Update article

Viral metagenomics and blood safety

La métagénomique virale : un nouvel outil au service de la sécurité transfusionnelle

V. Sauvage [a,*], M. Eloit [b,c]

[a] *Département d'études des agents transmissibles par le sang, Institut national de la transfusion sanguine (INTS), Centre national de référence des hépatites virales B et C et du VIH en transfusion, 75015 Paris, France*
[b] *PathoQuest, bâtiment François-Jacob, 25, rue du Dr-Roux, 75015 Paris, France*
[c] *Inserm U1117, Biology of Infection Unit, Laboratory of Pathogen Discovery, Institut Pasteur, 28, rue du Docteur-Roux, 75724 Paris, France*

Available online 9 January 2016

This manuscript has been written in memory to Jean Jacques Lefrère.

Abstract

The characterization of the human blood-associated viral community (also called blood virome) is essential for epidemiological surveillance and to anticipate new potential threats for blood transfusion safety. Currently, the risk of blood-borne agent transmission of well-known viruses (HBV, HCV, HIV and HTLV) can be considered as under control in high-resource countries. However, other viruses unknown or unsuspected may be transmitted to recipients by blood-derived products. This is particularly relevant considering that a significant proportion of transfused patients are immunocompromised and more frequently subjected to fatal outcomes. Several measures to prevent transfusion transmission of unknown viruses have been implemented including the exclusion of at-risk donors, leukocyte reduction of donor blood, and physicochemical treatment of the different blood components. However, up to now there is no universal method for pathogen inactivation, which would be applicable for all types of blood components and, equally effective for all viral families. In addition, among available inactivation procedures of viral genomes, some of them are recognized to be less effective on non-enveloped viruses, and inadequate to inactivate higher viral titers in plasma pools or derivatives. Given this, there is the need to implement new methodologies for the discovery of unknown viruses that may affect blood transfusion. Viral metagenomics combined with High Throughput Sequencing appears as a promising approach for the identification and global surveillance of new and/or unexpected viruses that could impair blood transfusion safety.
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Keywords: Viral metagenomics; High Throughput Sequencing; Viral discovery; Blood-borne viruses; Emerging viruses; Blood safety

Résumé

Le risque transfusionnel infectieux lié aux virus connus (VHB, VHC, VIH et HTLV) peut être considéré à ce jour comme maîtrisé, en raison des mesures préventives prises par la plupart des pays à ressources élevées. En revanche, le risque potentiel persistant est lié à l'émergence d'agents infectieux, et en particulier viraux, non encore identifiés. Ceci est d'autant plus important qu'une proportion significative des patients transfusés sont immunodéprimés et donc plus exposés à des formes infectieuses graves dont l'issue peut être fatale. Plusieurs mesures visant à prévenir la transmission par transfusion de virus inconnus sont mises en œuvre tels que l'exclusion des donneurs à risque, la déleucocytation des plasmas, et le traitement physico-chimique des différents composants sanguins. Cependant, il n'existe pas à ce jour de méthode universelle pour l'inactivation des pathogènes, qui soit applicable à tous les types de composants sanguins et, tout aussi efficace pour toutes les familles virales. Par ailleurs, parmi les procédures d'inactivation des génomes viraux disponibles, certaines d'entre elles sont reconnues pour être moins efficaces sur les virus non

* Corresponding author.
  E-mail address: vsauvage@ints.fr (V. Sauvage).

enveloppés, et non optimales pour inactiver des titres viraux élevés dans les pools de plasma ou les produits dérivés. Il apparaît donc indispensable de mettre en place de nouveaux outils pour la détection d'agents viraux inconnus susceptibles d'affecter la sécurité infectieuse du receveur. La métagénomique virale associée au séquençage haut débit constitue une approche prometteuse pour l'identification et la surveillance d'agents viraux nouveaux ou inattendus qui pourraient compromettre la sécurité transfusionnelle.

