

REVIEW

Next-generation sequencing technologies: Tool to study avian virus diversity

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Received June 19, 2014; accepted February 13, 2015

Summary. – Increased globalisation, climatic changes and wildlife-livestock interface led to emergence of novel viral pathogens or zoonoses that have become serious concern to avian, animal and human health. High biodiversity and bird migration facilitate spread of the pathogen and provide reservoirs for emerging infectious diseases. Current classical diagnostic methods designed to be virus-specific or aim to be limited to group of viral agents, hinder identifying of novel viruses or viral variants. Recently developed approaches of next-generation sequencing (NGS) provide culture-independent methods that are useful for understanding viral diversity and discovery of novel virus, thereby enabling a better diagnosis and disease control. This review discusses the different possible steps of a NGS study utilizing sequence-independent amplification, high-throughput sequencing and bioinformatics approaches to identify novel avian viruses and their diversity. NGS lead to the identification of a wide range of new viruses such as picobirnavirus, picornavirus, orthoreovirus and avian gamma coronavirus associated with fulminating disease in guinea fowl and is also used in describing viral diversity among avian species. The review also briefly discusses areas of viral-host interaction and disease associated causalities with newly identified avian viruses.

Keywords: next-generation sequencing; viral metagenomics; viral transcriptomics; virus diversity; DNA; RNA; genome; gene

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Abbreviations: AIV = avian influenza virus; AGV 2 = avian gyrovirus 2; aHEV = avian hepatitis E virus; APMV-1 = avian paramyxovirus 1; ILTV = infectious laryngotracheitis virus; ITV = Israel turkey meningoencephalitis virus; LPDV = lymphoproliferative disease virus; MDV = Marek's disease virus; NGS = next-generation sequencing; PPMV-1 = pigeon paramyxovirus 1; USUV = Usutu virus; WNV = West Nile virus

1. Introduction

Globalisation, climate change, wildlife-livestock interface, changes in agricultural practices (e.g. intensive farming), growth in live animal market are some of the drivers responsible for the emergence of novel pathogens and zoonoses (Jones *et al.*, 2013). Among the animal species, wild birds are well-known reservoirs of emerging infectious diseases such as avian influenza (AI) and West Nile (WN) in humans

(Mackenzie and Jeggo, 2013; Wang *et al.*, 2014a). Besides the zoonotic agents, the emergence of new serotypes and genotypic variants of known avian viruses are recognized as potential agents responsible for new infectious diseases (Jackwood and Handel, 2012). High biodiversity of the wild bird population can increase the risk of pathogen spill over to domesticated poultry. Additionally, bird migration also provides a facility to spread the pathogen or dispersing infected arthropod vectors at multiple geographic scales (Mackenzie and Jeggo, 2013). Understanding of virus diversity may be used for forecasting future transmission risks or eventual outbreaks of viral diseases. However, identification or monitoring of the circulation of unrecognized viruses is one of most important requirements for the response to disease outbreaks.

Many classical methods such as electron microscopy, tissue culture or serology were used for virus detection and discovery. Electron microscopy provides only morphologic clues; however many viruses are not culturable. Serological tests depend on the availability of quality antiserum, hindering identification and discovery of viral pathogens; however this can be achieved by molecular methods (Bexfield and Kellam, 2011). During recent decades, introduction of molecular methods has allowed the direct detection and characterisation of the genome sequences of various infectious agents. However, most of the conventional molecular assays are designed to be virus specific or aimed at a limited group of infectious agents. This makes them unsuitable for the detection of unexpected and/or completely new viruses, as well as novel viral variants.

The Sanger sequencing has been standard diagnostic tool for detecting and identifying various pathogens. Nevertheless use of Sanger sequencing provides less sequence information, high cost, and it is used to identify high titre pathogens. With development of novel modern molecular biology technologies, sequence-independent amplification and next-generation sequencing (NGS) offer higher sequencing capacity, speed and lower cost per nucleotide relatively to Sanger sequencing technique. Moreover, NGS allows unbiased detection of a very wide range of infectious agents in a culture-independent manner (Lipkin, 2013). In the field of virology, NGS technologies have been widely used for investigation of genome diversity, virus discovery, metagenomics, discovery of non-coding viral RNAs and gene expression profiling (Radford *et al.*, 2012). This review discusses the different possible steps of avian virus diversity and discovery using next-generation sequencing technologies.

