Review

Molecular diagnostics in virology

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Abstract

Molecular biology has significantly improved diagnosis in the field of clinical virology. Virus discovery and rapid implementation of diagnostic tests for newly discovered viruses has strongly beneficiated from the development of molecular techniques. Viral load and antiviral resistance or subtyping assays are now part of the biological monitoring of patients chronically infected by human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and CMV. It will be important to add to this panel assays for other viruses of the *herpesviridae* family. Qualitative assays for the detection of blood-borne viruses have increased safety of blood donation and organ transplantation. Screening of other blood-borne viruses (parvovirus B19, HAV), multiplexing of detection and test automation to improve practicability and reduce costs will be the next steps. A major evolution in the near future will be the generalization of NAT for the diagnosis of viral etiology in patients, mostly with respiratory, CNS or gastro-intestinal diseases. Major technical improvements have been made to avoid obstacles that still limit this generalization, i.e. genetic variability of viruses, multiplex detection, contamination risk. Commercial offers already exist but menus must be extended to limit the validation and documentation work associated with home-brew assays. Real-time amplification has allowed the development of new NAT platforms but automation and integration of all steps of the reaction are still required to reduce hands-on-time, time-to-result and costs, and to increase throughput.
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1. Introduction

Molecular biology has revolutionized all domains of viruses diagnosis including the rapid identification of emerging or re-emerging viruses, viral safety of blood products or organ transplants and viral disease management. One of the major driving forces for the introduction of molecular techniques in virology has been the absence of easy and performing multiplication techniques similar to those developed for bacteriology. The most striking illustration of the power of molecular techniques concerns blood transmitted viruses—human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) for which spectacular progresses in the detection and treatment of viral diseases have been made following the introduction of qualitative and quantitative nucleic acid tests (NAT). The recent discovery of a new human coronavirus responsible for severe acute respiratory syndrome (SARS) epidemic is another example. It is obvious that NAT will encourage the development of antiviral drugs which, compared to antibiotics, has been delayed partly because performing efficiency assessment techniques were lacking.

2. New, emerging or re-emerging viruses

During the last 15 years, many new human pathogens have been discovered among which eight were viruses with various pathogenicity. Molecular techniques have played a central role in their discovery (Table 1). The construction of cDNA libraries by cloning techniques has been used to identify HCV and hepatitis E Virus. Representational difference analysis (RDA) was successful in identifying human herpes virus 8 (HHV-8), and the hepatic viruses GBV-C and TTV. RDA allows the detection of viral sequences by comparing whole nucleic acids sequences in cells from humans or animals before and after infection. Reverse-trancription polymerase chain reaction (RT-PCR) with random or degenerate primers has been used to identify human metapneumovirus (hMPV), the virus SEN and the coronavirus associated to SARS (SARS-CoV). Molecular techniques alone

have allowed the characterization of very important human pathogens like HHV-8, responsible of Kaposi sarcoma or HCV which induce acute or chronic hepatitis, cirrhosis and hepatocarcinoma. However, other viruses detected in a similar way are still waiting for the demonstration of their clinical importance. This illustrates the need to verify Koch's postulate and the importance of keeping laboratory competencies for classical virology—tissue culture, electron microscopy and animal experiments—which plays a major role in virus discovery, together with epidemiological studies. Large epidemiological studies are also required to assess the clinical interest of new pathogens.

Molecular diagnostics have been very rapidly implemented in clinical virology laboratories following the discovery of hMPV (van den Hoogen et al., 2001; Peiris et al., 2003; Boivin et al., 2003) and SARS-CoV (Ksiazek et al., 2003; Drosten et al., 2003; Anderson, 2003). A prospective study on the prevalence of hMPV could be initiated as early as during the 2000–2001 winter, although the virus has been discovered in 2000 only. There has been only a few weeks lag between sequencing SARS-CoV and availability of the first commercial NAT.