1. Introduction

In developed countries, blood and organ donors are routinely screened for a range of blood-borne viruses (HIV, HBV, HCV and HTLV in some countries) with highly sensitive screening tests. This has dramatically improved the safety of blood supply. However, transmission by transfusion of unknown or unsuspected viruses, responsible for persistent and asymptomatic viremia, remains a continuing threat. This is particularly relevant considering that a significant proportion of transfused patients are immunocompromised and more frequently subjected to fatal outcomes. Several measures to prevent transfusion transmission of unknown viruses have been implemented including the exclusion of at-risk donors, leukocyte reduction of donor blood, and physicochemical treatments of the different blood components aimed to inactivate infectious agents potentially present in blood. However, up to now there is no universal method for pathogen inactivation, which would be applicable for all types of blood components (currently there is no method applicable for red blood cell concentrates) and, equally effective for all viral families [1]. Several inactivation procedures are available but some of them are recognized to be less effective on non-enveloped viruses, such as human parvovirus B19 (B19 V), Hepatitis A (HAV) or Hepatitis E (HEV) viruses [2]. Moreover, recent publications have also confirmed that some viral reduction methods (solvent/detergent and heat treatment) are inadequate to inactivate high viremic titers of these viruses in plasma pools or derivates [3,4]. Given this, the threat for blood safety due to emerging viruses is of crucial importance. Thus, it remains imperative to improve the blood product safety, not only by surveillance of well-characterized viral pathogens (HBV, HCV, HIV, B19 V) but also, by implementing new methodologies for discovery of unknown viruses that may affect blood transfusion safety.

The recorded emergence of infectious diseases has risen significantly since the last three decades. In a period of 64 years (1940–2004) the emergence of 335 infectious diseases (all pathogens combined) was reported. Emerging infectious diseases (EIDs) are dominated by zoonoses, the majority of which (72%) has wildlife origin. The increasing and ongoing documentation of novel animal viruses identified worldwide predicts that additional animal pathogens may jump species barrier to potentially become novel human infectious diseases in the near future, some perhaps highly pathogenic. Viruses account for 25–44% of all EIDs [5] and are the pathogen class most likely to emerge [6] with an estimated number of undiscovered mammalian viruses of at least 320,000 [7]. This estimation

suggests that zoonotic EIDs represent a serious threat to human health and emphasizes the importance to evaluate the factors that are involved in the contact between human and animals, responsible for the emergence of "zoonotic-human" infections [5]. Among some recent examples of viral emergence by host switching, with great impact on the public health, are the HIV pandemic (Europe, 1981; Central Africa, 1983), Severe Acute Respiratory Syndrome (SARS) caused by coronavirus (SARS-CoV, China, 2003) and Middle East Respiratory Syndrome (MERS) caused by MERS coronavirus (MERS-CoV, Saudi Arabia, 2013). Other examples include the extreme genetic variability of RNA influenza viruses like the emergence of highly virulent types like H5N1 (since 2003, China), pandemic H1N1 (Veracruz, Mexico, 2009–2010) and more recently a more virulent H7N9 influenza A virus (2013, China). These examples demonstrate that emergent diseases can easily overcome country boundaries, and even continents by means of infected travelers or international trade of livestock or plants. Many factors can contribute to the emergence and the spread of a novel infectious agent [8], as the increasing contact between humans and wildlife habitats mainly due to agricultural practices and the globalization of travel and trade. Other important factors include climate changes that promote vector expansion (mosquitoes, ticks) and mass human migrations (due to wars, natural disasters, poverty, and desertification) [9]. For these reasons, there is now a clear increased risk of arbovirus emergence in the future due to environmental and climate changes, extensive tropical urbanization and to the colonization of this expanding habitat by highly anthropophilic mosquitos (i.e. Chikungunya and dengue viruses both transmitted by *Aedes aegypti).* To date, dengue viruses are internationally recognized as an existing threat to blood safety due to a known transfusion-transmission and severe/fatal disease in recipients [10]. As all arboviruses are potentially transmissible by transfusion due to their capacity to induce an asymptomatic viremic phase, this represents new risks for blood safety.