2. Classical methods for study of virus diversity

Cell culture has been the gold standard method for virus discovery and downstream analysis of viruses with other

diagnostic methods, including electron microscopy, serology and PCR. These other methods accompanied by use of virus culturing have been reported in identification of chicken proventricular necrosis virus (CPNV) and adenoviral gizzard erosion in broiler chickens (Guy *et al.*, 2011; Schade *et al.*, 2013). Currently, a variety of singleplex serological and molecular assays are used for avian viral disease diagnosis. Multiplex assays can be more useful in differential diagnosis. Recently, multiplex fluorescent microsphere immunoassay has been developed for simultaneous detection of avian influenza virus (AIV) antibodies against (H1-H16) subtypes and avian paramyxovirus serotype 1 (APMV-1) (Pinette *et al.*, 2014). A wide range of genetic methods have been established for multiplex detection of various poultry viruses causing enteric and respiratory syndromes (Nguyen *et al.*, 2013). Even higher levels of multiplexing can be obtained with DNA microarrays and panviral microarrays that can comprise millions of individual probes to identify or discover divergent avian viruses (Kistler *et al.*, 2008; Maughan *et al.*, 2014).

Sequencing of gene or genome is playing an important role in virus characterisation, epidemiological studies and has also allowed the discovery of new viruses. Novel RNA sequences of an avian calicivirus were revealed from intestinal contents of poultry (Wolf *et al.*, 2012). Novel avian picornavirus species (turdivirus 1 and 2) from wild birds (Woo *et al.*, 2010), pigeon picornaviruses (Kofstad and Jonassen, 2011), quail picornavirus (Pankovics *et al.*, 2012) and two novel picornaviruses (*Gallivirus*) were discovered (Farkas *et al.*, 2012). Aalivirus, a novel duck origin picornavirus has been reported in China (Wang *et al.*, 2014b). The picobirnaviruses are recently described group of viruses in several mammal species that contain dsRNA, bi-segmented genomes and are classified into two distinct genogroups (I or II). Avian picobirnaviruses (AvPBV) genogroup I in Brazilian broiler chickens and distinct group of turkey origin picobirnavirus have been identified (Ribeiro Silva *et al.*, 2014; Day and Zsak, 2014). A novel avian hepatitis E virus (aHEV) genotype IV has been identified in Hungary and Taiwan (Banyai *et al.*, 2012; Hsu and Tsai, 2014). CPNV associated with transmissible viral proventriculitis in broiler chicken was identified as a novel avian birnavirus, representing a distinct genetic cluster within the *Birnaviridae* family (Guy *et al.*, 2011). Novel psittacid herpesvirus 3 and phasianid herpesvirus have been identified with respiratory disease in Bourke's parrots and pheasants with fatal infection, respectively (Shivaprasad and Phalen 2012; Seimon *et al.*, 2012). An existence of a distinct avian papillomavirus group has been reported in various birds (Johns *et al.*, 2009). A novel wood pigeon and feral pigeon astrovirus were identified in Norway (Kofstad and Jonassen, 2011). Rubbenstroth *et al.* (2014) discovered a new avian bornavirus genotype in estrildid finches (*Estrildidae*) in Germany. Emerging novel orthomyxovirus, named as

Wellfleet Bay virus has been reported in eider ducks (Pello and Olsen, 2013). A novel siadenovirus has been identified from polar seabird (Park *et al.*, 2012). During the surveillance for arboviral encephalitis, Travassos de Rosa *et al.* (2002) identified rhabdoviruses from wild birds.

