major drawback is the relatively high error rate (single pass accuracy ~86%, but the consensus accuracy >99.99%), which stems mostly from indel errors.

Currently Pacific Biosciences offers two devices, the Sequel System and the PacBio RS II [Ari and Arikan, 2016; Heather and Chain, 2016; Kulski, 2016; Liu et al., 2012; Metzker, 2010; <http://allseq.com/knowledge-bank/sequencing-platforms/pacific-biosciences/> (accessed: 2018.09.22)].

2.4.3.2 Nanopore sequencing

Oxford Nanopore Technologies developed a new, incomparable system. The first nanopore based sequencer was the MinION, in 2014. Recently, the company offers miniature handheld devices (MinION, SmidgION) and high-throughput installations (PromethION) as well. Nanopore devices perform DNA and RNA sequencing directly and in real time. A nanopore is a nano-scale hole, which is created by pore-forming proteins and they act as channels embedded on lipid bilayer or artificial membrane (Fig. 2.2). They are suitable for the detection and quantification of biological and chemical molecules. During the sequencing, a voltage is applied across the membrane continuously, causing ionic current. When the DNA fragment is passed through the nanopore, the conductivity of ion currents in the pore changes, since the nucleotides have different shapes and they have a different effect on the change of the ionic flow.

At this time, the smallest instrument commercially available is the MinION. It is a portable, pocket-sized device; when it is attach to a laptop or computer freshly generated data are displayed on the screen in real time. The technology provides long read length (up to 1 Mbp). The method does not require an amplification step and the detection of bases is fluorescent-tag free. However, the technology provides relatively high error rate which can be counterbalanced by increasing the sequencing depth [Ari and Arikan, 2016; Escobar-Zepeda et al., 2015; Heather and Chain, 2016; Kulski, 2016; Liu et al., 2012; <https://nanoporetech.com> (accessed:2018.09.22)].

2.5 Bioinformatics

NGS has dramatically increased the amount of sequenced data. Storage and handling of such data is challenging. Bioinformatics provide software and several workflows (bioinformatics pipelines) to analyze biological data. The main purposes include the conversion of NGS-based signals to sequence data that can be readily processed and the adequate interpretation of the processed sequence data to obtain the desired biological information (Fig. 2.3).

Nucleotide sequence analysis has a couple of steps, including base calling, the quality check of the sequencing runs. The read sequences are commonly stored in FASTQ file format or in the native raw data file formats depending on the sequencing instrument and the associated onboard software. This step is followed by quality control of the reads (searching for sequence errors and artifacts, platform-specific error profiles, etc.), then trimming (cleavage of adapters, tags, primers), and the alignment and assembly of contigs (mapping to reference sequences or de novo assembly). In general, there are several assembly tools and software (e.g., Geneious, Velvet, and ABySS); but nowadays viral genome specific genome assemblers, such as VICUNA, Viral Assembly Pipeline (VrAP), V-FAT, AV454, RIEMS, and metagenome assemblers like Omega, Genovo, MEGAHIT, MetaSpades, MetaVelvet are also available (Holzer and Marz, 2017;

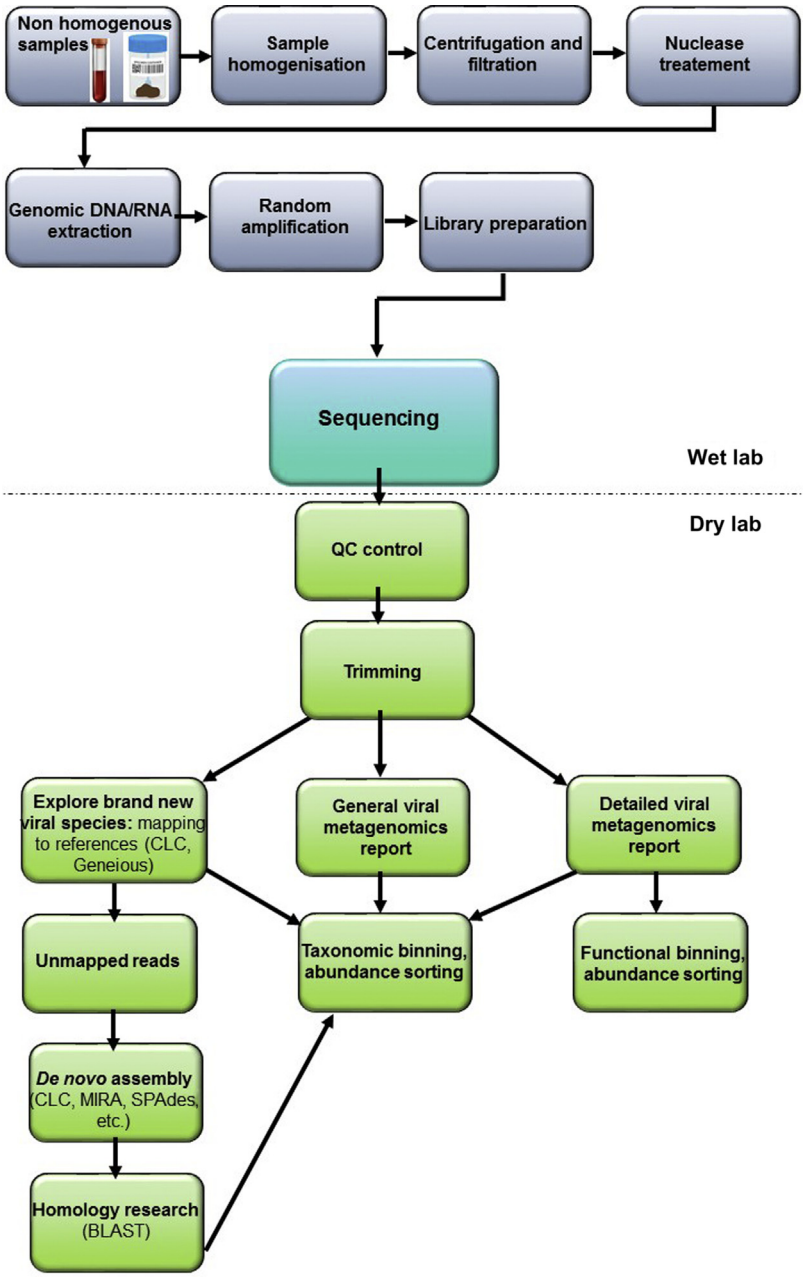

FIGURE 2.3 Schematic workflow for viral metagenomics.

Flowchart represent major steps of metagenomics analysis. For detailed protocol please see the manuscript, Conceição-Neto et al. (2015).

Rose et al., 2016; Roy et al., 2018). During de novo assembly, there is no available reference sequence for alignment; this technique is key to identify novel virus genome sequences. The next step is genome identification and annotation [open reading frame (ORF), the coding sequence, repeated elements, untranslated regions, etc.], search for single nucleotide polymorphism and insertion/deletion, and comparative sequence analysis. Commercially available analysis tools (e.g., CLCBIO Genomic Workbench, Avadis NGS, and Softgenetics Nextgene) offer user-friendly solutions for this purpose. Virus-specific data bases are useful to collect relevant information on virus families and also sequence data for subsequent taxonomic and phylogenetic classification (Holzer and Marz, 2017; Kulski, 2016; Rose et al., 2016; Roy et al., 2018). Online platforms are also available to make rapid viral taxonomy in a metagenomic data set [<http://kaiju.binf.ku.dk/> (accessed: 2018.09.22), <https://www.taxonomer.com/> (accessed: 2018.09.22)].