2.1. Viral safety

NAT are more and more used to exclude blood donations from patients infected by viruses (Allain, 2003). HCV and HIV testing has been implemented as part of routine screening in blood banks in 14 and 7 European countries respectively. In France, two commercial offers – Procleix (Gen-Probe, Inc. USA) and NucliSens Extractor (bioMerieux, France) associated with Cobas Ampliscreen (Roche Diagnostics, Switzerland)—are used to screen donations for HIV and HCV infections. This allowed the reduction of residual risk from 1 in 400,000 to 1 in 2.5 millions for HIV and 1 in 760,000 to 1 in 5 millions for HCV (Assal et al., 2003). However, there is room from improvement as few contaminated blood units are still missed due to the lack of sensitivity induced by pooling strategies. Cost constraints must also be considered if further improvements are to be considered. Cost effectiveness of HIV and HCV NAT addition to serology testing is already very low: in USA it has been calculated that the cost of each saved life is 4.7–11.2 millions US$ per year (Jackson et al., 2003).

Hepatitis B virus screening using molecular biology should also be included as even the most recent antigen assays (HbsAg) assays miss infected blood units. As an example, single-sample HBV testing would allow the detection of 35–50 additional contaminated units among 10^7 units tested (Biswas et al., 2003). Monovalent or trivalent assays (HIV, HBV, HCV) are proposed by Roche Diagnostics (Ampli NAT) and Gen-Probe (Procleix Ultrio) but blood units should not be pooled to provide sufficient sensitivity. Procleix Ultrio detects single seroconversions 20 days earlier than Abbott Prism-HBsAg but only 13 days in pools of 8 and 11.5 days in pools of 24 (Cambié, 2002).

Table 1
Role of molecular biology in virus discovery

Year	Virus	Method of discovery
1988	Hepatitis E	Animal transmission + cDNA library
1989	Hepatitis C	Random primed cDNA library
1995	Human herpes virus 8	RDA
1995	GBV-C	RDA
1997	TT virus	RDA
2001	Human Metapneumovirus	Tissue culture + RT-PCR with random primers
2001	SEN virus	RT-PCR with degenerate primers

RDA: representational difference analysis.

Alternatively, an ultracentrifugation step could be introduced following pooling to obtain a higher sensitivity compared to current antigen assays (Roth et al., 2002).

Screening for West-Nile virus contamination has been implemented in US blood banks. From late June to mid-September 2003, approximately 2.5 million donations were screened. Twelve hundred eighty-five (0.05%) were initially reactive for WNV by using nucleic acid-amplification tests and 601 (0.02% of the total donations) are considered presumptive viremic donations (i.e. a donation that is repeatedly reactive by the primary and/or alternate NAT assay or a primary NAT assay with a very high signal) (CDC, 2003). Other viruses (parvovirus B19, Hepatitis A virus) are also transmitted by blood donation and may be part of the screening in a near future. However, NAT may not always be the best diagnosis approach and antigen tests or antibody tests may be efficient and less expensive alternatives.

Automation and integration of NAT is necessary to reduce costs and quarantine delays for blood units supply. In this respect, the recent approval by US Food and Drug Administration (FDA) of the TIGRIS molecular diagnostic system (Gen-Probe, San Diego, USA) is a major breakthrough as it has been designed to process 500 samples in 8 h.

Integration and time-to-result are also very important parameters to be considered to insure viral safety of transplant organs, especially lung, heart and liver. It is very important to determine the status of transplants regarding infection by HIV, HBV, HCV and viruses from the *herpesviridae* family.

2.2. Viral disease management

NAT have significantly improved identification of viruses as etiologic agents of human diseases affecting various organs, especially respiratory and gastro-enteric tracts and central nervous system (CNS). As a consequence, rapid antiviral treatments can be initiated and considerably reduce morbidity and mortality as, for example, in the case of herpes encephalitis. The increasing number of available antiviral drugs will even accentuate the need for positive viral identification. Similarly, unnecessary antibiotic treatments can be avoided or reduced and hospitalisation durations shorten. Treatments of chronic viral diseases are very efficiently monitored by viral load assays.

However there are still obstacles that prevent a wider dissemination of molecular assays.

2.3. Technical constraints and recent improvements of molecular assays

The extreme genetic variability of some viruses, especially RNA viruses (which RNA-polymerases have no proof-reading activity), often makes their diagnosis difficult. The most striking examples are found in the norovirus family which contains viruses responsible for the vast majority of gastro-intestinal epidemics in adults. HIV diagnosis is also quite difficult to achieve due to its high genetic variability.