In the last years, very broad and sensitive high throughput techniques based on metagenomics were developed to detect and characterize previously unknown or variant viruses associated with several human diseases. These methods based on the random amplification of genomes include pan-viral microarrays and High Throughput Sequencing (HTS) [11]. By overcoming conventional methods of viral identification, metagenomics, which gives access to all nucleic acids present in a given sample, allows the description of viral communities and their diversity in environmental [12,13], human [14–16] and animal samples [17–19] using HTS. Metagenomic sequencing has also demonstrated its usefulness in investigations when infectious diseases have

unknown etiology, as reported in human Merkel cell carcinoma for which a polyomavirus was identified as responsible [20], for the thrombocytopenia and leukopenia syndrome in China associated with a new bunyavirus [21] and for acute hemorrhagic fever outbreaks in Uganda [22] and Central Africa [23] due to new Ebola virus and rhabdovirus, respectively. This approach has also been used to identify novel viruses responsible of severe or fatal post-transplant associated diseases in grafted recipients [24,25]. Besides the development of metagenomics as a diagnostic tool in clinical virology [26,27], this methodology was applied to viral safety for biotechnological and biological products [28,29]. Using metagenomics, it is then possible to provide manufacturers the exhaustive viral bioburden of raw materials [30–34] and to detect adventitious viruses in live-attenuated vaccines [35].

With more than 3 million blood components transfused annually in France alone, viral metagenomics appears as a promising approach for the identification and global surveillance of new and/or (re)-emerging viruses that could impair blood transfusion safety.

2. Pipeline of a metagenomic analysis: from the sample preparation until the bioinformatic analysis of raw sequencing data

Metagenomic sequencing offers the considerable advantage compared to traditional methods of pathogen identification of being a molecular approach able to detect simultaneously multiple organisms in a complex sample without culturing and a priori knowledge of the microorganism's nucleic acid sequences. However, the application of viral metagenomics to clinical samples is made difficult by the fact that viral sequences represent a very low proportion compared to host DNA sequences and transcripts, bacterial and other contaminating genetic material (e.g. from environment and reagent). This leads to the requirement of high depth of sequencing and intensive bioinformatics analyses to increase the probability of virus detection. Despite the fact that amplification method, library construction and sequencing platform impact metagenome composition [36–40], adequate sample preparation enhancing the ratio of viral to host nucleic acid loads remains a crucial step for biological samples. The pipeline presented below comprises key steps and recommendations for viral metagenomic analyses, especially as applied to plasma or serum sample (Fig. 1).

3. Sample preparation

In order to increase the proportion of viruses, different purification and enrichment strategies can be applied on plasma samples prior sequencing, such as clearing centrifugation at low speed, membrane filtration (0.45 μm and/or 0.2 μm filter), ultracentrifugation, polyethylene glycol (PEG) virus precipitation, digestion of host cell-free DNA/RNA by nuclease(s) treatment and the removal of ribosomal RNA. These different techniques may be combined to maximize the amount of viral nucleic acids after extraction [39,41–43]. All viral metagenomic studies agree on the fact that nuclease(s) pretreatment of biological sample is

an essential step to importantly reduce the amount of background nucleic acids.

Different manual and automated methods to extract viral nucleic acids can be used (Fig. 1) and require choosing between extracting DNA and RNA simultaneously or independently. Since (re)emerging viral threats are mainly caused by RNA viruses [5,44], viral nucleic acid isolation can be performed using TRIzol LS (Life Technologies) or reagents designed to obtain high quality RNA molecules in high yields. Following viral particles lysis and nucleic acid precipitation steps, viral genomic RNAs are purified using a spin column. Residual host and/or viral DNA are then removed by an additional DNAse digestion is performed directly on the silica membrane or the final eluate. In some protocol, unwanted ribosomal RNAs are also removed after RNA purification [43], but removing ribosomal RNA at this stage by capture probes hybridization may cause viral sequences loss, especially those from unknown viruses. In addition, TRIzol extraction method offers the possibility to recover DNA virus genomes from the organic phase.

Once nucleic acids are extracted, RNAs are converted into single-stranded complementary DNA (ss cDNA) and randomly amplified together with DNA (if DNA and RNA were extracted simultaneously) to obtain the concentration required for library construction (1 μg to 1 ng depending on library preparation kits and sequencing platform). Number of sequence-independent amplification methods have been developed and used in combination with HTS for virus discovery. The most common are the sequence-independent single-primer amplification (SISPA) [45,46], Multiple Displacement Amplification (MDA) [36,47] and cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) [48]. These three methodologies can amplify simultaneously cDNA and DNA, linear and circular, single- and double-stranded viral genomes in a same sample. The SISPA method is extensively used by a number of laboratories in the world and allowed the identification of many new viruses in animal [49–51] and human clinical samples in the last ten years [23,52–54]. Compared to SISPA, MDA uses bacteriophage Phi29 polymerase. The MDA process presents the disadvantage to preferentially amplify circular ss DNA than linear viral genomes and thereby introduced a bias of amplification. However, this limitation does not prevent the detection of known RNA viruses [29,36,55] even at low concentrations (around 500–1000 copies/mL), and the discovery of new ones in biological samples [56]. Recently, MDA was successfully used in combination with HTS to discover a novel human astrovirus associated with a case of progressive encephalitis in a boy with X-linked agammaglobulinemia [57]. The cDNA-AFLP method was developed by Bachem et al. [58] and adapted for virus discovery (VIDISCA method) by van der Hoek et al. [59] for successful identification of a novel human coronavirus, HCoV-NL63, in a child suffering from bronchiolitis and conjunctivitis. Later, VIDISCA was used to discover novel parvoviruses [60,61] and papillomavirus [62] in veterinary and clinical human samples, respectively.

