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Opinion

Next-Generation Sequencing: An Eye-Opener for the Surveillance of Antiviral Resistance in Influenza

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Next-generation sequencing (NGS) can enable a more effective response to a wide range of communicable disease threats, such as influenza, which is one of the leading causes of human morbidity and mortality worldwide. After vaccination, antivirals are the second line of defense against influenza. The use of currently available antivirals can lead to antiviral resistance mutations in the entire influenza genome. Therefore, the methods to detect these mutations should be developed and implemented. In this Opinion, we assess how NGS could be implemented to detect drug resistance mutations in clinical influenza virus isolates.

Highlights

Sanger sequencing remains sufficient when only a few samples are analyzed and only the NA gene is sequenced.

With the development of new antivirals that also target other proteins than the NA protein, resistance mutations could arise across the whole genome. In this case, sequencing only the NA segment will not be sufficient anymore and NGS will be the better option.

NGS opens the possibility to analyze the minority variants present in the sample. This could give the possibility to detect the emergence of antiviral resistance at an early stage.

What Is the Importance of Genetic Influenza Surveillance with Regard to Antiviral Resistance?

One of the major technological evolutions in life sciences of the past decade is **next-generation sequencing** (**NGS**; see Glossary). This technology could enable a more effective response to a wide range of communicable disease threats, such as influenza viruses. A major advantage of whole-genome sequencing (WGS) is that this one technique can provide broad and detailed data on the identify of pathogens that previously required multiple laboratory phenotypic and genotypic assays. Moreover, WGS provides higher resolution information than conventional genotyping tests. qRT-PCR and Sanger sequencing, for example, are often targeted to a limited fraction of the genome, meaning that crucial information can be missed. The additional genotypic information that WGS can provide, could be critical when tracking the origin of outbreaks and to forecast the spread of disease. Influenza A and B viruses are a major cause of respiratory tract infections in humans, resulting in significant morbidity and mortality [1]. The genome of these viruses consists of eight segments of single-stranded RNA of negative polarity. Influenza viruses can mutate rapidly when subjected to selection pressures, such as immunity induced by prior or vaccination [2] or antiviral drug use [3]. The **hemagglutinin (HA)** and **neuraminidase (NA)** proteins are often considered to be the most important viral antigens, because they are major targets of the immune system. HA and NA are used as targets for current anti-influenza strategies: HA is the prime target of the currently licensed influenza vaccines, and NA inhibition is the main mechanism of the most frequently used influenza antivirals. Although vaccines are considered the best way to prevent influenza, the limited use and their generally poor effectiveness in the elderly [4] (https://www.cdc.gov/flu/about/qa/vaccineeffect.htm) imply that efficient antiviral drugs are needed as a complementary or alternative line of defense.

Monitoring and detecting mutations in the influenza virus genome, especially those that confer antiviral resistance, is of paramount importance to public health surveillance (https://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/nai_genotyping_molecular/en/). As the use

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of antiviral drugs continues to grow, more cases of drug-resistant viruses are expected to occur. Because of this, and the fact that a limited number of anti-influenza drugs with different mechanisms are currently available, it is important to assess whether the use of NGS could add value for the preparedness and response to the emergence of antiviral resistance. The determination of full influenza genomes, which is possible by more extensive use of NGS, will allow for a better understanding of the genetic determinants of viral resistance, and may enable the detection of minority drug resistant viral populations.

In this Opinion, we provide an overview of the potential antiviral drug resistance mutations in influenza virus genome, based on a thorough review of the literature on antiviral drugs that target different stages of the viral life cycle (Figure 1).

Discovering Mutations That Confer Antiviral Resistance

Different methods can be used to identify the appearance of antiviral drug resistance. Screening viruses in clinical samples for antiviral resistance using a specific phenotypic assay is the first method that can be used, but this implies that the antiviral drug is already commercially available and in use. Phenotypic assays can evaluate the production of virus particles in the presence of the antiviral drug in comparison to mock-treated conditions (total amount of virus using for example ELISA-based methods, or infectious particles using for example plaque reduction assays), or the activity of the enzyme/protein targeted by the antiviral (NA-inhibition assay for example).