A wide variety of genetic and antigenic variants of novel avian coronaviruses have been reported in poultry (Jackwood and Handel, 2012). Interspecies transmission of avian coronaviruses and emergence of a novel gamma and delta coronavirus were identified in wild aquatic birds (Woo *et al.*, 2009). A wide genetic diversity of novel avian astroviruses detected in different species of wild birds which showed different groups of astroviruses had different host ranges (Chu *et al.*, 2012). Role of recombination has been documented to be a contributing factor in emergence of turkey coronavirus (TCoV); the S gene of infectious bronchitis virus (IBV) recombined with an unknown virus (likely of avian origin), which resulted in a host change (chicken to turkey) and a tropism switch (respiratory to enteric) (Jackwood *et al.*, 2010). Sarker *et al.* (2014) reported quasispecies variants and recombination events in an outbreak of emerging beak and feather disease virus (BFDV) that caused psittacine beak and feather disease. Chicken anemia virus (CAV) was the only known representative of the *Gyrovirus* genus, novel avian gyrovirus 2 (AGV2) and genetic variants of AGV2 were reported in chickens (Rijsewijk *et al.*, 2011; Dos Santos *et al.*, 2012). Avian orthoreoviruses have been associated with a variety of diseases in chickens. Dandar *et al.* (2013) characterized divergent avian reovirus strain from a broiler chicken with central nervous system disease. For the first time, Beserra *et al.* (2014) reported the occurrence of rotavirus group F in broiler from Brazil. Gyuranecz *et al.* (2013) established worldwide phylogenetic relationship of avian poxviruses and provided novel insights into the complex relationship between avian poxviruses and their hosts.

Evidence for intercontinental spread of APMV-1 class II genotype I was provided in migratory wild birds (Ramey *et al.*, 2013). A distinct class II genotype VI consisting of a globally diverse range of pigeon paramyxovirus 1 (PPMV-1) strains has been identified among feral and domestic pigeons (Snoeck *et al.*, 2013). Wild waterfowl is the major reservoir of all influenza A virus subtypes. Highly pathogenic AIV (HPAIV) has been reported to emerge after transmission of low-pathogenic AIV (LPAIV) from wild reservoir to gallinaceous birds. Wu *et al.* (2013) reported emergence of H7N9 viruses by sequential reassortments involving distinct H9N2 donor viruses from wild birds and domestic birds. Parvin *et al.* (2014) identified H9N2, a LPAIVs internal gene reassortment with H5N1, a HPAIV in commercial and backyard poultry. Emergence of H5N8 has been identified in domestic poultry and wild birds with high mortality in domestic poultry (Lee *et al.*, 2014). Recently, emerging H7, H6 and H10 AIV subtype viruses have been detected frequently

in the live poultry markets causing serious concerns to the poultry industry and human health (Wang *et al.*, 2014c; To *et al.*, 2014). Lymphoproliferative disease virus (LPDV), an oncogenic avian retrovirus has been reported in domestic turkeys in Europe and Israel for decades. Allison *et al.* (2014) described emerging LPDV in wild birds and genetic diversity and evolution of LPDV in the United States.

As natural reservoirs or amplifier hosts, birds play an important role in the maintenance and transmission of flaviviruses. Some flaviviruses cause disease in domestic poultry or captive game birds. The emergence of widespread flavivirus named, duck Tembusu Virus (DTMUV), with distinct genotypes has been demonstrated in domestic ducks in China (Yu *et al.*, 2013). Outbreaks of West Nile virus (WNV) have been reported in domestic duck flocks (Wojnarowicz *et al.*, 2007). Emergence and establishment of Usutu virus (USUV) infection was reported in wild and captive avian species in Europe (Steinmetz *et al.*, 2011). Two different species of *Flavivirus* genus, Israel turkey meningoencephalitis virus (ITV) in turkeys and Bagaza virus (BAGV) in wild partridges and pheasants were identified (Llorente *et al.*, 2013). Based on serological and phylogenetic comparison, Fernández-Pinero *et al.* (2014) reported ITV and Bagaza virus were the same virus species and proposed unified name avian meningoencephalomyelitis virus for both viruses.

3. NGS technologies

Several commercially available high-throughput sequencing platforms exist that vary by way of their sequencing principle, sequencing speed, expense, and read length. NGS essentially entails the following steps: sample preparation, sequence-independent amplification, high-throughput sequencing, and bioinformatics analysis as outlined in Fig. 1.

Due to low abundance of viral sequences relative to total host nucleic acids, viral enrichment and concentration procedure requires to remove non-viral nucleic acid. To enrich viral nucleic acid, several measures have been taken such as homogenization, filtration, chloroform treatment, ultracentrifugation, nuclease treatment or combinations of these methods prior to viral nucleic acid extraction (Hall *et al.*, 2014).