Despite the efficiency of nuclease(s) pretreatment to decrease the host background, a significant proportion of reads after deep sequencing always correspond to host genome and
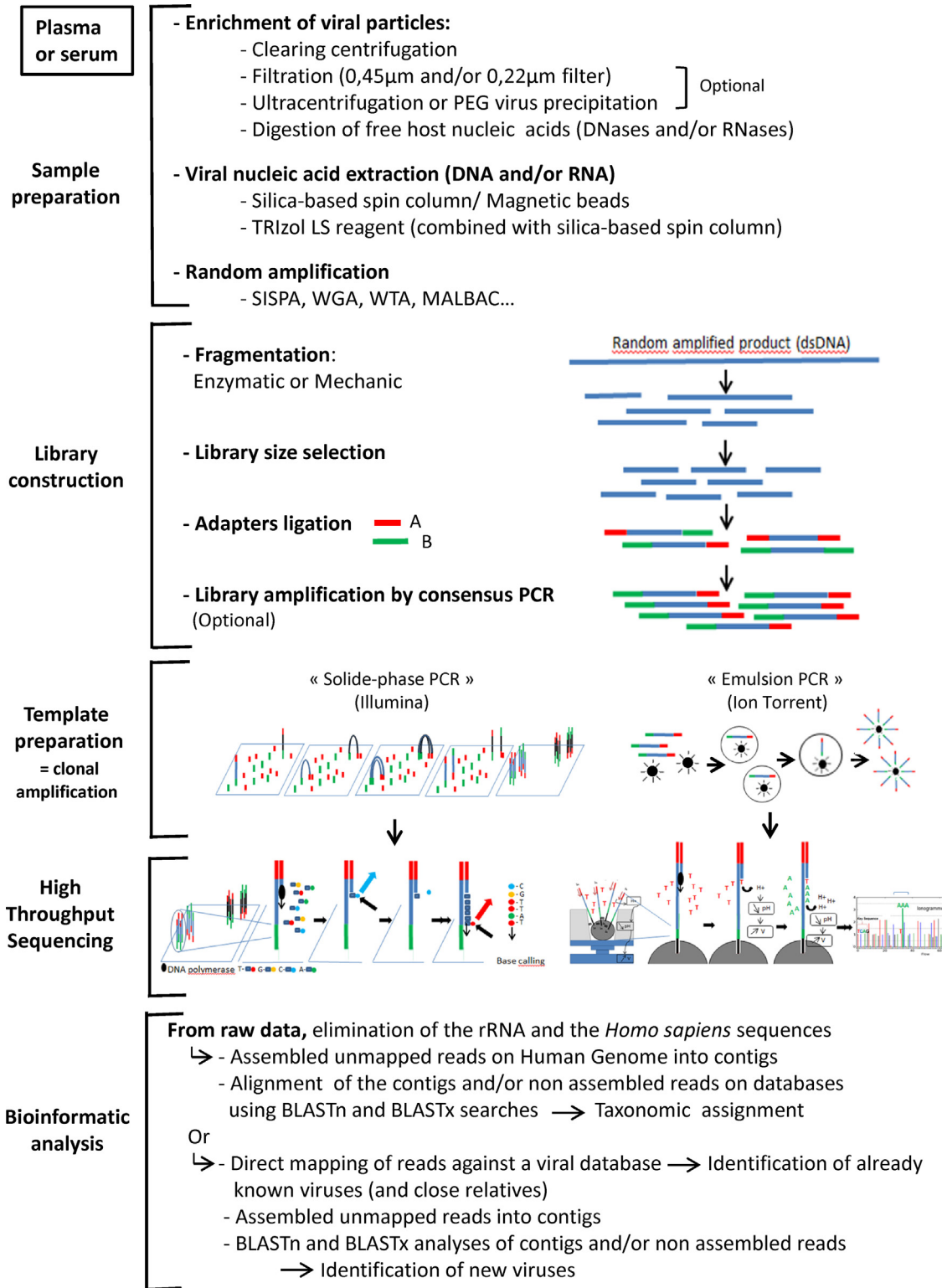
Fig. 1. Schematic overview of a typical viral metagenomics workflow applied to plasma and serum samples.