Other approaches, such as serial passages in cell culture or in animal models [5,6], are useful to evaluate new compounds and potential appearance of resistance. In both cases, the associated mutations can then be identified by sequencing the viral genome. Structural analyses can also be used to determine amino-acid substitutions that would likely confer resistance. Using **site-directed mutagenesis** and **reverse genetics** systems, these theoretical mutations can be tested to confirm the resistance in phenotypic assays [7].

More than 200 mutations in the influenza virus genome reportedly confer antiviral drug resistance. For each mutation (Table S1 in the supplemental information online), the mechanism that explains the conferred resistance (if known) and the origin of that used to identify these mutations (e.g., clinical sample, reverse genetics, or serial passaging) are provided. Oseltamivir and zanamivir, two NA-activity inhibitors, are currently the most commonly used antiviral drugs; it is thus not surprising that the majority of the mutations were found in the NA segment (see Figure S1 in the supplemental information online). Mutations detected in patients and reported in more than five papers that induce resistance against commercially available antivirals or antivirals in clinical trials were included in a table of antivirals (see Table S2 in the supplemental information online).

Detection of Antiviral Resistance Mutations: From Classical Surveillance to the Implementation of NGS

Once a mutation has been identified that confers resistance to an antiviral drug, it is important to be able to rapidly detect and follow its possible emergence in circulating strains. Although phenotypic tests remain the only way to confirm the resistance of the virus, genotypic assays are the most commonly used in clinical samples because they provide a rapid method to detect known mutations and eliminate the need for virus isolation and propagation in cell cultures (Table 1) [8–12]. In addition, if loaded into an in-house bioinformatics pipeline or another web-based application, these known mutations can be rapidly identified.

Genotyping by qRT-PCR is commonly used as a fast and relatively inexpensive method that can be performed directly on clinical specimens [8,13]. However, this approach can only detect one

Glossary

Coverage: sequenced fragments overlap a certain location in the genome. The number of times a nucleotide at a particular position in the genome has been read is defined as the coverage.
Hemagglutinin (H) and Neuraminidase (N): both influenza A and B have H and N proteins that cover the surface of the viral envelope. Influenza A viruses are divided into subtypes based on the structure and antigenicity of these two proteins. Among influenza A viruses there are 18 different types of hemagglutinin and 11 different types of neuraminidase. Each virus has one type of H (H1–H18) and one type of N (N1–N11).
Next-generation sequencing (NGS) or high-throughput or massively parallel or deep sequencing:: description of DNA sequencing technologies that can produce millions or billions of short reads in parallel, such as Illumina. Each position is sequenced multiple times, which provides a high depth of coverage to deliver accurate data and an insight into nucleotide sequence variation.
Reverse genetics: to understand the function of a gene, reverse genetics tries to identify which phenotypes arise because of particular genetic sequences. A gene sequence is known and then the phenotype is investigated.
Site-directed mutagenesis: specific and intentional introduction of nucleotide sequence changes in a DNA or RNA molecule. This way, the impact of certain genetic changes (insertions, deletions, substitutions) can be investigated with reverse genetics.
Viral quasispecies: subpopulations present in a biological sample that carries low frequency variants. The RNA viral genomes typically exhibits high mutation rates, which leads to a heterogeneous population within the same patient, each with specific evolutionary properties.
Virion: free viral particle that consists of an RNA or DNA core with a protein coat and sometimes with an external envelope, which is a lipid bilayer.