The amount of total nucleic acids isolated from viral particles is often too low for sequencing and may require amplifying of viral nucleic acid. The amplification has been aided by the development of sequence independent methodologies which simultaneously amplify several highly divergent viral genomes (Rosseel *et al.*, 2013). Sequence-independent single-primer amplification (SISPA), random PCR (rPCR), multiple displacement amplification (MDA) or virus discovery cDNA-amplified fragment length polymorphism (VIDISCA) are commonly used sequence-independent

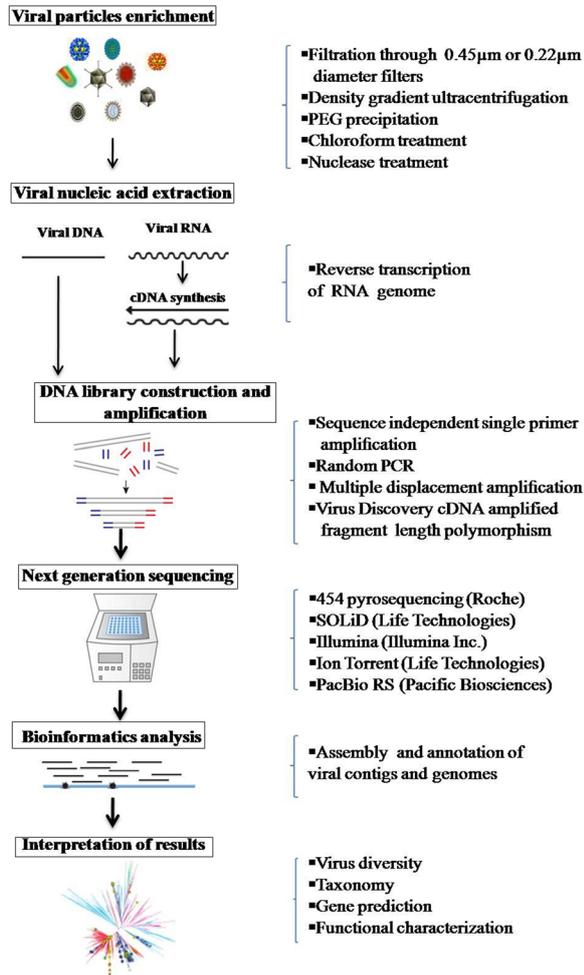


Fig. 1

Workflow for virus detection using next-generation sequencing

amplification methods (Johne *et al.*, 2009; de Vries *et al.*, 2011; Sujayanont *et al.*, 2014).

NGS platforms require the viral genomic sequences in the samples to be converted into sequencing libraries and later their amplification. Sequencing libraries are constructed by fragmenting the DNA (or cDNA) sample and ligating adapter sequences onto the ends of the DNA fragments and clonal amplification for sequencing. NGS technologies are based either on sequencing by synthesis or sequencing by ligation reaction controlled by polymerase or ligase. 454 pyrosequencing (Roche) is based on emulsion PCR (emPCR), in combination with a sequencing-by-synthesis approach. SOLiD (Applied Biosystems) also uses emPCR for clonal DNA amplification on beads to allow sequencing by ligation. Illumina (Illumina Inc.) is based on bridge-PCR on the surface of glass flow cell and uses sequencing-by-synthesis approach. Ion Torrent (Life Technologies) is sequencing-by-synthesis method that also uses emPCR. Ion Torrent

detection relies on ion-sensitive, field-effect transistor-based sensors to measure hydrogen ions released during polymerase-mediated incorporation. PacBio RS (Pacific Biosciences) is able to sequence single DNA molecule in real time without the need for clonal amplification. Currently, Nanopore sequencers (Oxford Nanopore Technologies) are being developed, which are based on the concept that single DNA molecule can be identified when passing through engineered nanopore protein immobilised on a solid surface. A detailed comparison and performance of various NGS instruments have been reviewed elsewhere (Mardis, 2013; Ku and Rouks, 2013).

The applications of NGS technologies in avian virology with examples are listed in Table 1.