transcripts. To increase the sensitivity of virus detection required for clinical applications, several methods were developed to selectively hybridize and amplify non-human sequences. Of them, the "virome capture sequencing platform for vertebrate viruses" (VirCapSeq-VERT) [63], which comprises around 2 million probes covering all viral taxa known to infect vertebrates, the "Preferential Amplification of Pathogenic Sequences" (PATHseq) based on oligonucleotides that do not match the most abundant human transcripts [64] and the modified VIDISCA method using random hexamers that do not anneal to rRNAs [65,66]. Today, VirCapSeq-VERT in combination with Illumina HiSeq appears to be the most sensitive

method as it enabled detection of HHV-1 and West Nile Virus genomes, and enterovirus D68 at concentrations corresponding to 100 and 10 copies/mL, respectively, in human blood and serum samples [63]. Furthermore, according to the authors, the method can detect a virus whose the genome is 40% different to the probe sequence, making this method a suitable tool for viral discovery. However, this method is extremely time-consuming (hybridization step > 12 h) and, therefore, non-appropriate for clinical diagnostic applications.

Finally, particular precautions to avoid contamination of samples, nucleic acid extracts and amplification products by exogenous nucleic acids must be implemented throughout the process including ultra-violet light treatment of some kit reagents, substitution of commercial carrier RNA kits by neutral chemical carriers such as linear polyacrylamide (LPA) and good laboratory practices compliance.

4. Library construction

Following reverse transcription and amplification steps, the resulting ds DNAs are sheared into defined DNA fragment size according to the sequencing platform by using enzymatic (e.g. Illumina Nextera® technology) or mechanical techniques (e.g. ultrasonic fragmentation with the Bioruptor [Diagenode] or the Covaris E210 [Covaris] systems). The extremities of fragmented DNA are end-repaired (mechanical shearing only) and ligated to specific sequencing adapters, which can be barcoded to allow multiplexing of several samples in a same sequencing run. Adapter-ligated DNAs are then size purified (e.g. Agencourt AMPure XP beads from Beckman Coulter) to select the properly ligated DNA. A reconditioning step corresponding to few cycles of amplification (5–16 cycles according to the sequencing chemistry) can be performed. This adaptor-mediated amplification step is used to increase product yield and enrich for DNA fragments with the appropriate adapters combination for optimal sequencing (Fig. 1). The library is then quantified by a fluorimetric-based method (e.g. Qubit®, Life Technologies or quantitative real-time PCR) and quality controlled with 2100 Bioanalyzer® (Agilent Technologies) to confirm the library size.

It should be noted that the choice of the library preparation method depends in part on the input DNA amount. For example, Illumina TruSeq® DNA PCR-Free library preparation kit (based on mechanical fragmentation) requires 1–2 µg of input DNA, while Illumina Nextera® technology uses 1–50 ng of DNA. For Ion Torrent Technology, the library preparation kit based on enzymatic shearing (Ion Xpress® from Life Technologies) requires a minimum of 50–100 ng. With the recent development of library preparation kits (e.g. Illumina Nextera XT® DNA) from very low input DNA (≤ 1 ng), non-amplified ds DNA can be directly fragmented, associated to adapters and PCR amplified to obtain the required DNA concentration for sequencing. Removing the non-specific preamplification step significantly reduces duplicated sequences, and thus increases the genome coverage and the number of viruses detected [67]. Likewise, the linker-amplified (LA) protocol in combination with optimal sample preparation for viruses enabled detection of viral

sequences from 1–10 pg of input DNA [37,68], even if is not recommended by the manufacturers.