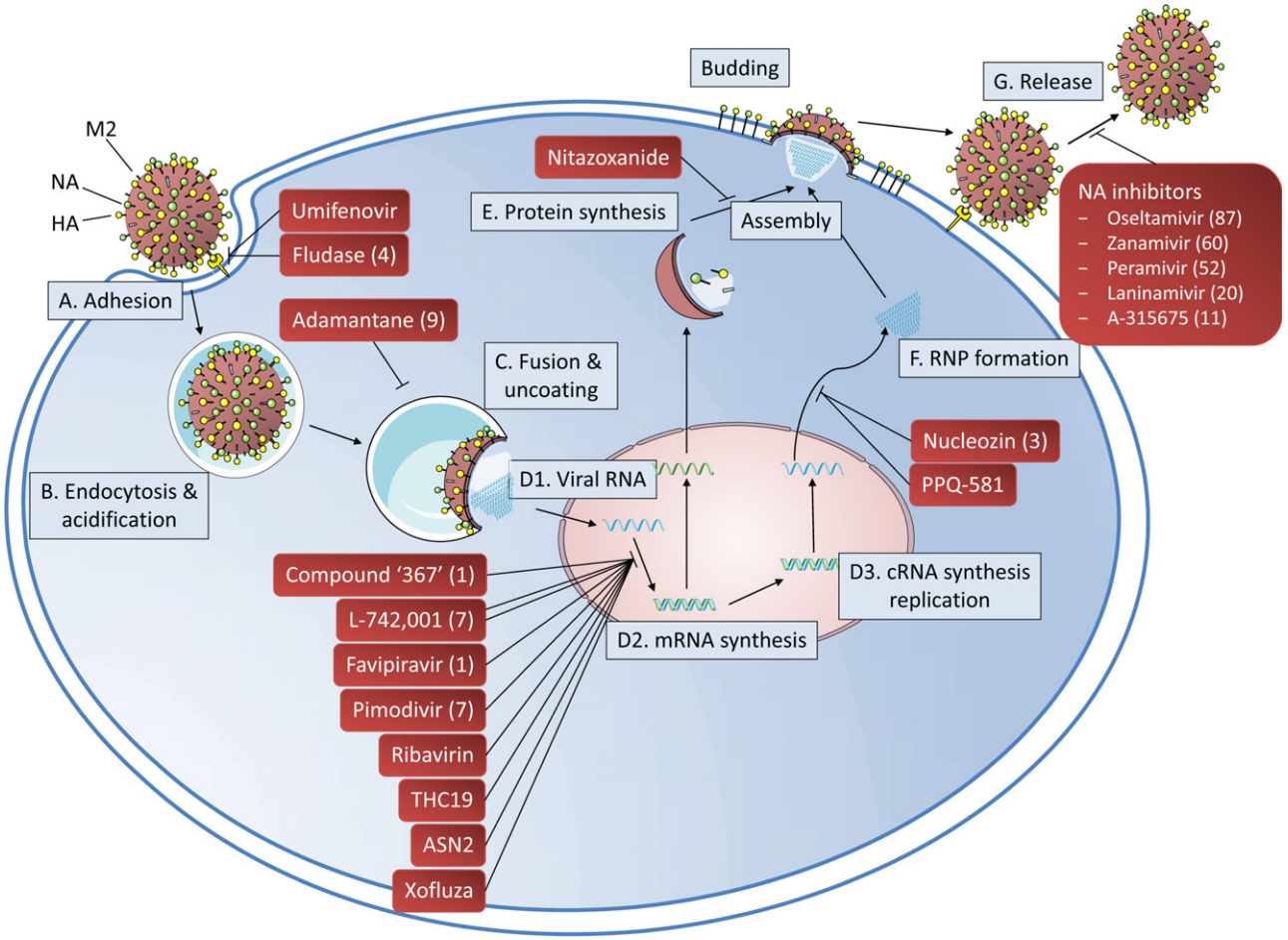

Figure 1. Antiviral Drugs against Influenza Virus and Their Target Sites in the Virus Cycle. The different stages of the viral cycle are the binding of HA to the sialic-acid containing host receptor (A), followed by the endocytosis and the acidification of the HA protein (B). This acidification in the early endosomes leads to fusion of the viral and endosomal membranes, and triggers the influx of H^+ ions through the M2 channel that results in the dissociation of the vRNPs and uncoating (C). After transport of the vRNPs to the nucleus, viral mRNA synthesis is initiated by the viral polymerase. The latter is also responsible for the unprimed replication of the vRNA through a cRNA intermediate (D). The viral mRNAs are exported to the cytoplasm and translated into viral proteins. In the endoplasmic reticulum, the surface proteins HA, M2, and NA are processed, glycosylated, and transported to the cell membrane (E). The newly synthesized vRNPs are transported to the cytoplasm, mediated by an M1–NS2 complex that is bound to the vRNP (F). At **virion** assembly and budding sites, the newly produced vRNPs are incorporated into new viruses. Finally, the NA cleaves these sialic acid residues and virions are released from the host cell (G). The figure illustrates the different antiviral drugs at the position where they interfere with the viral cycle. Between brackets the number of mutations that are known to be related to antiviral resistance are indicated to the different antiviral drugs. Abbreviations: HA, hemagglutinin; NA, neuraminidase: vRNP, viral ribonucleoprotein.

targeted mutation, as it relies on limited differences in the genome. qRT-PCR can thus be difficult to develop and it has limited benefits in the context of surveillance [8,14,15].