Gardner et al. (2003) have deduced from sequence alignments that a real-time TaqMan assay should contain not less than nine primer and probe sets to detect with the same sensitivity all HIV strains in a geographically representative panel. To reduce the impact of variability on amplification and detection efficiency, one can use primers and probes with 2′ *O*-methyl bases, degenerate bases or "universal" bases, such as inosine or nebularine. Touchdown PCR protocols, in which the annealing temperature slightly decreases during the successive amplification cycles to bracket the melting temperature Tm of the reaction, provides sensitivity even when primers have mismatches with target sequences of divergent species in a viral family. Finally, degenerate primers or probes, with mixtures of the bases found in sequence databases among various species, may be useful to detect all species of a viral family.

It is often desirable to provide the capability for panel detection, i.e. to detect several viruses that can be responsible for a disease. For example, Coyle et al. have described at the winter meeting of European Society for Clinical Virology (Copenhagen, January 2004) a molecular viral respiratory strip for the detection of 12 common respiratory viruses. Whenever possible, consensus primers able to detect all viruses from a family or genus must be used. There are several examples of such consensus primers for enterovirus (Kammerer et al., 1994), flavivirus (Scaramozzino et al., 2001) or *Herpesviridae* (Tenorio et al., 1993). However, their ability to amplify all viruses with the same efficiency must be carefully evaluated. If such an approach is not possible, two different possibilities exist for panel detection: multiplex detection in single tubes or parallel detection in individual tubes. Mixing primers in a single amplification tube to achieve multiplex detection of viruses usually results in decreased sensitivity of assays compared to single tests. For example, we have observed, using a DNA-microarray assay (see below), that the analytical sensitivity of multiplex RT-PCR detection of six viruses, i.e. influenza A, influenza B, RSV A/B, parainfluenza 1, 2 and 3 is reduced by a factor of <1–2 logs compared to single detections, depending on the virus. Nevertheless, this multiplex assay was able to identify correctly 21/22 infections in respiratory specimen (one RSV B infection was misidentified as RSV A; unpublished data). The formation of primer dimers is generally considered as the major cause of sensitivity loss but careful optimisation of all parameters of amplification including primer, enzymes, nucleotides and salts concentrations as well as protocol conditions are required to obtain expected performances. Real-time assays (see below) that monitor signal apparition during the amplification step are also limited in their capacity to realize multiplex detection by the number of available wavelengths in existing equipments which currently allows the detection of three viruses only. Instead of mixing several pairs of primers in a single tube, nucleic acids purified from the original clinical specimen can be distributed into several tubes for independent amplification and detection. Major drawbacks of this approach are the reduction of sensitivity because of lower amounts of nucleic acid available for each individual

amplification, higher hands-on times required to manipulate all the different tubes, difficulty to automate the distribution of small volumes of purified nucleic acids without introducing cross-contaminations and higher costs due to the need for enzymes in each tube.

Internal controls (IC) are important components to monitor each step of the assay from extraction of nucleic acids to detection. Because inhibitors of the amplification reaction, which are frequent in some specimen types, will also impact its amplification, the presence of an IC is a strong validation in case of negative result. Niesters (2002) has described an original approach for internal control of NAT: the use of viral universal controls that can be added to each specimen and be amplified with specific primers, preferably in a multiplex format with primers for the virus to detect. Seal herpes virus 1 and phocine distemper virus can be used to control NAT for DNA and RNA viruses respectively. Of course, animal viruses which can not infect humans are required. Other strategies for IC involve synthetic materials, i.e. plasmids or transcripts which contain sequences able to bind the test primers and a specific probe.

2.4. Automation of molecular assays

Clinical virology laboratories have high expectations in term of automation and integration of molecular assays.

A major bottleneck in the workflow of these laboratories is at the level of sample preparation. Table 2 shows automated systems for nucleic acid purification that are currently commercialised. Most of them use the nucleic acid binding properties of silica (Boom et al., 1990). As many as 96 samples can be handled in a single run on the BioRobot 9604 (Qiagen GmbH, Germany). Time-to-result and, more important, hands-on-time tend to decrease, with the most recent equipments requiring no longer than 20 min of technician time for more than 24 samples.