The resulting libraries can be sequenced following the manufacturer's protocol for each of the sequencing platforms.

5. Template preparation and High Throughput Sequencing

Novel sequencing technologies that are currently available comprise second- and third-single molecule generation of sequencers. Second-generation sequencers are currently the most widely used and include the Genome Analyzer (GA)/HiSeq/NextSeq/MiSeq from Illumina, GS FLX System/GS Junior from Roche (whose marketing will be stopped mid-2016) and the Ion Personal Genome Machine (PGM)/Ion Proton from Ion Torrent (Life Technologies) (Table 1). Note that Ion Torrent recently launched (September 2015) a newest sequencer, the Ion S5 instrument (Table 1), which offers in a unique device, chips with output (number of reads) and read lengths comparable to those of the Ion PGM and the Ion Proton (e.g. the 520 and 540 chips on S5 system are similar to the 318 chip on the PGM and the PI chip on the Proton, respectively). While the second-generation platforms present distinct sequencing chemistries and detection technologies [69], the sequencing workflow is based for all on libraries amplification to produce clonal clusters and sequencing using massive parallel synthesis (Fig. 1). On the other hand, given the number of second-generation sequencers, several factors must be considered in the choice of the technology, including cost performance, run time, output, flexibility, convenience and type of application. For example, the desktop sequencers, such as the Illumina MiSeq and the Ion Torrent PGM (both launched in 2011), are positioned for rapid targeted resequencing [70,71], and whole viral [72,73] and bacterial genome sequencing [74,75]. While they are both also used for metagenomic analysis [38,76–78], they offer a lower depth of sequencing (25 million reads per run for the MiSeq and 5–6 million reads for the Ion PGM) compared to the Illumina HiSeq (3 billion pair-end reads) and the Ion Proton (60–80 million reads with Ion PI chip), which therefore are more appropriate for whole metagenomics. Nowadays, the number of scientific publications using HiSeq instrument is considerably higher than those using Ion Proton, which is mainly attributable to its recent availability (September 2012) and lower capacity in terms of samples multiplexing (Ion PII chip announced for 2016 (but needs confirmation), will increase Ion Proton throughput to 300 million reads per run). However, the Ion Proton is a much more affordable, faster and compact system, theoretically capable of generating long reads (400b announced in 2016 but needs confirmation) compared to the HiSeq instrument. Recently, this platform was successfully used in viral metagenomics to identify two novel papillomaviruses in the vaginal microbiome of HIV positive women [79] and to detect different known viruses in plasma samples spiked at low viral concentrations [29].

To date, the use of the third generation of sequencers in viral metagenomics remains anecdotal [80,81]. Third-generation sequencers include the Single-Molecule Real-Time technology (SMRT) from Pacific Biosciences with the PacBio RS II

Table 1
Features of the most commonly used next-generation sequencing platforms in research and clinical diagnostic laboratories.

	Desktop sequencers					Massively parallel sequencers					
	From http://www.illumina.com					From https://www.thermofisher.com					From http://www.illumina.com
	MiSeq Illumina (2011)	NextSeq 500 Illumina (2014)	Ion PGM Ion Torrent (2011)			Ion S5 System Ion Torrent (2016)			Ion Proton Ion Torrent (2012)		HiSeq 2500 Illumina (upgrade HiSeq 2000)
	Single-lane flow cell	4-lane flow cell	Ion 314 chip	Ion 316 chip	Ion 318 chip	Ion 520 Chip	Ion 530 chip	Ion 540 chip	Ion PI chip	Ion PII chip (2016)	8-lane flow cell
Maximum read length	2 × 300b	2 × 150b	Up to 400b (up to 500b, 2016)	Up to 400b	Up to 400b	Up to 400b	Up to 400b	Up to 200b	Up to 200b (400b, 2016)	100b	2 × 100b
Number of single reads	25M[b]	130M[a]	Up to 0.6 M	Up to 3 M	Up to 5.5 M	Up to 5 M	Up to 20 M	Up to 80 M	Up to 80 M	Up to 330 M	3B[b]
Output/run	15Gb	39Gb	Up to 100Mb	Up to 1Gb	Up to 2Gb	Up to 2 Gb	Up to 8Gb	Up to 15 Gb	Up to 10 Gb	Up to 32 Gb	600Gb
Run time	55 hrs	26 hrs	2.5-4 hrs	3-5 hrs	4-7 hrs	2.5-4 hrs	2.5-4 hrs	2.5 hrs	2-4 hrs	4 hrs	11 days
Applications											
Human whole genome	x	O	x	x	x	x	x	x	x	O	O
Exome	O	O	x	x	x	x	x	O	O	O	O
Small genome	O	x	O	O	O	O	O	O	O	O	x
Targeted	O	x	O	O	O	O	O	O	O	O	O
Transcriptome	x	O	x	x	x	x	x	O	O	O	O
ChIP- Seq	O	O	x	x	x	x	O	O	O	O	O
16S Metagenomics	O	O	x	O	O	O	O	O	O	O	O
Whole metagenomics	O	O	x	x	x	x	x	O	O	O	O