3.1 Genome characterization and virus diversity

Molecular epidemiology using nucleotide or full genome sequence data is widely used to monitor transboundary movements of avian viruses which clarify our understanding of the genetic diversity and viral evolution. Moreover, genomic sequences of viral strains can be used to identify the molecular determinants that underpin important phenotypic traits such as virulence and pathogenicity and prediction of novel genes and viral features that are important for viral replication or pathogenesis. NSG techniques have been used to sequence and conduct comparative full genome analysis of district genotype of infectious laryngotracheitis virus (ILTV) (Spatz *et al.*, 2012). Complete genomes of fowl adenovirus A to E species (Marek *et al.*, 2013) and the non-chicken aviadenovirus species has been reported (Kajan *et al.*, 2010), which provided phylogeny and evolution of the genus *Aviadenovirus*. Recently, Marek *et al.* (2014) revealed the existence of two unknown lineages and common evolutionary origin of turkey adenovirus 5 (TAdV-5) and fowl adenovirus 1 (FAAdV-1). Bodewes *et al.* (2013) identified novel gull adenovirus and demonstrated that birds of the *Laridae* family are infected by family-specific adenoviruses.

Many RNA viruses exhibit extreme evolutionary dynamics and genetic diversity due to the high mutation rates and short generation times (Beerenwinkel *et al.*, 2012). APMVs are composed of the antigenically and genetically distinct avian paramyxovirus serotypes APMV 1 to 9 and two more APMV-10 and APMV-11 serotypes have been suggested (Miller *et al.*, 2010; Briand *et al.*, 2012). Based on antigenic and genetic characterization, Terregino *et al.* (2013) proposed new APMV-12 group. Forrester *et al.* (2013) revealed ancestral lineage of APMV 1 class II genotype XII isolated from Indonesia and provided an insight into historic Newcastle disease virus (NDV) evolution. Van Borm *et al.* (2013) identified genome sequence variability in PPMV-1 isolates from Belgium. Three APMVs, two different APMV-4 and one APMV-6 were identified (Rosseel

Table 1. Application of next generation sequencing technologies in avian virology

NGS methods	Application	Examples	References
Genome sequencing	Genomic characterization and gene discovery	Revealed the unique genome feature like internal ribosomal entry site and aphthovirus 2A-like sequence with 'ribosome-skipping' sites in avian picornaviruses	Boros <i>et al.</i> (2014)
		Identified a novel small-RNA population encoded from exogenous virus-specific region E (XSR) element of avian leukosis virus subgroup J (ALV-J)	Yao <i>et al.</i> (2014)
		Provided evidence for a role of Nsp14-Exonuclease (nsp14-ExoN) in RNA proof-reading during coronavirus replication	Eckerle <i>et al.</i> (2010)
	Molecular determinants associated with virulence	Identification of emergence of virulence-associated E627K substitution in polymerase basic protein of H7N7 as adaptation marker	Jonges <i>et al.</i> (2014)
		Identification of US10 gene and UL41 gene associated with reversion to virulence of low and high passages of tissue culture origin and chicken embryo origin infectious laryngotracheitis vaccine strains respectively	García <i>et al.</i> (2013)
	Characterization of viral quasispecies	Presented evidence of quasispecies and mixed infections among avian influenza A viruses	Ramakrishna <i>et al.</i> (2009)
		Characterized quasispecies composition and genomic diversity of pigeon paramyxovirus type-1	Van Borm <i>et al.</i> (2013)
	Novel pathogen discovery	Discovery of a novel orthomyxovirus, designated Cygnet river virus, within genus quarjavirus in Muscovy ducks	Kessell <i>et al.</i> (2012)
		Discovery of Farmington virus as a new species within the family <i>Rhabdoviridae</i> isolated from a wild bird	Palacios <i>et al.</i> (2013)
		Discovery of Tvarminne avian virus as a candidate new species within the genus orthoreovirus in a crow	Dandar <i>et al.</i> (2014)
	Viral metagenomics	Metagenomic identification of several viral families, such as <i>Picornaviridae</i> , <i>Caliciviridae</i> and <i>Picobirnaviridae</i> from turkey gut	Day <i>et al.</i> (2010)
		Characterization of enteric virome in droppings from wild pigeons	Phan <i>et al.</i> (2013)
	Virus associate with disease of unknown etiology	Identification of a novel avian gammacoronavirus associated with Fulminating disease ('X disease') of guinea fowl	Liais <i>et al.</i> (2014)
	Immune escape	Investigation of the emergence of escape mutants during serial in-vitro passaging of H5N1 AIV under immune pressure	Hooper <i>et al.</i> (2012)
	Quality control of live attenuated viral vaccines	Detection of nucleic acids from endogenous avian leukosis virus in live attenuated viral vaccines derived from chicken embryo fibroblasts	Victoria <i>et al.</i> (2010)
RNA sequencing	Discovery of viral non coding RNA	Identification of role of viral encoded microRNAs in tumour transformation and latency in Marek's disease virus	Hicks and Liu (2013)
		Identification of novel miRNAs encoded by duck enteritis in regards to their genomic positions	Yao <i>et al.</i> (2012)
	Host-pathogen interaction	Discovery of novel genes and signalling pathways associated with disease resistance to avian influenza virus infection in chickens	Wang <i>et al.</i> (2014d)
		Identification of MYC, TERT, and ZIC1 genes as common targets for viral integration and transcriptional deregulation in ALV-J induced myeloid leukosis	Li <i>et al.</i> (2014)
Chromatin immunoprecipitation sequencing	DNA-protein binding	Identification of binding sites occupied by Marek's disease virus oncoprotein, (Meq) and/or c-Jun within the chicken genome	Subramaniam <i>et al.</i> (2013)