Sanger sequencing is still used as a standard reference method for routine genetic surveillance of influenza viruses. However, the viral RNA genome must first be extracted, and the genomic segment of interest must be amplified by RT-PCR. Each Sanger sequencing reaction is based on one single primer pair and provides a sequence of 400–1000 bases in length [16,17]. To obtain the sequence of an entire segment or even the whole influenza genome (approximately 14 kb), multiple primers must be used in parallel reactions [18]. Sanger sequencing thus becomes labor intensive and can be expensive if the whole genome sequence of a large set of samples is

Table 1. Comparison of Different Genotypic Assays

	Benefits	Challenges	NGS platform	Time (h)	Read length (bases)	Raw error rate (%)	Cost per Gb
qRT-PCR	• Equipment often already present in laboratories • High sensitivity • Quick & simple workflow	• Limited set of variants • No identification of novel variants • Low scalability • Low variant resolution	NA	1–2	NA	NA	NA
Sanger sequencing	• Cost-effective for small stretches of DNA • Quick & simple workflow	• Low sensitivity • Low variant discovery power • Low scalability	NA	24	400–1000	0.001	US$ 10 000 000
NGS	• Identification of novel variants • Expanded discovery power • Higher analytical sensitivity • Great resolution • Higher sample throughput with multiplexing	• Less cost-effective for sequencing low numbers of samples • Time-consuming for sequencing low numbers of targets • Requires a dedicated data-handling workflow	Illumina	27–144	36, 75, 100, 150, 250, 300	0.1–1	US$ 7–2000
			Ion torrent	2–7.5	200–400	1–2	US$ 80–2000
			PacBio	0.5–60	10 000–20 000	14–15	US$ 600–1000
			Oxford nanopore	<48	<200 000	5–40	US$ 100–400

required [8,14,19]. Sanger-based technologies are also not the most suitable methodologies to detect polymorphisms or minority variants with a frequency lower than 20% [20–22].

The beginning of the 21st century has seen a gradual shift from Sanger sequencing towards newer, NGS (also known as second-generation sequencing) methods that allow a higher throughput at a relatively low cost [17,23]. For these technologies, targeted RT-PCR might still be necessary to amplify the influenza virus genome, especially to overcome the otherwise over-represented host sequences in the sample. In 2005, Bright and colleagues were the first to use a pyrosequencing platform to monitor the emergence and spread of adamantane resistance in circulating A(H3N2) influenza virus strains over a 10-year period [24]. Pyrosequencing has since been successfully used to detect already known mutations in NA and M2, responsible for antiviral resistance. However, different sets of primers are required to detect these mutations [25].

NGS platforms remain the best choice in terms of value for money for high-throughput sequencing. NGS can be accomplished with several methodologies, namely sequencing-by-ligation (SOLiD technology), sequencing-by-hybridization (resequencing microarray), and sequencing-by-synthesis (Illumina, Ion Torrent; now the dominant nucleic acid technology) [17,26,27]. NGS methods generate massive numbers of reads that are 75–700 bases in length but with a high **coverage** per base. Such parallel, deep sequencing allows for the reconstruction of the entire genome in a sample. NGS also provides the ability to detect **quasispecies** with a frequency below 20%. Van den Hoecke and colleagues arbitrarily proposed the use a threshold of 0.5% for Illumina MiSeq and Ion Torrent, below which it becomes too difficult to distinguish real mutations from background errors cumulatively introduced by the RT-PCR and the sequencing technology itself [28].

However, NGS methods come with several limitations: the short-read length requires powerful bioinformatic algorithms to assemble a consensus sequence, and the required amplification by RT-PCR may introduce biases. Third-generation sequencers may address these problems, by constructing longer reads and possibly eliminating the requirement for amplification of the virus [29]. However, the low abundance of virus in most clinical respiratory samples and the relative novelty of these approaches currently still makes it difficult to eliminate RT-PCR amplification. The two main approaches for third-generation sequencing [30] are the synthetic approach that

