

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



---

# **Molecular Diagnostics as an Indispensable Tool for the Diagnosis of Infectious Diseases of Viral Origin and Global Impact**

---

Nora Rosas-Murrieta, Irma Herrera-Camacho,  
Lourdes Millán-Pérez-Peña, Francisca Sosa-Jurado,  
Gerardo Santos-López, Verónica Vallejo-Ruíz and  
Julio Reyes-Leyva

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/57581>

---

## **1. Introduction**

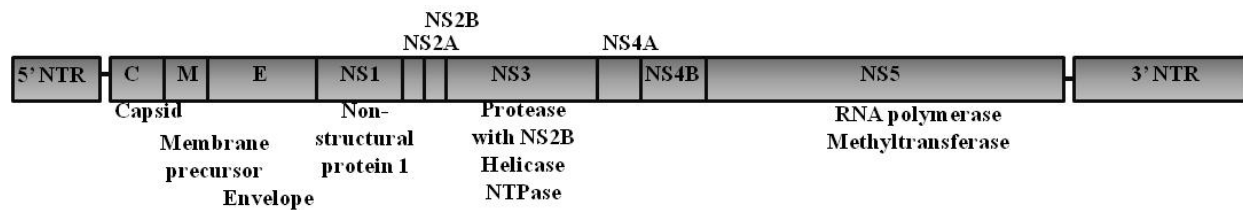
Infectious diseases are responsible for a considerable number of deaths in infectious in entire world. Infectious diseases are human diseases caused by viruses, bacteria, parasites, fungi and other microorganisms. Most of them have been controlled by vaccines or antimicrobials. However, some of them still represent global public health problems and are being monitored by the WHO and Center for Disease Control and Prevention. This chapter provides an overview of the applications of molecular methods for infectious diseases caused by viruses (intracellular obligate parasites) of global impact such as Dengue virus, Hepatitis B virus or influenza A virus. The infectious diseases not only represent a potential danger to the life of all human beings but also a significant investment in its detection, treatment and control of their spread. The increase in opportunities of infection by globalization, high rates of mobility among most countries around the world, the patient susceptibility to diseases due to genetic variation in populations [1], the ability of the microorganisms to evade the host immune response has forced the World Health Organization (WHO) to establish better methods of detection, prevention and control of infectious diseases caused by viruses as influenza A virus, coronaviruses, dengue virus, among others [2]. On the other hand, some types of cancer are the result of chronic viral infections caused by human papillomavirus, hepatitis B and C virus. Other infectious diseases are related to the development of neurological disorders caused by the measles virus, or human immunodeficiency virus [3]. In the determination of the etiology

of such diseases has made extensive use of clinical procedures internationally validated as methods based on viral cultures and serological assays. However, it is increasing the use of nucleic acid tests in the diagnosis of infectious disease of viral etiology, considering that a critical step to proper treatment and control of any virus infection is a correct diagnosis. Diagnostic tests based on nucleic acid (NAT, nucleic acid tests) more used are the nucleic acid sequence-based amplification (NASBA), polymerase chain reaction (PCR) or real-time PCR for virus detection, genotyping and quantification. In addition, the automation of these techniques decrease in test time, low contamination risk, ease of performance, speed and have lower detection limits [4] show the relevance of their use. The detection of the infectious agent can be done by detection of the genomic DNA, genomic ARN and the viral messenger RNA (mRNA) using the follow techniques: Nucleic acids hybridization (Solid-phase, Liquid-phase or *in situ* hybridization), amplification of the signal of nucleic acids (branched-DNA assays and Hybrid capture assays), nucleic acid amplification (PCR, Real-Time PCR, Nested PCR, Multiplex PCR, Transcriptional-based amplification methods coupled to qPCR or NASBA, Strand displacement amplification), Microarrays (DNA microarrays and Multiplexed microsphere-base array) [5]. Currently there are some variants of the above techniques aimed to screening for detection or simultaneous discrimination of various etiological agents using multiplex PCR techniques [6], MassTag PCR, a PCR platform coupled to a mass spectrometer which allows simultaneous detection of >20 different pathogens [7] or microarrays pathogen detection (Virochip) [8]. The routine use of molecular techniques for the fast differential diagnosis of viral infections is vital for a high quality care of the patient with an infectious disease, directs the best therapeutic scheme, thus reducing the likelihood of complications, the proper choice of antiviral drug or the best strategy for control of viral replication, reduces resistance to antivirals and prevent the worsening of the clinical picture, the spread of the disease and the death of the patient [9]. This chapter will present molecular techniques applied to the diagnosis of infectious disease of viral etiology and incidence worldwide. A critical first step to proper treatment and control of any virus infection is a correct diagnosis. Conventional diagnostic tests for viruses it based on amplification of conserved portions of the viral genome, detection of antibodies against to viral proteins, or replication of the virus in cell cultures.