New labelling technologies that do not need solid-phase separation have allowed the development of real-time molecular assays in which the detection of amplicons is done as soon as they appear during amplification. The most simple real-time detection chemistry uses the SYBR green dye which specifically binds during double-stranded DNA generated during PCR. Several probe technologies (Fig. 1)

have also been designed for real-time assays like TaqMan (Fig. 1A) or molecular beacons (Fig. 1B) in which a quencher molecule is removed from the vicinity of the fluorescent marker upon binding to RNA or DNA generated during amplification cycles. In the FRET technology (Fig. 1C), two probes, one with a fluorescence donor and one with a fluorescence acceptor molecule are designed to bind adjacent sequences of the amplified material to generate signal (Mackay et al., 2002). Real-time techniques have been designed for PCR or NASBA amplification and have several advantages which facilitate automation and reduce time-to-result (30–120 min) and hands-on-times. They are performed in closed devices which do not need to be opened to transfer amplified material for end-point detection, thus reducing the risk of laboratory or samples cross-contamination. The use of real-time platforms makes the general organisation of molecular biology laboratories easier by reducing the constraints on activities segregation in different rooms to control contaminations. Table 3 shows existing real-time automates.

The next generation of NAT platforms will integrate sample preparation, amplification and detection in a single test device GeneXpert (CEPHEID, USA) is the first fully integrated system which allows the detection of *Bacillus anthracis* or group B streptococcus in approximately 45 min directly from clinical specimen. Several viral assays based on real-time NASBA on this platform will soon be available from bioMerieux.

Several IVD companies offer commercial NAT for the diagnosis of viral diseases.

2.5. Regulatory considerations

Besides technical difficulties, another obstacle to the development of molecular assays is the importance of resources needed to optimise, produce and validate home-brew assays and to build up documentation required for qualification of techniques and laboratories. New European Community regulations will even increase this need. It is the role of in vitro diagnostic (IVD) companies to provide reagents which are the results of careful optimisation and are produced according to high quality manufacturing procedures. They have clinical and regulatory affairs departments that conduct validation studies and assemble documentation required to get approval

Table 2
Automated systems for the purification of nucleic acids

| Manufacturer	Technology	Instruments	Samples/run	Run duration	Hands on time
Qiagen	Magnetic particles/silica extraction	BioRobot 9604	96	<2.5 h	20 min
	Magnetic particles/silica extraction	BioRobot EZ1	1–6	20 min	
Roche diagnostics	Magnetic particle/specific oligonucleotide capture probes	MagNA Pure	32 (capillaries or plates)	2 h/24 samples	35 min/24 samples
	Filter-based/silica extraction	Ampliprep	72	2.5 h	30 min/24 samples
bioMerieux	Magnetic particles/silica extraction	Nuclisens extractor	10	45 min	20 min
	Magnetic particles/silica extraction	Nuclisens EasyMag (in development)	24	1 h	<20 min
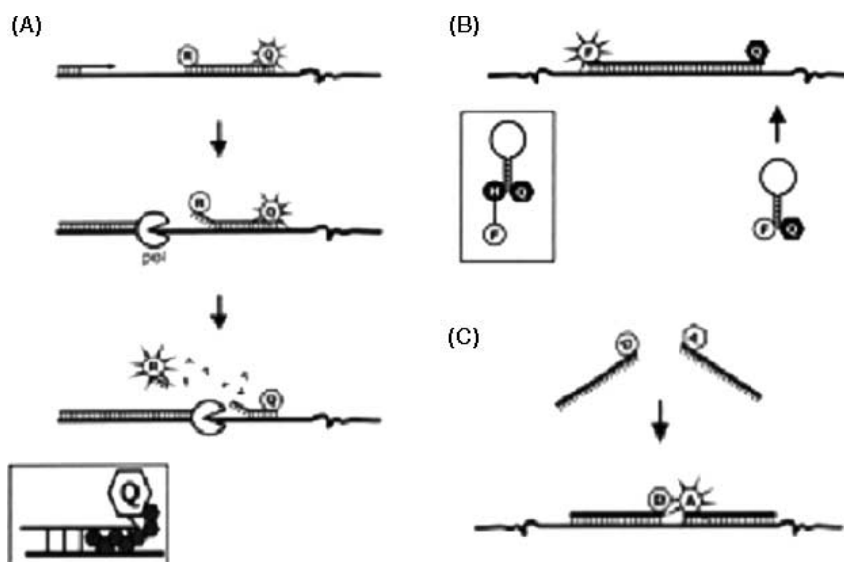
Fig. 1. Probes used for real-time detection. From Mackay et al., 2002. A: TaqMan 5′-nuclease oligoprobes. As the DNA-polymerase progresses along the amplicon, it displaces and hydrolyses the oligoprobe via its 5′–3′ endonuclease activity. When the reporter group (R) is removed from the amplicon it is no longer under the inhibition effect of the quencher group (Q). B: molecular beacons. Hybridisation of the complementary part of the probe on the amplicon separates the fluorophore (F) from the quencher group (Q). C: LightCycler FRET probes. Two probes, one with a donor group (D) and one with an acceptor group (A) bind in adjacent positions on the amplicon. The donor group transfers the energy acquired upon excitation to the acceptor groups which generates fluorescence.