et al., 2011) and their genetic diversity within the serotypes was demonstrated. NGS approaches have been approved for use in routine diagnostics to monitor the genomic diversity of AIV, early emergences and transmission of these viruses from waterfowl to domestic poultry (Croville *et al.*, 2012). Based on ultra-deep sequencing approaches, evidence of emerging HPAIV from a LPAIV progenitor (H7N1) has been provided (Monne *et al.*, 2014). USUV and WNV are members of *Flavivirus* genus belonging to the Japanese

encephalitis antigenic complex and are phylogenetically closely related. First epizootic emergence of USUV in wild and captive birds in Germany and WNV genetic variability has been reported from birds in USA (Becker *et al.*, 2012; Grinev *et al.*, 2013). Divergent avian origin picornavirus genera such as *Avisivirus*, *Megrivirus* and *Mesivirus* have been identified from turkeys and chickens (Ng *et al.*, 2013; Boros *et al.*, 2014). Two novel picornaviruses (mesivirus 1 and 2) were identified from wild pigeons (Pham *et al.*, 2013).

Most recently, Bullman *et al.* (2014) identified picornavirus (*Siccinivirus*) from chickens which form a monophyletic group with *Passerivirus* (formerly turdivirus 1) and proposed novel genus within *Picornaviridae* family.

The highly variable mixture of closely related genomes within a given host is referred as quasispecies. NGS technologies which sequence viruses with ultra-deep coverage ('ultra-deep sequencing') are able to track genomic changes over time and are high enough to capture rare variants. NGS approaches have significantly improved our understanding of the intra-host viral population dynamics. The feasibility of using ultra-deep sequencing to reveal the genomic diversity of PPMV-1 and AIV quasispecies has been demonstrated (Ramakrishna *et al.*, 2009; Van Borm *et al.*, 2013).

Using ultra-deep sequencing, Jonges *et al.* (2014) investigated the viral quasispecies dynamics associated with AIV subtype H7N7 infection and identified emergence of virulence-associated E627K substitution in polymerase basic protein of H7N7 as adaptation marker. To detect the evolution of HPAI virus from LPAI virus within the infected host, Iqbal *et al.* (2014) reported ultra deep sequencing of hemagglutinin (HA) gene polybasic cleavage site and failed to detect any evidence of HPAI virus signatures.

New evidence obtained from the analysis of whole genome sequences of ILTV vaccine and field strains indicated that recombination between attenuated vaccines belonging to different genotypic lineages in the field resulted in the emergence of novel virulent strains of ILTV (Lee *et al.*, 2013). Evidence of interspecies transmission and reassortment among avian group A rotaviruses has been reported (Schumann *et al.*, 2009). NGS has been employed to characterise an avian group A rotavirus genome containing a novel viral protein (VP) 4 gene which is closely related to mammalian rotaviruses representing an avian/mammalian rotavirus reassortment (Trojnar *et al.*, 2013).