## 2. Dengue virus

Dengue virus (DENV) infection is the most common arthropodborne viral disease of humans; *Aedes* mosquitoes, principally *Aedes aegypti*, transmit this disease. According World Health Organization DENV is an emerging infectious agent that infects with an estimated 50–100 million clinical infections occurring annually worldwide [10]. DENV belongs to the family Flaviviridae, genus Flavivirus. DENV is a small are spherical and enveloped virus that contain a positive strand RNA genome of approximately 10,600 nucleotides coding for three structural proteins (capsid C, membrane, M, and the envelope, E) and seven non-structural proteins (NS1, xlinkA, xlinkB, NS3, NS4A, NS4B, NS5) [11], (Figure 1). The envelope protein (E) plays a key role in several important processes including receptor binding, blood cell hemagglutination, and induction of a protective immune response, membrane fusion and virion assembly. Two types of virions are recognized: mature extracellular virions contain M protein, while imma-

ture intracellular virions contain prM, which is processed proteolytically during maturation to yield M protein. The envelope of the virus contains the viral surface proteins E and M [12,13].



**Figure 1.** Schematic diagram of dengue virus genome. The DENV genome of positive-sense RNA and single-stranded, comprises approximately 10,600 nucleotides. Any region of genome can be used for genome detection by molecular techniques.

There are 4 antigenically and genetically distinct serotypes (DENV-1,-2,-3 and-4), being the “Asian” genotypes of DEN-2 and DEN-3 the most frequently associated with severe disease accompanying secondary dengue infections [14]. In human, the virus can cause a spectrum of illness, 75% of DENV infections are asymptomatic. But in persons with symptomatic DENV infection (dengue), the illness occurs in three phases. Acute phase, with 2–7 days of fever or self-limiting influenza-like illness (dengue fever or DF), accompanied by headache, retro-orbital eye pain, joint pain, muscle and/or bone pain, rash, mild bleeding manifestations and low white cell count. The critical phase of dengue which marks a 24 to 48 hours, period in which can occur the named severe dengue associated with vascular leakage, hemorrhage (dengue hemorrhagic fever or DHF), potentially leading to vascular shock (dengue shock syndrome or DSS), without appropriate treatment, patients with severe dengue are at risk of death. The convalescent phase of dengue lasts for 4-7 days [15-18]. During the past five decades, the incidence of dengue has increased 30-fold. In 2012, dengue was the most important mosquito-borne viral disease in the world. The emergence and spread of all four dengue viruses (“serotypes”) from Asia to the Americas, Africa and the Eastern Mediterranean regions represent a global pandemic threat. Because epidemics of dengue result in human suffering, strained health services and massive economic losses, an international effort to reduce morbidity and mortality is long overdue coordinated by WHO named Global Strategy for Dengue Prevention and Control 2012-2020 [19]. The goal of the global strategy is to reduce the burden of dengue by to reduce mortality and morbidity from dengue by 2020 by at least 50% and 25% respectively. The laboratory diagnosis of dengue can detect severe cases, case confirmation and differential diagnosis with other infectious diseases. Diagnosis of dengue is made by detecting the infective virus, virus genome, dengue antigen or by analyzing, the serological responses (IgM or IgG) present after infection. Serology is currently the most widely applied in routine diagnosis [20] (Table 1). After the mosquito bites occurs an incubation period of 4–10 days, resulting in an asymptomatic or symptomatic dengue infection. In this period, the virus replicates and an antibody response is developed (Figure 2). The development of IgM antibody is coincident with the disappearance of fever and viraemia. In a primary infection (when an individual is infected for the first time with a flavivirus), viraemia develops from 1–2 days before the onset of fever until 4–5 days after and anti-dengue IgM specific antibodies can be detected 3–6 days after fever onset. Low levels of IgM are still detectable around one to