of the reagents. However, even for IVD companies, the conception and validation process is time-consuming and time-to-market may be long. This is especially a problem when a diagnostic tool is urgently needed in case of emergence of a new virus. One possibility to reduce time-to-market is to release "research use only" (RUO) assays or assays that have the "CE analytical" approval in Europe or the status of "analyte specific reagent" (ASR) in the USA. In this case, commercial products that have excellent analytical sensitivity and are manufactured according to quality standards of the IVD industry can be used by clinical virology laboratories, which have the responsibility to validate their use as diagnostic tools and obtain authorization to use them.

2.6. Reagents for the diagnosis of viral infections

Infections by HIV and hepatitis B and C viruses are usually well diagnosed using serology. Only diagnosis of early primo-infections may benefit from NAT. Platforms like Amplicor Amplicor from Roche Diagnostics or EasyQ from bioMerieux that are usually used for viral load measurement during therapy (see below) are also suitable for the early detection of HIV or HCV infections. Many other infections and especially acute infections for which IgM appear only several days after onset of symptoms cannot be efficiently diagnosed using serology assays. Forty to sixty percent of community acquired pneumonia that require hospitalisation

Table 3
Real-time amplification platforms

Manufacturer	Platform	Number of samples	Number of wavelengths
Applied	ABI PRISM 7700	96	Continuous wavelength detection from 500 to 660 nm allows the use of multiple fluorophores in a single reaction
Biosciences	ABI PRISM 7900	394	
Roche diagnostics	LightCycler	32	3
	COBAS TaqMan 48	24 per processing block 2 processing blocks	4
bioMerieux	EasyQ	48	4
Stratagene	Mx3000/Mx4000	96	4
Bio-Rad	iCycler iQ	96	4
Cepheid	Smart Cycler II	16 independent channels per processing block, 1–6 processing blocks can be interconnected	4
Cepheid/bioMérieux[a]	GeneXpert	4	4

[a] Integrated real-time platform (sample preparation and amplification).

Table 4
QCMD programs in 2002 (from Wallace, 2003)

Program type	Number of participants	Number of data sets	Total tests	Correct (%)	Equivocal (%)	False +ve (%)	False −ve (%)	Commercial assays (%)
CMV	89	105	1260	81.6	1.0	2.9	20.3	81.0
EBV	61	67	603	80.6	0.0	3.0	19.0	29.9
Enterovirus	93	100	1200	79.0	1.6	5.5	27.7	93.0
HBV	70	97	776	89.2	0.1	2.1	11.9	51.5
HCV	80	124	992	91.8	0.1	0.0	9.2	88.0
HIV	64	90	720	84.8	0.6	0.0	14.6	90.0
HSV	98	109	1308	87.8	0.9	0.9	14.8	9.2

have no known aetiology despite intensive investigation and this percentage is even higher when less severe lower respiratory tract infections (LRTI) are considered (L. Kaiser, personal communication). In a recent study, Henrickson et al. (2004), have shown, using multiplex RT-PCR that 40% of children hospitalised for LRTI are infected with the seven most common respiratory viruses. Similarly, a recent survey of encephalitis leading to hospitalisation in the USA from 1988 to 1997 has revealed that nearly 60% had no aetiology (Khetsuriani et al., 2002). Absence of specific antiviral treatments for most viruses, which limits prescription of biological tests and weak performances of diagnostic tests based on viral culture or serology are major explanations of this situation.