In response to the need for effective detection, assembly, and classification of pathogens, several tools were put on the market in recent times for ease of bioinformatics analysis. The analysis of extremely high-throughput data is very time consuming, therefore development of faster and more user-friendly software will be essential in the near future.

2.6 Practical aspects of viral metagenomics

The application of viral metagenomics is being continuously adopted and used in veterinary medicine. In clinical diagnostics, the recognition and the treatment of novel and rare pathogens is a real challenge. With the new technical inventions, this problem could be solved. Another application is the real-time investigation of outbreaks caused by viral pathogens and the prevention of potential epidemics. The environmental monitoring of pathogens by viral metagenomics may also have an important role in efforts of infectious disease control. In this case, the quick reaction, the regulation, and risk assessment based on laboratory confirmed evidence is very important. Moreover, broad-range detection and identification of novel viruses or novel types of known viruses will be able to help us to broaden our range of vision, facilitate protection against viral agents, and understand viral diversity (Table 2.1). The origin of novel viruses or novel variants is diverse, one of the potential sources is the cross-species transfer, hence surveying of potential reservoirs and characterizing the intra-host variability is necessary (Barzon et al., 2013; Bibby, 2013; Capobianchi et al., 2013; Mokili et al., 2012). In several cases, the co-occurrence of the newly identified virus with a disease symptom does not proof the causation, although experimental and epidemiological verification is required. To provide disease causation, Mokili et al. proposed the metagenomics Koch's postulates. Viral metagenomics has the opportunity to identify the components of multifactorial infectious diseases (Belak et al., 2013; Kapgate et al., 2015).

Table 2.1 Selection of novel animal viruses identified with NGS technologies.

Host		Virus	Taxonomy	Disease	References	Sequencing
Pig	RNA virus	Porcine stool-associated RNA virus 1	Picornavirales	E	Shan et al. (2011)	454 pyrosequencing
		Porcine stool-associated RNA virus 2	Picornavirales	E	Shan et al. (2011)	454 pyrosequencing
		Swine pasivirus 1 (SPaV1)	Picornaviridae	?	Sauvage et al. (2012)	Illumina Hiseq 2000
		Atypical porcine pestivirus	Flaviviridae	O	Hause et al. (2015)	Illumina MiSeq
		Enterovirus species G (EVG) (EVG 08/NC_USA/2015)	Picornaviridae	E	Shang et al. (2017)	Illumina MiSeq
		Ndumu virus	Togaviridae	O	Masembe et al. (2012)	454 GS-FLX platform
		Porcine astrovirus 4	Astroviridae	E	Padmanabhan and Hause (2016)	Illumina MiSeq
	DNA virus	Pig stool-associated circular ssDNA virus	Unclassified CRESS	E	Sachsenröder et al. (2012)	454 pyrosequencing
		Porcine parvovirus 7	Parvoviridae	E	Palinski et al. (2016)	Illumina MiSeq
		Porcine circovirus 3	Circoviridae	O	Phan et al. (2016)	Illumina MiSeq
		Porcine parvovirus 2,4,5,6	Parvoviridae	E	Bovo et al. (2017)	IonTorrent PGM
		Porcine bocavirus 1-H18	Parvoviridae	E	Bovo et al. (2017)	IonTorrent PGM
Cattle	RNA virus	Bovine astrovirus-NeuroS1	Astroviridae	E & O	Li et al. (2013c)	Illumina MiSeq
		Bovine astrovirus-CH13	Astroviridae	O	Bouzalas et al. (2014)	Illumina HiSeq2500
		Bovine astrovirus-BH89/14	Astroviridae	O	Schlottau et al. (2016)	Illumina MiSeq

		Yak astrovirus S8	Astroviridae	E	Chen et al. (2015)	Illumina HiSeq 2000
		Bovine enterovirus AN12/Bostaurus/JPN/2014	Picornaviridae	E	Mitra et al. (2016)	Illumina MiSeq
		Rotavirus B	Reoviridae	E	Hayashi-Miyamoto et al. (2017)	Illumina MiSeq
		Bovine beta-retrovirus (BoRV-CH15)	Retroviridae	O	Wüthrich et al. (2016)	Illumina HiSeq2500
		Aichivirus B	Picornaviridae	O	Moreira et al. (2017)	Illumina MiSeq
	DNA virus	Bovine papillomaviruses BPV22	Papillomaviridae	O	Bauermann et al. (2017)	Illumina MiSeq
		Bovine polyomavirus BPyV2-SF	Polyomaviridae	O	Zhang et al. (2014a,b)	Illumina MiSeq
Small ruminants	RNA virus	Ovine astrovirus	Astroviridae	O	Pfaff et al. (2017)	454 pyrosequencing
		Ovine astrovirus type 2	Astroviridae	O	Reuter et al. (2012)	454 GS-FLX platform
		Statovirus D1	Unclassified RNA virus (phylogenetically related to Tombusviridae and Flaviviridae)	O	Janowski et al. (2017)	455 GS-FLX platform
Chicken	RNA virus	Sicinivirus 1	Picornaviridae	O	Bullman et al. (2014)	Illumina Miseq
		Picornavirus QIA01	Picornaviridae	O	Kim et al. (2015a,b)	Illumina
		Chicken phacovirus 1	Picornaviridae	E	Boros et al. (2016)	Illumina MiSeq
		Sunguru virus	Rhabdoviridae	O	Ledermann et al. (2014)	IonTorrent PGM
		Chicken astrovirus PL/G059/2014	Astroviridae	O	Sajewicz-Krukowska and Domanska-Blicharz (2016)	Illumina MiSeq
	DNA virus	Gyrovirus GyV7-SF	Anelloviridae	O	Zhang et al. (2014a,b)	Illumina Miseq

(*Continued*)

Table 2.1 Selection of novel animal viruses identified with NGS technologies. *Continued*

Host		Virus	Taxonomy	Disease	References	Sequencing
Turkey	RNA virus	Turkey heptatitis virus	Picornaviridae	E&O	Honkavuori et al. (2011)	454 GS-FLX platform
		Turkey gallivirus strain turkey/M176/2011/HUN	Picornaviridae	E	Boros et al. (2012)	454 GS-FLX platform
		Turkey avisivirus strain turkey/M176-TuASV/ 2011/HUN	Picornaviridae	E	Boros et al. (2013)	454 GS-FLX platform
		TuASV-USA-IN1	Picornaviridae	O	Ng et al. (2013a,b)	Illumina
		Picobirnaviruses	Picobirnaviridae	E	Verma et al. (2015)	Illumina HiSeq
	DNA virus	Turkey stool-associated circular virus	Unclassified single-stranded (ss) circular small DNA viruses	E	Reuter et al. (2014)	454 GS-FLX platform
		Turkey poxvirus-HU1124/2011	Poxviridae	O	Banyai et al. (2015)	IonTorrent PGM
Other birds	RNA virus	Mesivirus-1 and −2	Picornaviridae	O	Phan et al. (2013)	Illumina MiSeq
		Bornavirus	Bornaviridae	O	Honkavuori et al. (2008)	454 GS-FLX platform
		Avian coronaviruses	Coronaviridae	R	Chen et al. (2013)	IonTorrent PGM
		Avian gammacoronavirues	Coronaviridae	O	Liais et al. (2014)	Illumina MiSeq
		Avian orthoreoviruses	Reoviridae	O	Farkas et al. (2018)	IonTorrent PGM
		Bunyavirus	Bunyavirales	O	Alkovskhovskii et al. (2013)	Illumina
	DNA virus	Pigeon adenovirus 2	Adenoviridae	O	Teske et al. (2017)	Illumina MiSeq
		Aviparvovirus	Parvoviridae	O	Phan et al. (2013)	Illumina MiSeq
		Gyrovirus V8	Anelloviridae	O	Li et al. (2015a,b)	Illumina MiSeq