three months after fever. In addition, the primary infection is characterized by slowly increasing but low levels of dengue-specific IgG, becoming elevated at days 9–10. Low IgG levels persist for decades, an indication of a past dengue infection. In a secondary infection, there is a rapid and higher increase of anti-dengue specific IgG antibodies. High IgG levels remain for 30–40 days [21]. The serological tests have been used for the diagnosis of dengue infection: hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), immunoglobulin M (IgM) capture enzyme linked immunosorbent assay (MAC-ELISA) and indirect immunoglobulin G ELISA. High cross-reactivity was observed with these tests. MAC-ELISA and rapid tests are the most frequent methods for IgM detection and in the detection of IgG are used HIA or ELISA [22–25]. Dengue non-structural protein 1 (NS1) is secreted from infected cells and produces a very strong humoral response. NS1 is a useful early serum marker for primary and secondary dengue infections, and is typically present between days 1–9 after onset of clinical signs [23]. In febrile patients, the early diagnosis of dengue virus infection on based on NS1 viral antigen [26–27]. At the end of the acute phase of infection, serology is the method of choice for diagnosis by detection anti-DENV IgM and IgG antibodies using MAC-ELISA. High sensitivity for NS1 antigen detection was observed by the association of MAC-ELISA with a commercial anti-DENV IgM/IgG rapid test (PanbioH Dengue Early Rapid test) [28]. A DENV NS1 capture assay using a test NS1 Ag Strip (BioRad Laboratories) is a valuable tool to postmortem dengue confirmation [29]. There are several commercial kits to dengue diagnostic such as the SD Bioline Dengue Duo device (Standard Diagnostic Inc., Korea) this test is composed of 2 tests to detect DENV NS1 antigen (first test) and anti-DENV IgM/IgG (second test) in serum, plasma or whole blood. With this test, still after the onset of illness, the virus can detected in serum, plasma, circulating blood cells and other tissues for 4–5 days.

	SAMPLE	DIAGNOSTIC METHOD	METHODOLOGY
Serological response	Paired sera (acute serum from 1-5 days and second serum 15-21 days after)	IgM or IgG seroconversion	ELISA
			HIA
			Neutralization test
	Serum after day 5 of fever	IgM detection (recent infection)	ELISA
			Rapid tests
Virus detection and its components	Acute serum (1-5 days of fever) and necropsy tissues	IgG detection	IgG ELISA
			HIA
		Viral isolation	Mosquito cell culture inoculation
		Nucleic acid detection	RT-PCR and real time RT-PCR
	necropsy tissues	Antigen detection	NS1 Ag rapid tests
			NS1 Ag ELISA
			Immuno-histochemistry

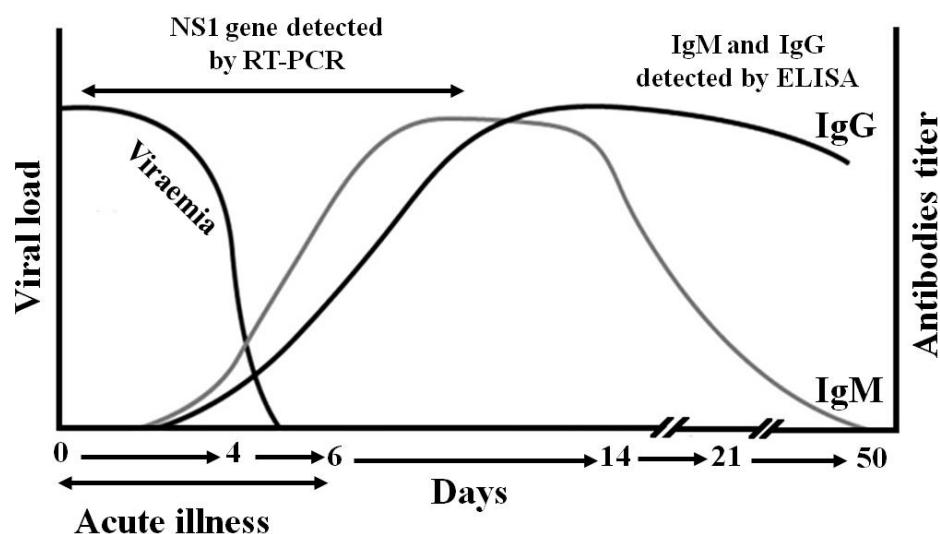
ELISA (Enzyme-linked immunosorbent assay). HIA (Haemagglutination inhibition assay). IgG (Immunoglobulin G). IgM (Immunoglobulin M). NS1 Ag (Non-structural protein 1). RT-PCR (Reverse transcriptase polymerase chain reaction). Information based on Chapter 4. Laboratory Diagnosis and Diagnostic Tests. Dengue Guidelines for Diagnosis, Treatment, Prevention and Control. Geneva. TDR/World Health Organization. 2012 [21].