b: base; M: million; b: billion.

[a] Mid mode.

[b] High output mode with v3 chemistry.

system and the Nanopore sequencing technology from Oxford Nanopore Technologies with the MinION and PromethION devices (currently in prerelease testing through the MinION and PromethION Access Programmes). Compared to the previous generation, the third generation is characterized by the absence of template amplification prior sequencing (and for this reason it's also called single-molecule sequencing), a signal registered in real-time and the ability to read long DNA fragments (several kilobases). Although obtaining long reads is revolutionary, these platforms still suffer from lower accuracy and the need of high input amounts of DNA (at least for MinION device according to our experience). However, the newest SMRT sequencer, the Sequel System (announced in Septembre 2015 by Pacifc Biosciences), may provide higher accuracy and throughput compared to PacBioRS II. The continuous performance improvements made by these two companies should secure an important place for these technologies in the field of viral metagenomics in the near future.

6. Bioinformatic analysis of raw reads

There are a lot of different pipelines that can be used for viral metagenomics. Basically, reads are first trimmed for quality and often, at least for the Illumina technology, duplicates (i.e. identical reads) are suppressed. In a second step, reads are filtered against the host human genome with a high stringency, meaning that in principle only human sequences are removed. There are then two possibilities.

There is often a step of assembly of single reads into contigs. Use of contigs allows for identifying hits with distant identities that would not have been distinguished from background on short single reads. As reads from new generation of sequencers become longer and longer, the need for assembly should decrease. Assembly of metagenomic reads into contigs poses specific difficulties due to the uneven abundance and thus coverage of viral species; moreover, random amplification also leads to uneven coverage of each genomes, the combination of both heterogeneity increasing the difficulty for assembly (see discussions on the tools in [82,83]). The following step is to conduct a blast analysis against a comprehensive database like the NCBI nr database and to identify sequences with a virus as best hit (a less ressource demanding protocol is to blast first a viral database like NCBI vrl and then confirm viral best hits by checking them against the NCBI nr). Blast analysis can be done with singletons and also with reads assembled into contigs. For contigs non assigned at the nucleotide level, a protein Blast (Blastx) can be used. Results need to be carefully screened by a virologist with some practical experience in metagenomic analysis, as there are frequent false positive results when the blast is conducted with a high e-value. Coverage of the putative viral genome is a good criterion to assess the pertinence of identification of new viral sequences.

A second possibility is to map the reads against a database of viruses using stringent rules and thus identify known viruses (already present in databases) and close relatives. This type of pipeline is quicker as less resource-demanding and is particularly well suited for diagnostic or as a first comprehensive filter before more demanding Blast analysis (see, for example, the computational pipeline SURPI [84]).

7. Viral metagenomics applied to plasma samples from blood donors and recipients

Viral metagenomics associated with HTS has successfully demonstrated its capacity to detect and discover viruses in complex biological samples such as serum and plasma, and therefore appears as a promising approach for the identification and surveillance of unknown or unexpected viruses that may be transmitted to recipients by blood products.