Dog	RNA virus	Kobuvirus	Picornaviridae	E	Li et al. (2011)	454 GS-FLX platform
		Norovirus	Caliciviridae	E	Martella et al. (2008)	?
		Sapovirus	Caliciviridae	E	Li et al. (2011)	454 GS-FLX platform
		Vesivirus	Caliciviridae	E/R	Martella et al. (2015)	?
		Astrovirus	Astroviridae	E	Toffan et al. (2009)	Primer walking!!
		Hepacivirus	Flaviviridae	O	Kapoor et al. (2011)	Primer walking!!
		Rotavirus I	Reoviridae	E	Mihalov-Kovács et al. (2015)	IonTorrent PGM
		Rotavirus C	Reoviridae	E	Marton et al. (2015)	IonTorrent PGM
	DNA virus	Bocaparvovirus 2	Parvoviridae	R	Kapoor et al. (2012b)	?
		Bocaparvovirus 4	Parvoviridae	R	Li et al. (2013b)	454 GS-FLX platform
		Protoparvovirus 2	Parvoviridae	R/E	Martella et al. (2018)	IonTorrent PGM
		Bocaparvovirus, NC	Parvoviridae	E	Conceição-Neto et al. (2017)	Illumina Hiseq 2500
		Polyomavirus	Polyomaviridae	R	Delwart et al. (2017)	Illumina MiSeq
		Papillomavirus 1–18	Papillomaviridae	O	Lange et al. (2016)	?
Cat	RNA virus	Sakobuvirus A	Picornaviridae	E	Ng et al. (2014)	Illumina MiSeq
		Astrovirus	Astroviridae	E	Ng et al. (2014)	Illumina MiSeq
		Astrovirus	Astroviridae	E	Zhang et al. (2014a,b)	Illumina MiSeq
		Rotavirus I	Reoviridae	E	Ng et al. (2014), Phan et al. (2017)	Illumina MiSeq, Illumina HiSeq
		Picobirnavirus	Picobirnaviridae	E	Ng et al. (2014)	Illumina Miseq
		Morbillivirus	Paramyxoviridae	O	Marcacci et al. (2016)	Illumina NextSeq 500
		Hepadnavirus	Hepadnaviridae	O	Aghazadeh et al. (2018)	Illumina Hiseq 2500

(*Continued*)

Table 2.1 Selection of novel animal viruses identified with NGS technologies. *Continued*

Host		Virus	Taxonomy	Disease	References	Sequencing
	DNA virus	Cyclovirus	Circoviridae	E	Zhang et al. (2014a,b)	Illumina MiSeq
		Bocaparvovirus 2	Parvoviridae	E	Ng et al. (2014)	Illumina MiSeq
		Bocaparvovirus 3	Parvoviridae	E	Zhang et al. (2014a,b)	Illumina MiSeq
		Bocaparvovirus	Parvoviridae	O	Garigliany et al. (2016)	IonTorrent PGM
		Cowpoxvirus	Poxviridae	O	Dabrowski et al. (2013). Mauldin et al. (2017)	454 GS-FLX platform, Illumina Hiseq 2000
		Ectromelia-like virus	Poxviridae	O	Lanave et al. (2018)	Illumina MiSeq
Fish	RNA virus	Cyprinid herpesviruses, CyHV-1, -2, -3	Herpesviridae	O	Davison et al. (2013)	Illumina
		Anguillid herpesvirus, AngHV-1	Herpesviridae	O	van Beurden et al. (2010)	Illumina
		Ictalurid herpesvirus 2	Alloherpesviridae	O	Borzak et al. (2018)	Illumina HiSeq2000
		Fisavirus 1	Picornavirales	O	Reuter et al. (2015)	454 GS-FLX platform
		Piscine reovirus	Reoviridae	O	Palacios et al. (2010)	454 GS-FLX platform
		Tilapia lake virus	Orthomyxoviridae	O	Bacharach et al. (2016)	Illumina HiSeq 2500, Ion Torrent
	DNA virus	European sheatfish virus	Iridoviridae	O	Feher et al. (2016)	IonTorrent PGM
		Totivirus	Totiviridae	O	Lovoll et al. (2010)	454 GS-FLX platform
		Sparus aurata papillomavirus 1	Papillomaviridae	O	Lopez-Bueno et al. (2016)	454 GS-FLX platform, Illumina
		Sparus aurata polyomavirus 1	Polyomaviridae	O	Lopez-Bueno et al. (2016)	454 GS-FLX platform, Illumina

Disease: E, *enteric;* R, *respiratory;* O, *other.*

2.7 Viral metagenomics and discovery of new viruses in livestock

2.7.1 New viruses in pigs

Several new astro- and boca-viruses were detected as well as RNA virus's posavirus 1 and 2 in porcine fecal samples from North Carolina using 454 pyrosequencing (Shan et al., 2011). Similarly, a novel virus alike Chimpanzee stool-associated circular ssDNA virus (ChiSCV) was discovered on 454 pyrosequencing platform and presumptively named pig stool-associated circular ssDNA virus (PigSCV) (Sachsenröder et al., 2012). In yet another study, a novel virus was discovered in the fecal samples of healthy piglets, on sequencer Illumina Hiseq 2000, and was named swine pasivirus 1 (SPaV1) (Sauvage et al., 2012). Pestiviruses are prevalent among varied animal species ranging from all farm animals to wild animals, like wild boar and deer species. They were initially discovered through the classical and conventional methods. Now their detection rate has increased with the aid of metagenomics. In the United States, recently discovered pestiviruses have been through the metagenomic studies, under the porcine reproductive and respiratory syndrome virus metagenomic sequencing project (Hause et al., 2015). One of the novel viruses discovered was named atypical porcine pestivirus. Through critical and various cross-validating analysis, identity of a novel virus is set. Later, this virus was found to be widespread in European countries as well (Beer et al., 2017; de Groof et al., 2016; Postel et al., 2016; Schwarz et al., 2017). Metagenomic analysis deciphers for a varied and extended viral flora from single-stranded RNA to circular DNA viruses.

A new parvovirus (PPV7) in rectal swabs of porcine was discovered having a significant identity 42.4% and 37.9%, with fruit bat (EhPV2) and turkey parvovirus, respectively, with NS1 gene (Palinski et al., 2016) and deciphered prevalence in US swine. A new enterovirus G (EVG) (EVG 08/NC_USA/2015) in diarrheic pigs has surfaced with the expressive feature of Torovirus Deubiquitinase (Shang et al., 2017). A putative novel rotavirus C VP6 genotype has been reported in Belgian piglets (Theuns et al., 2016). Novel picornavirus has also been reported in swine feces in Japan (Naoi et al., 2016) and another one having similarity with Posavirus (Hause et al., 2016).

In pigs, several novel viruses are being constantly detected through metagenomic analysis like circovirus PCV3 (Phan et al., 2016), parvovirus (strain swine/Zsana3/2013/HUN) (Hargitai et al., 2016), Parvovirus 7 (Palinski et al., 2016), etc. Porcine parvovirus 2 (PPV2), PPV4, PPV5, PPV6, and porcine bocavirus 1-H18 isolate (PBoV1-H18) (Bovo et al., 2017), first report for Ndumu virus in Uganda (Masembe et al., 2012), 2009 H1N1, pandemic influenza A virus (Greninger et al., 2010), Torque Teno virus, Porcine boca-like virus (Blomstrom et al., 2010), a new variant of Porcine astrovirus (Karlsson et al., 2015), all have been reported by metagenomics approach. Novel genotype of astrovirus 4 in nasal swab of pigs (Padmanabhan and Hause, 2016) has also been reported.