relies on existing short-reads technologies to construct long reads [26,31] and the single-molecule-real-time sequencing approach (SMRT) [32]. The SMRT approach is the most widely used and is represented by sequencing technologies developed by Pacific Biosciences (PacBio) and Oxford Nanopore [33–35]. PacBio is able to generate long reads, which enables the analysis of difficult regions with multiple repeats. In addition, the real-time acquisition of the signal means that there is no lag between each nucleotide addition. However, the PacBio flow cell is not as high-throughput as the Illumina platform and the error rate is still relatively high in comparison to the Illumina platform [26,30,36–39]. So far, only a limited number of publications have reported on the use of PacBio on clinical human influenza virus samples or on influenza viruses generated with reverse genetics [39–41]. The MinION from Oxford Nanopore is a small device that has a relatively low cost and can provide very long reads. Currently, one MinION study has used direct RNA sequencing of the influenza virus genome, without prior amplification by RT-PCR, but this was only feasible because of a very high viral load, which is rarely found in clinical samples [42–46]. The elimination of RT-PCR amplification may lead to a lower cost and a shorter execution time. MinION reads are still characterized by a lower quality with high error rates, and therefore a high depth coverage is necessary to detect antiviral resistance mutations with confidence [45].

Cases When NGS Could Have Added Value for Detection of Drug-Resistance Mutations

High-throughput molecular approaches offer new possibilities for influenza virus surveillance. By determining the whole-genome of the influenza virus, higher resolution evolutionary patterns can be revealed, knowledge of reassortment events and emerging mutations across all genes can be provided and information on intrahost diversity of the virus (quasispecies) can be obtained. This information can lead to a better understanding of genetic changes in all segments for various seasons, tropism markers, antigenic characteristics, virulence, reassortment events, and of course antiviral resistance [40,42,47–51].

Monitoring and Surveillance of Resistance to New Antiviral Drugs

Currently, adamantanes and NA inhibitors, which includes oseltamivir and zanamivir, or a combination of antivirals, are the only antiviral drugs for influenza viruses licensed in Europe. Adamantanes, however, are not used anymore due to the presence of resistance mutations in almost all currently circulating influenza strains. As long as only NA inhibitors are used, whole-genome information may not be required, as Sanger sequencing of the NA segment is probably sufficient for the surveillance of antiviral emergence.

Although NA inhibitors are licensed to treat uncomplicated influenza infection, they are, in most European countries, only used to treat patients at risk of developing more serious complications, such as the elderly or people with underlying conditions (https://ecdc.europa.eu/en/seasonal-influenza/prevention-and-control/antivirals/faq). However, the need to carefully monitor seasonal influenza virus susceptibility to NA inhibitors remain a priority for public health agencies. At present, the percentage of detected circulating NA inhibitor-resistant viruses is low, but as seen in the 2007–2008 influenza season in Europe for the seasonal A(H1N1) strain, there can be a sporadic emergence of resistance that spreads rapidly in the population; 14% of the A (H1N1) virus samples from that season were resistant to oseltamivir [52]. By the 2008–2009 season the number of resistant influenza A(H1N1) strains had increased to 98% [53]. This raised concerns until the extinction of this A(H1N1) subtype following the emergence of the susceptible A(H1N1pdm09) pandemic virus in 2009. Some mutations that confer antiviral resistance cause a decrease in fitness of the resistant viruses. However, several studies have shown that compensatory mutations may coemerge and improve the fitness of the resistant viruses. These mutations can also arise during cell culture, which led to the CDC's Influenza Division and

WHO Collaborative Centers to shift to a sequencing first approach using NGS before performing isolation and phenotypic characterization on a subset of samples [54].

As antivirals directed against other influenza virus proteins gradually become approved and used in the population, the need to monitor possible resistance mutations in other parts of the viral genome becomes more important (Table S1 and S2 in supplemental information online). For example, Xofluza (baloxavir marboxil) was recently approved in the US and Japan. This drug is a cap-dependent endonuclease inhibitor of the viral polymerase acidic (PA), for which a resistance mutation in the PA segment has already been found in clinical samples. Favipiravir, which targets the viral RNA-dependent RNA polymerase (RdRP), is available in Japan for patients infected by an influenza virus that is resistant to other available influenza drugs and it is in the third phase of clinical trials in the US and Europe. Recently, a substitution in the PB1 segment, namely K229R, was reported in an *in vitro* study to confer resistance to favipiravir. This substitutions was accompanied with a PA P653L substitution, which restores the fitness of the virus [55]. In the Russian Federation and China, umifenovir (Arbidol) is used to treat influenza. This broad-spectrum antiviral can prevent virus entry into the host cell and is believed to target the HA protein. Other antivirals, such as nitazoxanide, an HA maturation inhibitor and pimodivir, a PB2 inhibitor, are being evaluated in phase III clinical trials. No specific, easy, standardized phenotypic tests have been developed yet to monitor the susceptibility of influenza viruses to these new antiviral drugs, making sequencing almost indispensable. WGS is of interest since most of these new drugs target viral proteins involved in the replication pathways where multiple viral proteins usually cooperate. In a few *in vitro* studies, resistance substitutions appeared in other proteins rather than in the target protein of the antiviral drug (Figure S1 in supplemental information online) [56].