NAT, which have been implemented by many large European hospitals, significantly improve viral diagnosis. However, there are several obstacles to the generalization of molecular diagnostics in smaller, decentralized laboratories.

A major obstacle is the fact that, except for HIV, HBV, HCV and CMV, virology NAT are most often home-brew assays which sometimes suffer from bad performances and poor batch to batch consistency. However, quality of NAT is improving as illustrated by results of the 2002–2003 Quality Control Molecular Diagnostics (QCMD) proficiency testing (Table 4). Panels for different viruses have been sent to 61–111 European laboratories. The percentage of correct results ranged from 79% for enterovirus to 91.8% for HCV. The percentage of false-positive results which reflects laboratory or cross-contaminations and used to be high, dropped to 5.5% (Wallace et al., 2003).

Table 5 shows some products currently commercialised by major companies for viruses other than HIV, HBV and HCV, although the list may not be exhaustive. Most of them are ASR or RUO kits although some are EC marked. The majority of these reagents have been designed to run on real-time platforms.

Results shown by Liolios et al. in 2001 illustrate the interest of multiplex detection of respiratory viruses by NAT. One hundred forty-three clinical specimen were tested using the Hexaplex assay from Prodesse Inc. (USA) which detects six viruses in a single tube using PCR and detection with microplate capture and peroxidase-labelled probes. 9 samples were detected with the Prodesse assay only and not with immunofluorescence or viral culture (Table 6).

Table 5
Commercially available virology NAT (other than HIV, HBV, HCV)

	Argene biosoft PCR	Artus GmbH Real-time PCR	bioMerieux Real-time NASBA	Digene Corp. PCR	Prodesse Inc. PCR and real-time PCR	Roche diagnostics Real-time PCR
Respiratory viruses	NA	Coronavirus, Sars-CoV	Sars-CoV	NA	Hexaplex (Influenza A and B, parinfluenza A, 2 and 3, RSV A and B, hMPV	Sars-CoV
Herpesviridae	HSV 1 and 2 (generic and typing)	HSV 1 and 2, EBV, VZV	CMVq (pp67 mRNA)	NA	Herpes multiplex (HSV 1 and 2, CMV, EBV, VZV, HHV6)	HSV 1 and 2, EBVq,
Neurotropic viruses	Enterovirus	Enterovirus, West-Nile virus	Enterovirus	NA	NA	NA
Adenovirus	NA	NA	NA	NA	Adenoplex: (adenovirus A-F)	NA
Papilloma virus	NA	NA	NA	HPV (IVD assay)	NA	HPV (IVD assay)
Others	NA	Dengue virus, parvovirus B19	NA	NA	NA	Parvovirus B19, HAVq

IVD: in vitro diagnostic. The letter "q" means a quantitative assay.

Table 6
Multiplex Nat for the detection of respiratory viruses (Hexaplex, Prodesse Inc.)

RT-PCR/viral culture and/or immunofluorescence	PIV-1	PIV-2	PIV-3	Inf A	Inf B	RSV
+/+	0	0	0	6	1	1
+/−	0	0	1	7	1	0
0−/−	143	143	142	130	141	142
−/+	0	0	0	0	0	0

From Liolios et al., 2001.

2.7. DNA-microarrays in clinical virology

DNA-microarrays or DNA-Chips are very powerful detection tools that can be combined with amplification techniques to detect viruses or virus variants (reviewed in Clewley, 2004). Wang et al. (2002) have described a microarray spotted with 70-mer oligonucleotides which represent the five most conserved sequences (more than 20/70 bases are conserved among all representative sequences in a virus sequence alignment) of each virus of interest. The Chip contains 1600 different probes. Following PCR amplification of genetic material in the clinical specimen using random primers, hybridisation onto the microarray was able to identify respiratory viruses in the enterovirus, rhinovirus, paramyxovirus, adenovirus and herpesvirus families. However, this technique has not yet been extensively validated for routine diagnosis in a clinical virology laboratory.