2.7.2 New viruses in cattle

Novel bovine astroviruses (BoAstV-NeuroS1) in California (Li et al., 2013c), BoAstV-CH13 in Switzerland (Bouzalas et al., 2014), and BoAstV-BH89/14 (Schlottau et al., 2016) have been identified in the brain tissues of bovine with encephalitis, having phylogenetic similarity with that of ovine astrovirus, were sequenced through metagenomics. Metagenomics helped in establishing the relationships between various neurologic diseases among various farm animals like bovine, mink, sheep, etc., and comparison was drawn with the outbreak in Denmark, Finland, and Sweden (Quan et al., 2010; Blomstrom et al., 2010). Initially, BoAstV-NeuroS1 etiology was misdiagnosed. But metagenomics led to the proper diagnosis of an astrovirus-related neurologic disorder. A novel astrovirus has also been reported in diarrheic Yak having genetic closeness with deer in Qinghai-Tibetan plateau (Chen et al., 2015).

Schmallenberg virus is among the first novel viruses discovered through metagenomic analysis. It was identified from Germany in cattle suffering from pyrexia, milk drop syndrome, and intermittent diarrhea. The virus belonged to the Simbu serogroup Orthobunyavirus (Hoffmann et al., 2012).

Complete genome of bovine enterovirus (BEV) (BEV AN12/*Bostaurus*/JPN/2014), an enterovirus (EV) has been sequenced in bovines and the distant relation with EVs has been established on the basis of capsid VP1 protein-coding region (Mitra et al., 2016). A new strain of rotavirus A (RVA) G15P [14] in adult cows has been detected through next generation sequencing in Japan during epizootic diarrhea. This strain was associated with decreased milk production and a conclusion was drawn for its derivation from multiple reassortments among Japanese cattle. Various diverse genotypes have been discovered in Japanese cattle in VP3, NSP3, and NSP4 genes of rotavirus B (RVB). Their identity was found to be between around 52%−68% between human, murine, and porcine RVBs. Analysis and comparative studies were revealed for the independent evolutions of bovine RVBs (Hayashi-Miyamoto et al., 2017). In the Asia-Pacific region, a number of picornaviruses have been discovered in various hosts like bats, canines, felines, etc., in quick succession. Similarly, a number of picornaviruses have been found in bovines in Japan and named bovine Japanese Picornaviruses, due to having close relations with Chinese picornaviruses (Nagai et al., 2015).

Bovine papillomavirus type in the genus *Dyokappa papillomavirus* (Bauermann et al., 2017), bovine beta-retrovirus termed BoRV-CH15 (Wüthrich et al., 2016), have been identified in bovines. On Illumina MiSeq, a novel bovine polyomavirus species (BPyV2-SF) was detected along with other known viruses, in store purchased beef from stores in San Francisco (Zhang et al., 2014a,b). In Holstein bull calf, Aichivirus B, was tentatively identified in cerebrospinal fluid by next generation DNA sequencing (Moreira et al., 2017), only after its initial misdiagnosis for infection with RV and *Cryptosporidium*.

2.7.3 New viruses in small ruminants

Tunesian sheep pestiviruses strains Aydin/04 and Burdur/05, among ruminants discovered through next generation sequencing in Turkey, have been linked to have a close relation with classical swine fever virus (Becher et al., 2012). New variants of bluetongue virus have also been reported through metagenomics. A novel boca parvovirus 6 has been documented. Similarly, novel astroviruses have been detected in domestic sheep with nonsuppurative encephalitis and ganglionitis (Pfaff et al., 2017), and one was discovered serendipitously in feces of sheep in Hungary (Reuter et al., 2012).

Various other viruses have also been identified in farm animals and a new taxa of RNA viruses harbored in the gastrointestinal tracts were found to be related to *Tombusviridae* and *Flaviviridae* have been classified. Statoviruses (stool-associated Tombus-like viruses) is one such virus and have diverse range of mammal hosts from humans to bovine (Janowski et al., 2017).

2.7.4 Novel viruses in chickens

In 2014, Zhang et al. detected viral sequences in chicken meat purchased from stores in San Francisco by using a combination of MiSeq Illumina platform and Sanger method. In this study chicken meat contained a novel divergent gyrovirus species (GyV7-SF) besides other known gyrovirus sequences (chicken anemia virus; chicken gyrovirus 3,2,4; humangyrovirus 1).

Bullman et al. (2014) characterized a novel picornavirus (sicinivirus) from fecal samples of broilers which was proposed to represent a novel genus within *Picornaviridae* family. It was considered as a putative avian pathogen, because only a few picornavirus have been detected in birds. Zhou et al. (2015) identified the first sicinivirus isolate (strain JSY) from layer chickens by using viral metagenomics technique. The chickens showed serious symptoms associated with high mortality. Later, Kim et al. (2015a,b) identified the QIA01 strain of the novel picornavirus from chicken. It is similar to two chickens' megriviruses from Hungary. It was suggested that both viruses belong to the same species, Melegrivirus A, but based on the nucleotide identity analysis they may represent different serotypes. The young commercial chickens often suffer from transmissible viral proventriculitis and the novel picornavirus is thought to be responsible for this disease. Boros et al. (2016) reported the complete genome of six known picornaviruses and a novel virus, called chicken phacovirus 1, from a diarrheic chicken.

Ledermann et al. (2014) characterized the complete nucleotide sequence a novel Sunguru virus (family *Rhabdoviridae*), which was first isolated from the blood of domestic chickens from a market in Uganda in 2011. The semiconductor sequencer, Ion Torrent PGM, was used in this study. There is no evidence for this agent to be able to cause disease, moreover the mode of transmission is unknown; thus, further investigations will be needed to determine these missing but important details.

In 2016, a novel chicken astrovirus has been discovered within the genus *Avastrovirus* in Poland, which caused "white chicks" condition. This disease may be associated with economic losses, because the infected embryos and chicks have increased mortality (Sajewicz-Krukowska and Domanska-Blicharz, 2016).

2.7.5 Novel viruses in turkeys

Day et al. (2010) performed metagenomics analysis on RNA virus community in the turkey gut by using Genome Sequencer. Their study targeted the presence of turkey-origin picornaviruses, picobirnaviruses, and caliciviruses. The detection of putative novel enteric viruses in turkey is very important for the poultry industry, as these discoveries may allow new insight into the etiology of multifactorial diseases, such as severe enteric disease which is associated with high mortality in turkey flocks.

Liver samples were analyzed from eight flocks in California to identify an etiologic agent for turkey viral hepatitis, which is a highly infectious disease among young turkeys. Picornavirus sequences, which are probably responsible for the disease, were revealed by Honkavuori et al. (2011). Boros et al. (2012) identified and characterized a complete genome of novel picornavirus in a fecal sample from commercial turkey meat by using the 454 GS-FLX technology. Also Boros et al. (2013) characterized another novel picornavirus, detected in Hungarian flocks of turkeys, which is distantly related to the members of the genus Avihepatovirus. Newly established genera of picornaviruses, avisivirus, megriviruses, and mesiviruses, were also described from turkey (Boros et al., 2014; Ng et al., 2013a,b).

Day and Zsak (2014) analyzed a novel turkey-origin picobirnavirus by pyrosequencing. In that study turkey intestinal tracts served as samples. The picobirnaviruses were identified in Minnesota turkeys (Verma et al., 2015) with "light turkey syndrome" (LTS). They examined fecal samples by metagenomics analysis (Illumina HiSeq) and characterized picobirnavirus strains associated with LTS and non-LTS turkeys. In 2016, Day et al. characterized enteric picornaviruses in turkey and chicken samples also, which may participate in the development of the enteric disease syndromes.