3.2 Virus discovery and viral metagenomics

The introduction of culture-independent NGS technologies, termed as viral metagenomics (community genomics), made powerful diagnostic technology to investigate the complete viral genetic populations of a sample. Viral metagenomics is nowadays routinely used for viral detection, novel virus discovery, and divergent virus genome recovery. This approach has been reported in detection of AGV2, avirulent NDV co-infection in chickens with neurological symptoms and high mortality, avipolyomavirus and avipoxvirus co-infection in periocular skin lesion of gery butcherbird (Abolnik and Wandrag, 2014; Bennett and Gillett, 2014). Based on metagenomics data, Belak *et al.* (2013) reported interstitial nephritis associated with a variant of NDV-V4 vaccine strain. To explore the role of live poultry markets in the origin of the novel human H7N9 virus, Yu *et al.* (2014)

provided direct evidence of coexistence of influenza H7N9 and H9N2 in poultry linked to human H7N9 infection. Reuter *et al.* (2014) identified novel group of unclassified single-stranded circular DNA viruses called stool-associated circular virus in a faecal sample from a domestic turkey. Tvarminne avian virus (TVAV), avian origin reovirus was identified in a crow showing neurological disorders. Dandar *et al.* (2014) reported TVAV as a candidate new species within the genus *Orthoreovirus*. A novel duck specific avian coronavirus genetically closer to some coronaviruses circulating in wild water fowl has been identified (Chen *et al.*, 2013). A novel delta coronavirus and astrovirus were identified in shorebird samples from the Delaware Bay, an important feeding ground for thousands of migratory birds (Honkavuori *et al.*, 2014). Durham and Farmington viruses as new species from wild birds (Allison *et al.* 2011; Palacios *et al.*, 2013) and novel Sunguru virus within *Rhahdoviridae* family have been discovered from domestic chickens in Uganda (Ledermann *et al.*, 2014). Khurdun virus a new representative of the *Orthobunyavirus* has been identified from coot (Alkovskhovskii *et al.*, 2013).

Metagenomics have the capacity to detect viruses either as single agents or as syndromes to identify the etiologic agents. Honkavuori *et al.* (2008) discovered two strains of a novel Borna viruses in psittacine birds with proventriculus dilatation syndrome (PDS) characterised by gastrointestinal dysfunction and neurological signs. To identify a candidate of etiologic agent for turkey viral hepatitis (TVH), Honkavuori *et al.* (2011) reported picornavirus in liver samples from diseased turkey poults. Fulminating disease (X disease) of guinea fowl is acute enteritis characterised by intense prostration and a very high mortality. Although its viral origin was previously suspected, virus remained unknown. Liais *et al.* (2014) identified a novel avian gamma coronavirus associated with this disease which was distantly related to TCoV. To decipher the RNA virus community from turkey flocks experiencing enteric disease, Day *et al.* (2010) analyzed turkey gut RNA virus metagenomics and demonstrated the presence of turkey-origin members of the *Picornavirales*, and the genus *Caliciviridae*, and the turkey picobirnaviruses. In order to better understand the viruses shed by pigeons, Phan *et al.* (2013) characterized enteric virome in droppings from wild pigeons and identified sequences of novel aviparvovirus, picornaviruses and described group G rotavirus from pigeons.

4. Transcriptomics and virus-host interaction

Initially, microarrays have been used to quantify gene expression. Introduction of NGS technologies revolutionized transcriptome profiling, an approach referred to as RNA sequencing (RNA-seq). NGS offers an opportunity