We have developed assays combining RT-PCR and DNA-microarrays for the detection of viruses. The arrays are manufactured using the photolithography in situ synthesis technology (Affymetrix, USA) and contain 20-mer oligonucleotides. Sequence signatures are identified using extensive sequence databases because they are conserved in all viruses of a genus or family or in all subtypes or isolates of a virus and are not found in other viruses. For each base of the signature, probes perfectly matching the target and probes with a mismatch at the interrogated position are present on the array. If polymorphisms are present in or near the signature, variant probes may also be present. Consensus primers have been designed for enterovirus, flavivirus, *herpesviridae*, parainfluenza and influenza virus, RSV and adenovirus. They can be combined in multiplex detection PCR or RT-PCR assays for the diagnosis of viral respiratory or CNS infections. Following amplification, DNA is labelled using a newly developed diazomethyl chemistry (Laayoun et al., 2003). A complete line of instruments (sample preparation, thermocycler, hybridisation station and laser scanner) is available to perform the assay. As described above, a respiratory assay designed to detect six major viruses (parainfluenza 1, 2 and 3, RSV A/B, influenza A and B) in a single specimen has demonstrated high clinical sensitivity in preliminary evaluation. Fig. 2 illustrates the discriminatory capacity of this technology for CNS viruses.

Assays for the identification of human papilloma virus (HPV) are commercialised by several companies. The detection of a highly pathogenic HPV type has a very high predictive value for cervical carcinoma. However, the number of HPV types that are more or less closely associated to cervical cancer is high. DNA-microarrays may thus be appropriate for the multiplex detection of all these types. Such an assay has been described by An et al. (2003) and is distributed by biomedlab Co. (South-Korea). It is based on a consensus amplification and on 30-mer probes that are able to detect and discriminate 22 HPV types and has shown an association of 92.5–97.5% between HPV positivity and lesions of different severity or carcinoma whereas HPV infection was only found in 35.1% of cases when cytology was normal.

2.8. Treatment monitoring

Viral load platforms are available from several IVD companies (Versant from Bayer Diagnostics; Cobas Ampliprep/Amplicor from Roche Diagnostics, MiniMag/NucliSens EasyQ from bioMerieux). Significant progress have been made in the ability of most HIV assays to detect all subtypes of HIV-1 but no commercial assay exist for HIV-2. The analytical sensitivity of HBV viral load assays should be increased to reach the same performances as those of HIV assays, especially in the case of infections by variants with low replication competencies. For efficient treatment monitoring of immunosuppressed patients, for example in the case of organ transplantation, viral load assays should also be developed for Epstein-Barr virus, Varicella-Zooster Virus and HHV6.

Genotyping tests are now commercially available and are part of the biological follow-up of treated patients although home-brew assays are still used in most laboratories (Korn et al., 2003). HIV and HBV resistance assays as well as HCV subtyping assays are generally based on the sequencing technology (Trugene HIV and HBV from Bayer HealthCare; ViroSeq HIV-1 from Celera Diagnostics; 5′ genotyping HCV kit from Bayer healthcare). Hybridisation techniques, such as LiPA assays (Innogenetics, Belgium) are also used. However, the number of probes that can be spotted on nitrocellulose strips is limited and only few polymorphisms can be detected which is convenient for HCV subtyping but does not for resistance tests which are based on the detection of a high number of mutations. In addition, HIV and HBV have highly variable genomes and naturally occurring polymorphisms that are present in the vicinity of resistance mutations may affect the binding efficiency of probes.

DNA-microarrays are an alternative for resistance or typing reagents for viruses or bacteria (Vernet et al., 2004). We have developed an assay based on RT-PCR and detection with