Based on metagenomics data, Reuter et al. (2014) characterized a novel single-stranded DNA virus, called turkey stool-associated circular virus in a fecal sample from a 1-year-old domestic turkey with diarrhea in Hungary.

In 2015, Bányai et al. described a novel clade of avipoxviruses (turkey poxvirus-HU1124/2011) in turkeys associated with cutaneous and oral cavity lesions. Interestingly, the flock was vaccinated by commercial fowlpox virus vaccines. This study reaffirms that for better prevention, it is important to characterize genetic differences between strains and monitor avipoxviruses in birds.

2.7.6 Novel viruses in other birds

Pigeons are reared as pet birds, and pigeon racing is a popular sport. These birds also have close relationship with humans. Phan et al. (2013) collected fecal

specimens from feral pigeons. They identified and proposed a new genus, namely aviparvovirus. Two related picornaviruses, Mesivirus-1 and -2, were also characterized. Moreover, they described group G RV from pigeons. Teske et al. (2017) identified novel aviadenovirus (pigeon adenovirus 2) in the fecal virome of domestic pigeons, sequenced on Illumina MiSeq platform. This novel virus may have immunosuppressive potential, which has a role in the evolution of a multifactorial disease, the young pigeon disease syndrome.

Honkavuori et al. (2008), after pyrosequencing-based analysis, reported two strains of novel bornaviruses from brain samples of parrots with proventriculus dilatation syndrome.

Chen et al. (2013) identified a potential novel species of avian coronaviruses in the genus of Gammacoronavirus specific to ducks or some water fowls. The samples were originated from feces from ducks and swab samples from domestic fowls in poultry farms, backyard flocks, slaughtering houses, and live bird markets in 17 provinces in China. More recently, putative new orthoreovirus species were detected in Pekin ducks in Germany (Farkas et al., 2018). In France, novel avian gammacoronavirus has been identified, which was distantly related to turkey coronavirus. Sequencing of the intestinal contents from guinea fowls was performed by using MiSeq platform. The guinea fowls showed the symptoms of fulminating disease (Liais et al., 2014).

Alkovskhovskii et al. (2013) reported the Khurdun virus genome (on Illumina platform) and classified it as a novel prototypic bunyavirus. The virus was isolated from coot in 2001, in the Volga River delta. Li et al. (2015a,b) characterized the genome of highly divergent gyrovirus (GyV8) from fulmar (*Fulmarus glacialis*). For analysis, spleen and uropygial gland tissues were used. It is the first gyrovirus detected in other avian than chicken.

2.8 Viral metagenomics and discovery of new viruses in pets

2.8.1 Novel viruses in dogs

The first study describing the use of high-throughput sequencing for exploring the virome of dogs dates back to 2011 (Li et al., 2011). Deep sequencing of nucleic acids from the feces of 18 diarrheic dogs were identified as known canine parvoviruses, coronaviruses, and RVs. Also, the study discovered the genomes of canine kobuvirus (*Picornaviridae*) and sapovirus (*Caliciviridae*). Canine sapovirus constituted a novel genogroup within the genus *Sapovirus*, a group of viruses also associated with human and animal diarrhea.

Deep sequencing of pooled stools of wild and domestic canids (wolfs and dogs) in Portugal (Conceição-Neto et al., 2017) has revealed at least 15 different viruses of 12 virus families, including *Parvoviridae* (densovirus, protoparvovirus, dependoparvovirus, and bocaparvovirus), *Picornaviridae* (kobuvirus),

Nodaviridae, *Mimiviridae*, *Totiviridae*, *Paramixoviridae* (canine distemper virus), *Mycodnaviridae*, *Picornaviridae*, *Vigaviridae*, and a number of unassigned viruses. Some of the identified viruses (caninovirus, gemycircularvirus, and densovirus), which are commonly found in arthropods, were likely associated with diet, as wild canids may feed on carcasses infested with a wide range of insects. A novel wolf bocavirus was identified, distantly related to other canine and feline bocaparvoviruses.

A shotgun metagenomics approach was also used to explore the enteric virome diversity of dogs in a case−control study in Australia (Moreno et al., 2017). Eight eukaryotic viral families were detected, including *Astroviridae*, *Coronaviridae*, *Reoviridae*, *Picornaviridae*, *Caliciviridae*, *Parvoviridae*, *Adenoviridae*, and *Papillomaviridae*. Families *Astroviridae*, *Picornaviridae*, and *Caliciviridae* were found only in dogs with acute diarrhea, with *Astroviridae* being the most common family identified in this group.

A beta polyomavirus (dsDNA) was identified in 2017 by metagenomics in respiratory secretions of two dogs with severe pneumonia, which tested negative for all canine respiratory pathogens except *Mycoplasma cynos* (Delwart et al., 2017).

Canine circovirus (ssDNA) was first identified in serum samples from six out of 205 dogs in 2012 (Kapoor et al., 2012a). Subsequently, a similar virus was found in the liver of a dog with severe hemorrhagic gastroenteritis, vasculitis, and granulomatous lymphadenitis (Li et al., 2013a). Screening with specific molecular assays failed to find a firm association with any disease in dogs, although the virus appeared as a common component of canine virome (Anderson et al., 2017; Dowgier et al., 2017; Hsu et al., 2016; Gentil et al., 2017). Porcine-like circoviruses have also been identified in dogs in China (Zhang et al., 2018).

At least four different novel canine parvoviruses (ssDNA +) have been identified starting from 2011 in canids. Bocaparvoviruses distantly related to minute virus of canine (canine parvovirus 1) have been identified in dog respiratory samples (Kapoor et al., 2012b) and in the liver (Li et al., 2013b). Another unclassified bocaparvovirus was identified in pooled stools of wolves from Portugal (Conceição-Neto et al., 2017). A novel protoparvovirus, distantly related to canine parvovirus 2, was identified in young dogs with respiratory disease that tested negative to a panel of respiratory pathogens. The virus was also detected in collections of respiratory and enteric swabs, and its presence was significantly associated only in young animals (<6 months) with respiratory disease (Martella et al., 2018).

Papillomaviruses (PVs) are nonenveloped, dsDNA viruses with a circular genome of about 8000 base pairs. They are generally host species-specific with some exceptions. Numerous of known PVs are associated with benign and malignant neoplasias of the skin and mucous membranes in humans and animals, but there is evidence that asymptomatic infections are more common. More than 200 human and 140 animal PVs have been characterized, illustrating broad genetic diversity (http://pave.niaid.nih.gov/) (Bernard et al., 2010; de Villiers, 2013;

Howley et al., 2013). At least 18 PV types have been identified in dogs (Lange et al., 2016). Canine PVs have been found associated with classical exophytic papillomas such as the common canine oral papillomatosis, with endophytic papillomas, with pigmented plaques and in rare cases with squamous cell carcinomas (Lange and Favrot, 2011).

RVs, family *Reoviridae*, are important enteric pathogens causing gastroenteritis in many mammals and birds (Martella et al., 2010; Bernstein, 2009; Dhama et al., 2015). Non-group A rotaviruses have been identified in young dogs with enteritis in Hungary in 2015. The genome of a RV strain was genetically related to bovine and porcine rotavirus C strains (Marton et al., 2015) whilst two pups were infected by a novel rotavirus species (defined as rotavirus I) (Mihalov-Kovács et al., 2015).