Surveillance of Emergence of Resistance Mutations in Quasispecies

With traditional sequencing approaches, it is difficult to detect and quantify minority genomes present in viral quasispecies. NGS provides, for each patient, the possibility to investigate previously inaccessible aspects of viral dynamics [57]. The challenge in characterizing quasispecies composition remains to define a cut-off between real mutations and false positives.

Influenza virus quasispecies analysis in the context of antiviral resistance has already been performed in clinical samples in a few studies. Trebbien and collaborators [58] followed for 6 months an immunocompromised patient treated with oseltamivir and zanamivir. They concluded that NGS was necessary to properly investigate the complex population at the sites that are considered important for antiviral resistance. Similarly, Pichon and colleagues [59] followed a child with severe combined immunodeficiency. The authors concluded that NGS allowed for the characterization of viral variant evolution and that the quasispecies analysis could reveal a risk of decreased antiviral efficacy. These study cases clearly indicate that the characterization of the quasispecies composition of influenza virus genome could reveal the emergence of antiviral resistance. NGS technologies provide the necessary tools to detect the appearance and emergence of resistance mutations as quasispecies, either by studying samples from a patient under treatment or by analyzing a large set of circulating viruses, and thus to identify these mutations before they reach a proportion where they can affect the antiviral susceptibility phenotype or before they become dominant.

Concluding Remarks and Future Perspectives

Monitoring the antiviral drug susceptibility of influenza virus has long focused on the NA protein, because NA inhibitors have historically been the most extensively used anti-influenza drugs. However, as new antivirals that target different viral proteins become available and used to treat influenza patients, the need to obtain information about the whole genome increases.

Outstanding Questions

Is it possible to reduce the cost and develop easy-to-use bioinformatics tools to help implement NGS in routine influenza surveillance in the near future?

Is it possible to integrate the genetic sequence of influenza virus obtained by the NGS with the patient information for a better individual follow-up of the patient?

Will the detection of minority variants allow to foresee if a patient will rapidly develop mutations conferring antiviral resistance?

How could the limitations of third generation sequencers be overcome to allow the analysis of clinical viral samples like influenza in the future?

Therefore, NGS represents a more informative approach than Sanger sequencing in the context of routine surveillance.

This routine surveillance using WGS remains challenging regarding the complexity of data analysis for non-bioinformatics experts. Although, there are many tools available to analyze NGS results, many of these require substantial bioinformatic expertise because they are only available using the command line on Linux. Therefore, the spread of more web-based platforms with a user-friendly interface within the scientific community would be an advance in the use of WGS for non-bioinformatic experts [60].

The development of these NGS methods has provided an opportunity to obtain information about all the genomic segments and about the minority genomes present in viral quasispecies. Investigation of this quasispecies nature of influenza viruses thus improves preparedness by potentially forecasting the emergence of resistance substitutions.

However, despite intensive research on influenza viruses, little is still known about the role, dynamics, and spread of viral quasispecies. More work is needed to understand whether and how the quasispecies nature of influenza viruses plays a role in antiviral escape and in immune selection pressure, or whether this could be a contributing factor to disease severity. The use of WGS in routine surveillance will enable a better understanding of the association of the viral quasispecies and the host characteristics of a patient. It could also enable a quicker response when certain mutations that confer antiviral resistance are emerging within the patient. The interpretation of this genomic data is of course highly dependent on how complete and structured the epidemiological and clinical metadata is.

Use of NGS technology also comes with limitations including the cost, the requirement to amplify the genome, which introduces PCR errors, and the short-read lengths that require powerful bioinformatic tools to assemble a consensus sequence. Soon third-generation sequencers may become available and address some of these limitations by simplifying data analysis and lowering costs (see Outstanding Questions).

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