for detailed examination of transcriptomics at both host and pathogen level during an infection, thereby, elucidating the mechanisms of disease and pinpointing the functional pathways involved in the host response to infection. Comprehensive analysis of AIV-infected lung tissue suggested that several microRNA (miRNAs) and mRNAs were likely to be involved in regulating the host response (Wang *et al.*, 2012). Allele-specific expression (SNPs, genes and pathways) that was associated with transcriptional response to Marek's disease virus (MDV) infection was identified to underlay genetic basis for disease resistance to MDV infection in both broilers and layers (Perumbakkam *et al.*, 2013). Gene expression patterns of normal and duck hepatitis A virus genotype C (DHAV-C) infected duck livers were explored and compared, and new insights into the molecular mechanism of host-DHAV-C interaction were provided (Tang *et al.*, 2013). Huang *et al.* (2013) performed deep transcriptome analysis responsive to AIV using the lung of control ducks and ducks infected with highly pathogenic and weakly pathogenic H5N1 virus. The analysis revealed that β -defensin and butyrophilin-like gene repertoires were involved in host immune response. The information gained by using RNA-seq to obtain an overview of pathogen-induced host responses or to follow temporal expression changes during the infectious cycle can be exploited to design better control strategies as well as help in identification of genetic markers for disease resistance.

Chromatin immunoprecipitation (ChIP) was first developed to discover DNA-protein binding sites. ChIP coupled with high-throughput sequencing (ChIP-Seq) has been applied to quantitatively analyze binding sites of DNA associated proteins across the entire genome. ChIP-Seq has been reported to identify the binding sites in host genomes or transcription factors involved in virus infection and pathogenicity. Using chromatin immunoprecipitation-sequencing analysis, Subramaniam *et al.* (2013) identified binding sites occupied by MDV oncoprotein, (Meq) and/or c-Jun within the chicken genome and found enriched Meq binding sites in the promoter regions of upregulated genes. These technologies will contribute in achieving a comprehensive understanding of the DNA-binding profiles and epigenetic modification patterns associated with immune responses to both pathogens and vaccines.

5. Evidence of causal virus-disease relationship

NGS has provided a powerful tool for novel virus discovery but, it is only the first step to determine the etiology of a disease. To assign etiological agent, attempts should be made to accomplish Koch's postulates, which require the agent to be isolated in culture, or River's modifications, which recognize the added presence of neutralizing antibodies to

an agent in evidence of infection or relationship between the presence of viral sequences and disease (Lipkin, 2013). Moreover, *in vitro* culture assays may be performed to assess the host range, cell tropism and entry receptors. Recent successes in identifying avian viruses and associating them with disease include DTMUV associated with severe egg drop syndrome (Huang *et al.*, 2013). In case of unculturable viruses, the ability to construct infectious clones of novel virus, directly from clinical samples using either NGS de-novo sequencing or by standard methods such as primer walking can be helpful in identifying etiological agents and characterization of the virus. For example, due to the lack of an efficient cell culture system for aHEV, Kwon *et al.* (2011) developed an infectious cDNA clone of aHEV strain recovered and assessed causality, pathogenicity and sero-conversion in SPF chickens. To prove etiological agent using metagenomics, the metagenomic Koch's postulates have previously been introduced. They focus on the identification of metagenomic traits in diseased subjects (Mokili *et al.*, 2012). However, this adaptation still requires the introduction of disease to prove causal virus-disease relationship (Bibby, 2013). Complicating disease association studies are the extent of viral genetic diversity and co-infections with other agents may influence clinical outcome. Deciphering such complex interactions will require viral metagenomics that query all virus families or genotypes in such cases, therefore, number of samples and repeatability in independent studies should be considered (Delwart, 2012).

6. Conclusions and perspectives

NGS platforms have been recognized as important tools in veterinary medicine which have enabled the discovery of viruses, viral diversity and development of viral metagenomics. Although adoption of NGS in routine clinical laboratory has enormous challenges due to the high costs of instruments and supplies, and requirements of skills and expert personnel to analyse the large datasets and their interpretation. As the cost and complexity of sequencing platforms are declining the routine use of NGS technology in the microbiology laboratory particularly in diagnostic clinical microbiology will become a routine practice. The discovery of novel viruses has been reported from wild and domestic birds. The discovery of these viruses will enable the development of diagnostic assays for the future detection or epidemiological investigation of viruses. Based on viral metagenomics approaches, complex gut viral community related to enteric syndrome in poultry and avian related gyroviruses were identified on human skin or in diarrheal samples. However, the identification of the etiological agents which are not proven yet, requires isolation of agent to fulfil Koch's postulates.

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