Picornaviruses (ssRNA +) constitute one of the largest virus families and include several human and animal pathogens. However, picornaviruses were not known in the canine host until 2011, when a canine kobuvirus, genetically related to human Aichi virus, was discovered in fecal samples of dogs (Li et al., 2011). Later, the virus has been subsequently discovered in studies in Europe (Carmona-Vicente et al., 2013; Di Martino et al., 2013). In 2012, a novel canine picornavirus, strain 209, was identified in Asia; the picodicistrovirus was classified as a novel species, Cadicivirus A, in the new genus *Dicipivirus* (Woo et al., 2012a). In the same year, another canine picornavirus, strain 325F, still unclassified, was identified in Asia (Woo et al., 2012b). The virus is related to members of the *Sapelovirus* and *Enterovirus* genera.

Several advances have been gathered using sequence-independent assays and deep sequencing for caliciviruses (ssRNA +) of pets. Caliciviruses include important enteric pathogens of humans (noroviruses and sapoviruses) (Lindsay et al., 2015; Oka et al., 2015) and animal viruses (Harrison et al., 2007; Radford et al., 2009; Ohlinger et al., 1990). In 2008 noroviruses were first discovered in dogs (Martella et al., 2008). The virus was genetically related to Alphatron-like (GIV) human noroviruses. In the subsequent years, norovirus strains genetically heterogeneous have been reported in several countries (Mesquita et al., 2010; Martella et al., 2009; Tse et al., 2012) and exposure of dogs to noroviruses has been confirmed in several epidemiological investigations (Di Martino et al., 2017; Caddy et al., 2013; Mesquita et al., 2014). In 2011 the genome of a canine sapovirus was reconstructed in a metagenomic study in the United States (Li et al., 2011). Similar sapoviruses have been identified later on in Italy (Bodnar et al., 2016) and Japan (Soma et al., 2015). Also, specific antibodies and age-related patterns of prevalence have been identified in dogs, confirming that the virus is common in dogs (Melegari et al., 2018). In 2015, a novel vesivirus was reported in Italy (Martella et al., 2015). The canine vesivirus was highly related to caliciviruses found as contaminants of Chinese hamster ovary cells in the United States and Europe (strain 2117-like) and distantly related to the prototype canine calicivirus strain 48. The virus was detected at a low frequency in household dogs but at a high frequency in kennel dogs. Genome sequencing of old (1960s−70s) canine

calicivirus isolates in the United States has characterized the isolates as either 2117-like or strain 48-like vesiviruses (Binn et al., 2018). Also, an acute hemorrhagic gastroenteritis outbreak caused by vesiviruses has been described in the United States in 2015 (Renshaw et al., 2018). The outbreak affected 11 dogs following a stay in a pet housing facility and four dogs died.

A novel flavivirus (ssRNA +) with considerable genomic similarity to human hepatitis C virus was discovered in 2011 in respiratory samples of domestic dogs and tentatively named as canine hepacivirus (Kapoor et al., 2011). More recently it has also been detected in horse sera (Burbelo et al., 2012), and both viruses are currently referred to as nonprimate hepacivirus (NPHV). Epidemiological investigations in the UK dog population identified NPHV by reverse-transcription polymerase chain reaction (RT-PCR) in tracheal tissues of 48 out of 210 dogs and in the liver, lung, and/or tracheal tissues of 12 out of 20 dogs. The presence of NPHV RNA was confirmed by in situ hybridization. Histopathological examination demonstrated a trend toward higher histopathological scores in NPHV-positive respiratory tissues, although, this was not statistically significant (El-Attar et al., 2015).

2.8.2 Novel viruses in cats

Metagenomic studies have also made attempts to explore the composition of feline virome (Table 2.1). Analysis of the stools of a healthy cat from Portugal (Ng et al., 2014) has revealed at least five different viruses of five virus families, including *Picornaviridae* (*Sakobuvirus*), *Astroviridae* (*Astrovirus*), *Parvoviridae* (*Bocaparvovirus*), *Reoviridae* (*Rotavirus*), and *Picobirnaviridae* (*Picobirnavirus*). The presence of these viruses was confirmed by PCR screening in 10 out of 55 cats and coinfections were common. Almost all (nine out of10) cats positive for enteric viruses appeared healthy.

A novel picornavirus, named feline sakobuvirus A, was identified and appeared distantly related to other feline picornaviruses of the genera *Sapelovirus* (Lau et al., 2012b) and *Kobuvirus* (Chung et al., 2013). The feline sakobuvirus represents the prototype species for a novel genus, tentatively proposed as *Sakobuvirus* (Ng et al., 2014).

In the same study, the authors identified a novel feline bocaparvovirus (Ng et al., 2014), distantly related to other bocaparvoviruses identified in cats from Hong Kong (Lau et al., 2012a). The pathogenic role of bocaparvovirues is unclear. The nearly complete genome sequence of a feline bocaparvovirus has been subsequently retrieved in the brain tissues of a cat also infected with feline panleukopenia virus. This was the first evidence of nervous system infection by bocaparvovirus (Garigliany et al., 2016).

Also, a novel feline non-A rotavirus, strain Viseu, was identified in the Portuguese study. The virus was phylogenetically related to RV species B, G, and H (Ng et al., 2014; Mihalov-Kovács et al., 2015). Similar non-A rotaviruses have been identified in a distinct metagenomic study in the United States (Phan et al., 2017).

The etiology of diarrhea in three cats was investigated. The cats tested negative for a panel of bacteria, viruses, and protozoans. NGS analysis revealed the presence of rotavirus RNA and the virus was found to resemble species I rotaviruses detected in sheltered dogs in Hungary (Mihalov-Kovács et al., 2015).

The study by Ng et al. (2014) also identified a feline picobirnavirus closely related to a GII human picobirnaviruses.

A 2014 metagenomic investigation has provided insights into the enteric virome of 10 different small carnivores of the *Mustelidae*, *Canidae*, *Viverridae*, and *Felidae* families in wildlife areas of northern Spain (Bodewes et al., 2014). Viruses belonging to the families of *Anelloviridae*, *Astroviridae*, *Bunyaviridae*, *Caliciviridae*, *Circoviridiae*, *Parvoviridae*, *Picobirnaviridae*, *Picornaviridae*, *Rhabdoviridae*, and *Retroviridae* were detected in 26 out of 42 of the analyzed samples.

A comprehensive picture of the viruses shed in the feces of 25 healthy cats from a shelter in China (Zhang et al., 2014a,b) has identified either complete or partial viral genomes of astroviruses, bocaparvoviruses, and cycloviruses. The near complete genome sequences of three astrovirus strains, D1, D2, and D3, were obtained (Zhang et al., 2014a,b), with strain D1 being distantly related to other feline astroviruses (Lau et al., 2013; Ng et al., 2014). In the same study, the nearly complete genomes of two bocaparvovirus strains, FBD1 and FBD2, were retrieved (Zhang et al., 2014a,b), with FBD1 representing a novel parvovirus species (Lau et al., 2012a; Cotmore et al., 2014). Also, a cyclovirus (ssDNA) (family *Circoviridae*) strain, FD, was identified. Upon sequence and phylogenetic analysis, the virus differed from other cyclovirus species (Zhang et al., 2014a,b). Cycloviruses have been found recently in different biological samples from a number of host species, including mammals and insects (Li et al., 2010a,b; Ge et al., 2011; Rosario et al., 2011; Dayaram et al., 2013; Padilla-Rodriguez et al., 2013; Phan et al., 2014).