To further assess the performance and the limits of this methodology, we recently conducted a metagenomic analysis on plasma-pooled samples collected from at risk subjects, including recipients of multiple blood transfusions and HBV-, HCV- and HIV-infected blood donors. A large amount of various viral sequences has been found in all pools, among which, anelloviruses and human pegivirus (HPgV), formerly known as GB virus C, were the most frequently identified agents. The presence of HBV DNA and HCV RNA was confirmed in plasma pools from blood donors previously found HBV or HCV infected. In these plasma pools, HBV DNA and HCV RNA accounted for 3.8×10^3 IU/mL and 1.7×10^5 IU/mL, respectively. In contrast, HIV RNA accounting for 150 copies/mL in the pool, was not detected (data not published). We also detected numerous short nucleotide sequences distantly related to viral sequences reported in the current databases, and for which it was not possible to know if they represent *bona fide* viruses or they derive from other organisms. Despite the inclusion of at risk individuals and the efficacy of the pipeline to discover new viruses [36,56,85,86], no sequence suggestive of a new viral species was identified in the plasma samples analyzed in the study. Hence, these findings confirmed that viral metagenomics combined to HTS is able to identify the viral diversity present in blood samples, but also highlighted the difficulty to detect low levels of virus (lower than 500 equivalent genome/mL at the time of the study) and underlined some other issues including uncertainty of taxonomic assignment of short sequences and reagent contaminants. For instance, we retrieved sequences from the *Parvovirus*-like hybrid genome (PHV/NIH-CQV) in all pools, which although initially described as a possible new causative agent of seronegative hepatitis [87], was actually found as a background contaminant in water eluted from some nucleic acid extraction silica-binding columns [88,89]. Other viral contaminants from spin columns including circoviruses/densoviruses and iridoviruses were also previously reported [15]. All together, these findings demonstrate that HTS results must be interpreted with caution before discovery of a new virus is claimed.

Interestingly, Laupoucheung et al., [90] reported during the XXVIIth National Congress of the French Society of Blood Transfusion, the detection of Human astrovirus-MLB2 sequences in transfusion plasma. Human astroviruses are known as important agents of viral gastroenteritis, especially in young children, elderly and immunocompromised individuals [91]. The Human astrovirus-MLB2 was initially discovered in diarrheal stools of children from India and the United States [92],

and it has since been detected in the plasma of a febrile child [93], suggesting that astrovirus-MLB2 may spread from gastrointestinal tract to other tissues via the bloodstream. Several recent studies have reported the potential association of a new clade of astroviruses with neurological diseases and especially with encephalitis cases in both animals [94] and immunocompromised individuals [95,96]. In humans, the latest reported case concerned a boy with X-linked agammaglobulinemia associated with progressive global motor decline, cerebellar syndrome, and epilepsy, who was found to be infected with a new astrovirus strain from the VA1/HMO clade [57]. Reports of astrovirus-associated encephalitis were also described in allogeneic hematopoietic stem cells [97] and bone marrow transplant recipients [98]. These examples clearly demonstrate the usefulness of viral metagenomic sequencing for the diagnosis of unexpected viral infections.

Other examples of viruses recently described in human blood samples by metagenomics and HTS can be cited such as the human hepegivirus 1 (HHpgV-1), discovered in post-transfusion serum samples from blood recipients [99]; a divergent Gemycirculavirus (GemyC1c), found in an HIV1-positive blood donation [100]; and Giant Blood Marseillevirus (GMB), discovered initially in a pool of samples collected from asymptomatic blood donors from the South of France [101] and subsequently found by PCR in 4% of 174 studied healthy donors and in 9.1% of post-transfusion sera from thalassemia patients [102]. However, in two recent studies reported (339 [103] and 187 [104] subjects, respectively), GMB viral DNA was not detected by PCR in any of the human plasma samples analyzed. Further investigations are needed to confirm the reality of these novel viral infections in humans, their potential pathogenicity and transfusion transmissibility.

8. Conclusion

Despite the undeniable analytical strength of this methodology, further investigations of the blood virome in a research context are needed to enforce the potential use of viral metagenomics and HTS for the discovery and surveillance of viruses of concern in blood safety.

As it is unlikely that the first occurrence of an emerging infection will be seen in transfused recipients, it is of paramount importance to implement a proactive surveillance of emerging viral infections in blood donor populations from different parts of the world especially from geographic areas with the highest rates of EIDs. After the identification of a new agent, if any, a process for evaluating its transmission routes by blood transfusion and its potential pathogenicity according to the immune status of the recipients will be implemented. Moreover, the risk assessment will help to define the need for implementation of new preventive strategies to reduce the transfusion transmission risk associated to this emerging agent. Such project will generate several recommendations to help health authorities to issue guidelines. This approach will become part of blood safety vigilance and will be extended to address more general public health issues regarding emerging infectious agents.

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