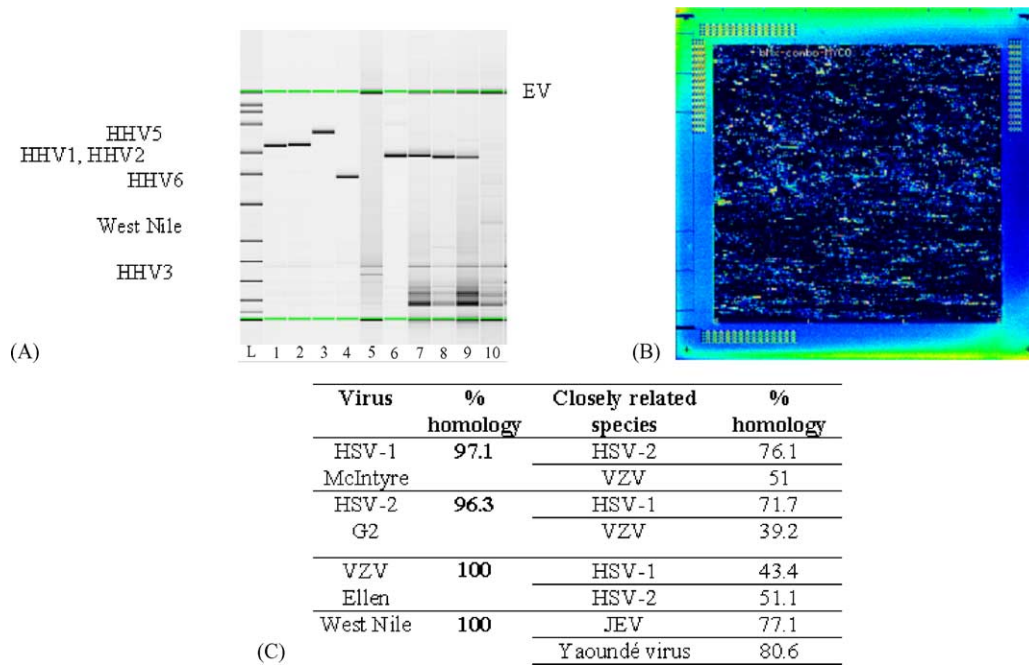| Virus	% homology	Closely related species	% homology
HSV-1	97.1	HSV-2	76.1
McIntyre		VZV	51
HSV-2	96.3	HSV-1	71.7
G2		VZV	39.2
VZV	100	HSV-1	43.4
Ellen		HSV-2	51.1
West Nile	100	JEV	77.1
		Yaoundé virus	80.6

(C)

Fig. 2. bioMerieux DNA-microarray for the detection of neurotropic viruses. Following nucleic acid purification, amplification is performed by PCR using a single touchdown protocol in three tubes, one for *herpesviridae* (one primer pair for HHV 1, 2, 3, 5 and 6), one for enteroviruses (one primer pair for all serotypes) and one for flaviviruses (one primer pair for all viruses). Amplification products are combined and labelled using diazomethyl chemistry and hybridised on a DNA-microaaray which contains 20-mer oligonucleotides. Two or four probes are used for the detection of each base of sequence signatures determined for each virus. A total of 20,000 probes are synthesised on this DNA-microarray which has been designed for the simultaneous detection of viruses from the *herpesviridae* family and from the flavivirus, enterovirus, paramyxovirus, poliomavirus, bunyavirus and orthopoxvirus genus. A: amplicons generated resolved using the bioanalyzer (Agilent Technologies). B: image of the DNA-microarray obtained with a confocal laser reader. C: resolution capability of the array. Closely related viruses hybridise with very different efficiency on heterologous probes.

high density probe arrays, designed to detect 204 antiretroviral resistance mutations simultaneously in gag cleavage sites, protease, reverse transcriptase, integrase and gp41. This assay has been tested on a panel of 99 HIV-1 patients on a total of 4465 relevant codons in comparison with the classic sequence-based method. Key resistance mutations were correctly identified in 95 and 92% of codons in protease and reverse transcriptase, respectively (Gonzalez et al., 2004). We have also developed a similar assay for the detection of polymorphisms in the complete HBV genome: 78 antiviral resistance mutations in pol gene, 146 vaccine, diagnostic or immunotherapy mutation in s gene and 209 mutations in basic core promoter, pre-core, core, X, pre-S1 and pre-S2 regions that may have an impact on disease evolution or treatment efficiency. This assay is currently under evaluation in the frame of HepBVar a European collaborative group for the study of emerging variants of Hepatitis B virus.

3. Conclusion

In the coming years, more and more laboratories will offer to clinicians viral diagnosis based on nucleic acid tests. Many biological and instrumentation problems that have slowed the generalization of molecular assays have been resolved but several others remain and need to be addressed. Major im-

provements are expected in the integration and automation of NAT diagnostic platforms to reduce hands-on-time, time-to-result and costs and to increase throughput. IVD companies have engaged in development programs to provide clinical virologists with equipments and application menus adapted to their diagnosis needs.

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