At least five papillomavirus types have been described so far in domestic cats. Feline papillomavirus type-1 was originally detected in a cutaneous viral plaque (Tachezy et al., 2002). Feline papillomavirus type-2 was detected in a preneoplastic feline Bowenoid in situ carcinoma (BISC) (Munday et al., 2007). The full genome sequence of a third papillomavirus, type-3, has been obtained by NGS from a cat presenting multiple BISCs (Munday et al., 2013), closely related to canine PVs from the genus Taupapillomavirus. Other two feline PVs, types 4 (Dunowska et al., 2014) and 5 (Munday et al., 2017b), were retrieved from a cat presenting multifocal ulcerative gingivitis (Dunowska et al., 2014) and from a cat with preneoplastic facial lesions (Munday et al., 2017a), respectively.

Morbilliviruses (genus *Morbillivirus*, family Paramyxoviridae) cause severe and often fatal infections of humans and animals including measles, canine distemper, and pest of small ruminants (Lamb and Parks, 2013). Morbilliviruses have been very recently identified in cats. Feline morbillivirus was first detected in Hong Kong (Woo et al., 2012c) and associated with tubule-interstitial nephritis. Further studies demonstrated the presence of the virus in domestic cats in Japan

(Sakaguchi et al., 2014; Furuya et al., 2014), Europe (Sieg et al., 2015; Lorusso et al., 2015; Marcacci et al., 2016), and the United States from healthy cats and from cats with chronic kidney disease (Sharp et al., 2016).

Orthopoxviruses (OPXVs), family *Poxviridae*, are viruses of high interest for scientists because of their potential use as bioterroristic agents and in gene therapy. Reports of OPXV infections in animals and humans have been increasing during the last few decades leading to the perception of an increasing risk for humans (Vorou et al., 2008). In cats, there are a number of reports of poxvirus infections, but the causative agent has been characterized mostly as cow OPXV (Schaudien et al., 2007; Schöniger et al., 2007; Schulze et al., 2007; Johnson et al., 2009; Kaysser et al., 2010; Herder et al., 2011). The first full genome sequences of two feline ortopoxviruses were obtained by NGS approach (Dabrowski et al., 2013) from strains collected as part of the routine diagnostic work at the German consultant laboratory for poxviruses. Phylogenetic analysis showed that the two cat strains clustered into cowpox clade. Later, five novel feline OPXV whole genome nucleotide sequences were generated by NGS from a collection of isolates obtained from several localities in the United Kingdom and Norway (Mauldin et al., 2017). Phylogenetic analyses demonstrated the presence of five different clades (A−E) from cowpox. More recently, a cat with multicentric nodular ulcerative dermatitis was found positive for OPXV (Lanave et al., 2018). The virus differed from cowpoxes common in cats and was grouped with an OPXV strain identified from captive macaques in Italy (Cardeti et al., 2017), related to ectromelia viruses (Chen et al., 2003; Mendez-Rios et al., 2012; Mavian et al., 2014).

A recent evolution toward the diagnostic application of NGS takes advantage of an enrichment/selection step of cDNA with a mixture of oligonucleotide DNA and RNA probes (targeted genome capture, TGC) (Gnirke et al., 2009). TGC coupled with NGS has been performed to detect and generate sequence data of multiple pathogens from three felid species (domestic cats, bobcats, and mountain lions) (Lee et al., 2017). Thirty-one pathogens in 28 samples representing nine of 11 targeted taxa were retrieved from the NGS experiments. Eleven out of 31 pathogens had not been previously detected despite being subjected to standard diagnostic methods. Seven of 11 pathogens were completely or partially sequenced, including feline immunodeficiency virus (FIV), feline coronavirus, feline foamy virus, felid alphaherpesvirus 1, and feline leukemia virus.

Viruses of the genus Orthohepadnavirus, family *Hepadnaviridae*, are partially ds DNA viruses that infect a variety of mammals including primates, bats, and rodents (Seeger et al., 2013). The type species, human hepatitis B virus (HBV), is a major public health problem, and an estimated 257 million people are currently living with HBV. A new feline hepadnavirus was retrieved in a virus discovery project in Australia (Aghazadeh et al., 2018) from a domestic cat presented with multicentric, large cell, high-grade, B-cell lymphoma, and infected with FIV. A molecular survey for hepadnavirus revealed the presence of the novel hepadnavirus in the blood of 6/60 (10%) FIV-infected cats and two out of 63 (3.2%) uninfected cats.

2.9 Metagenomics revealing new virus species in aquaculture

Diseases caused by viruses have a devastating economic effect on aquaculture worldwide, which could be estimated in billions of US dollars annually. NGShas speeded up and revolutionized the viral research of aquatic viruses, as well. The high abundance of aquatic viruses has been known since the 1990s (Bergh et al., 1989; Hara et al., 1991; Proctor and Fuhrman, 1990). Subsequent studies revealed that viruses are the most abundant organisms in the marine environment (Angly et al., 2006; Suttle 2005; Wen et al., 2004). The large number of viral ORFs showing no homologies to any known sequences in databases suggest that the diversity of aquatic viruses may be even greater than imagined (Yin and Fischer, 2008). However, these aquatic viruses infect a much wider range of marine and freshwater species than fishes and shrimps, which are the economically most important species for humans, majority of the detected viruses have hosts among phyto- and zooplankton, and some of them were found in marine mammals, as well. Four major application of NGS in aquaculture could be distinguished and will be discussed below in this order: (1) Metagenomic characterization of the viromes of aquatic environment. (2) Whole genome sequencing of already known/isolated viruses. (3) Discovering novel viruses in diseased or asymptomatic hosts. (4) Molecular epidemiology (tracking the mutations causing changes in the virulence/pathogenicity of viruses), and studying the evolution of viruses (Nkili-Meyong et al., 2016).

2.9.1 Virome characterization

Several papers were published on the microbial ecology of different seas and coral atolls in the last decade (Hwang et al., 2016, 2017; Mahmoud and Jose, 2017; Tseng et al., 2013; Zeigler Allen et al., 2017). In these studies, the overwhelming majority of the viral sequences detected by NGS are unknown, while among the viruses identified, bacteriophages (order *Caudovirales*) infecting a wide range of microbial hosts (*Cyanobacteria*, *Proteobacteria*, and *Bacteroidetes*) and members of the family *Phycodnaviridae* (large eukaryotic phytoplankton viruses) showed the highest abundance. For example in a study on the virome of the marine environment in Goseong Bay (Korea), the BLAST searches showed that only 0.74% of the sequences were associated with known viruses, and 87% of these known sequences were originating from bacteriophages and only 13% showed homology to viruses of eukaryotes (Hwang et al., 2016). Nonetheless, already known DNA and RNA viruses of fish were also detected in these studies, these viral sequences were similar to those of members of diverse families, such as *Nodoviridae*, *Retroviridae*, *Iridoviridae* (Zeigler Allen et al., 2017), and *Alloherpesviridae* (Wood-Charlson et al., 2015; Hwang et al., 2016).

In the last decade, a huge amount of circular replication-associated protein encoding single-stranded (CRESS) DNA viruses were discovered in different ecosystems including marine and freshwater environments (Angly et al., 2006; Kim et al., 2015a,b; Labonte and Suttle, 2013; Rosario et al., 2009; Rosario and Breitbart, 2011). CRESS DNA viruses have a very broad host-range: vertebrates, mollusc, arthropods, etc. Some of them, classified into the family *Circoviridae*, were described from diseased fish, however the connection between the circoviruses and the diseases were not yet proved (Doszpoly et al., 2014; Lorincz et al., 2011, 2012).

An interesting study, demonstrating the potential significance of a ship-mediated virus spread was carried out lately (Kim et al., 2015a,b). In this survey, the composition of viral communities in ballast and harbor waters were determined by metagenomic methods. The results showed that a majority of the viral sequences do not have any homologs in public databases, hence they could not be assigned to any taxa. The minority, the assigned viruses, were prevailed by dsDNA viruses, especially bacteriophages. However, sequences associated with emerging, important viruses of fish and shrimp were also detected, for example, the koi herpesvirus (KHV), infectious spleen, and kidney necrosis virus (ISKNV), white spot syndrome virus Thus, drawing attention to the fact, that ballast water of cargo ships could be an important vector for the transport of nonnative species to new aquatic environments including viruses (Kim et al., 2015a,b).

2.9.2 Complete genome sequencing by next generation sequencing

NGS could be used for the full-length sequencing of already known viruses, this method proved to be very useful for the complete genome characterization of large dsDNA viruses (adeno-, herpes-, iridovirus). For example, the complete genomes of all members of the genus *Cyprinivirus* (Cyprinid herpesviruses, CyHV-1, -2, -3 and Anguillid herpesvirus, AngHV-1) were sequenced by NGS, their genome sizes proved to be the largest among herpesviruses (248−295 kbp). Cyprinid herpesvirus 3 (KHV) is the most ill-famed among the above mentioned viruses, revealing its complete genome was necessary for developing reliable diagnostic methods (PCR, qPCR) and designing DNA vaccine for KHV (El-Matbouli et al., 2007; Zhou et al., 2014). In another study, the deep sequencing of poly(A) RNA of the formerly mentioned AngHV-1 was carried out, providing the first data about the transcriptome of a fish herpesvirus. They found surprisingly lots of RNA splicing, and a low level (1.5/%) of antisense transcription in predicted protein-coding regions unlike in the genome of mammalian herpesviruses (Van Beurden et al., 2012). Another herpesvirus genomes belonging to the genus *Ictalurivirus* were also sequenced, namely the Ictalurid herpesvirus 2 (Borzak et al., 2018) and the Acipenserid herpesvirus 2 (Doszpoly et al., unpublished). These viruses have a significantly shorter genome (143−165 kbp) than that of the cypriniviruses.

Ranaviruses of fish (family *Iridoviridae*) were also sequenced by NGS. The European sheatfish virus was isolated from wels catfish (*Silurus glanis*) and from brown bullhead (*Ameiurus nebulosus*) in Europe (Ahne et al., 1998; Feher et al., 2016), while the epizootic hematopoietic necrosis virus, another ranavirus was described in Australia earlier (Steiner et al., 1991). These viruses share very high nucleotide identity alongside their entire genome (99%). After sequencing their whole genome by NGS, regions showing insertions/deletions were identified, which allows us to design PCR-based diagnostic that differentiates between the viruses without sequencing them (Mavian et al., 2012). NGS could also be used, if the isolation of a virus fails.

Another well-known virus family that encompasses large dsDNA viruses, among them the feared pathogen of smallpox, is *Poxviridae*. This virus family has a rather wide host-range spanning from invertebrates to human, including fish. The presence of pox-like viruses in fish was discovered in carp (*Cyprinus carpio*) 40 years ago (Murakami et al., 1976) causing the carp edema disease (Ono et al., 1986). The causative agent could not be isolated yet, in spite of the several attempts, however its complete genome sequencing using metagenomics is in the pipeline (Haenen et al., 2016). Another poxvirus (salmon gill poxvirus) was reported from salmon (*Salmo salar*), and its full genome was determined by NGS using the organs of diseased fish. Phylogeny inference suggested that the fish poxvirus represented the most basal branch of the subfamily *Chordopoxvirinae* (Gjessing et al., 2015).

2.9.3 Discovery of novel viruses

If all the traditional methods to identify viruses have failed and when the viral pathogen is not culturable in vitro, then NGS is a very powerful tool to discover novel viruses. For example, a novel ssRNA virus was detected from carp (*Cyprinus carpio*) in Hungary by metagenomics. Its genome proved to be 8712 nucleotide in lengths. The virus was named fisavirus, phylogenetic calculations clustered it under the order *Picornavirales*, and it shows a distant relation to posaviruses, however a novel family should probably be established for this virus (Reuter et al., 2015).

Another example is the heart and skeletal muscle inflammation, it was reported from salmon farms in Norway and the United Kingdom in 1999 causing high mortality, but the causative agent remained undiscovered for more than a decade. In 2010, a virus was identified using NGS, the phylogenetic reconstructions clustered the virus into the family *Reoviridae*, and it was named piscine reovirus (Palacios et al., 2010).

A new virus belonging to the genus *Totivirus* (family *Totiviridae*) was also identified in salmon affected by cardiomyopathy syndrome (Haugland et al., 2011; Lovoll et al., 2010).

Tilapia lake virus, an orthomyxo-like virus, was also detected by NGS from brain tissues of diseased fish (Bacharach et al., 2016). In an interesting case, three

viruses from three different virus families were described in a single sea bream (*Sparus aurata*) using NGS. Fish showed the symptoms of lymphocystis disease, usually caused by an iridovirus (lymphocystis disease virus, LCDV). However, in this case a papilloma- and a polyomavirus were also detected in the same specimen, their complete genome sequences were recovered, providing the first description of a papillomavirus in fish. Moreover, the genome of the sequenced iridovirus (termed as LCDV-Sa) proved to be much longer (the largest known vertebrate iridovirus genome) then the formerly described LCDVs, thus LCDV-Sa should be considered a novel and distinct virus species within the genus *Lymphocystivirus* (Lopez-Bueno et al., 2016).

As for crustacean, NGS also contributed for discovering novel viral pathogens (Van Aerle and Santos, 2017). Recently, an iridescent virus causing severe disease and high mortality in farmed whiteleg shrimp (*Litopenaeus vannamei*) has been reported and termed as shrimp hemocyte iridescent virus (SHIV). Partial sequences were obtained by viral metagenomics, and phylogenetic calculations based on the sequences of major capsid protein and ATPase showed that SHIV is a new iridescent virus, which could not be clustered into any of the five known genera of *Iridoviridae*. Based on the sequences a diagnostic PCR was developed for detecting this novel virus threatening shrimp farming industry in China (Qiu et al., 2017).

Novel viral sequences were obtained from another shrimp species, the northern pink shrimp (*Farfantepenaeus duorarum*), using NGS on the hepatopancreas of 12 healthy shrimp captured from the Gulf of Mexico. Among the sequences, a novel nodavirus (*F. duorarum* nodavirus, FdNV) and a new CRESS DNA virus showing highest similarity to circoviruses were identified. The genome analysis of the FdNV suggested that the virus is closely related to nodaviruses causing white tail disease in giant river prawn (*Macrobrachium rosenbergii*) and muscle necrosis disease in whiteleg shrimp (Ng et al., 2013a,b). These studies underline the potential of metagenomic approaches in fisheries and other aquaculture industries to identify unknown pathogens causing diseases or even in asymptomatic animal stocks.

2.10 Conclusion

Viral metagenomics based on sequence-independent amplification techniques coupled with massive parallel sequencing on new generation sequencers have led to the discovery of numerous novel animal DNA and RNA viruses in intensively reared livestock and poultry, pets, and selected aquaculture species over the past decade. In most cases, the pathogenic role, if any, of the newly discovered viruses has remained unclear. Direct and indirect epidemiological investigations, coupled with animal experiments, will be necessary to obtain clearer indications for an etiologic and clinical connection. Nonetheless, new portable next generation

sequencers are on the horizon and MinIon sequencer has proven to serve a promising approach in the field application of disease outbreaks. Currently, the application of this laboratory approach is still in the developmental stage and will likely remain so until per sample sequencing costs becomes acceptable for farmers or pet owners, clinical sensitivity approaches the sensitivity of other multiplexed molecular methods commonly used in diagnostic laboratories and, importantly, bioinformatics pipelines become an easy task not requiring special skills.

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