Table 1. Dengue diagnostic methods.



Dengue diagnosis also relies on viral cell culture methods [30,31]. Specimens for virus isolation should be collected early in the course of the infection, during the period of viraemia (usually before day 5). Virus may be recovered from serum, plasma and peripheral blood mononuclear cells. Dengue virus is heat-labile; specimens awaiting transport to the laboratory should be kept in a refrigerator or packed in wet ice. Cell culture is the most widely used method for dengue virus isolation usually takes several days. Four methods of viral isolation have been routinely used for dengue viruses: intracerebral inoculation of newborn mice, inoculation on mammalian cell cultures, intrathoracic inoculation of adult mosquitoes, and inoculation on mosquito cell cultures [32]. The mosquito cell line C6/36 (cloned from *Ae. albopictus*) or AP61 (cell line from *Ae. pseudoscutellaris*) are the host cells of choice for isolation of virus. Indirect fluorescent antibody staining of the infected cell culture is often regarded as the “gold standard” in dengue diagnostics. However, it is tedious, time-consuming, and requires cell culture. All these studies have shown that this can be a valuable approach, especially in the early phase of infection; however, in some cases, they do not identify the viral serotype and these assays may not be as sensitive as the detection of viral RNA by nucleic acid amplification tests (NAAT) using reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR may assume a very important role in dengue diagnosis at confirms an acute dengue infection [33, 20, 21]. Molecular diagnosis methods are usually rapid, sensitive, and simple when correctly standardized and can be used for serotype identification and quantification of genome copies in human clinical samples, biopsies, autopsy tissues, or mosquitoes. NAAT assays may identify viral RNA within 24–48 hours. Although, also be able to detect dengue viruses up to the 10th day after the onset of the symptoms. RNA extraction from clinical samples can be performed with the QIAamp Viral RNA kit [34], VERSANT Molecular System SP (Siemens) or HighPureViral Nucleic Acid Kit (Roche). Several RT-PCR procedures to detect and identify dengue serotypes in clinical specimens have been reported such as one-step, two-step, nested RT-PCR or real time RT-PCR [35]. These PCR assays vary in the amplified gene regions of the genome, in the detection method of RT-PCR products, and the virus typing methods. Real-time RT-PCR assays “singleplex” or “multiplex” can identify all four serotypes from a single sample, in a single reaction and is useful to determine viral titer in a clinical sample. Real-Time-PCR detection and typing of DENV usually addresses to the partially conserved 3'-UTR region of the genome. Due to the typical RNA-viral sequence variations it is difficult to identify one particular probe to be strictly related to one type or which could be used for a melting curve based analysis [36].

Many laboratories utilize a nested RT-PCR assay, using universal dengue primers targeting the C/prM region of the genome for an initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific. The analysis of the amplification efficiencies of an in-house quantitative real time-PCR (qPCR) assay of DENV, between the region protein-5 (NS5) versus the capsid/pre-membrane region (C-prM) and the 3'-noncoding region (3'NC) showed that the non-structural conserved genomic region the NS5 genomic region provides the best genomic region for optimal detection and typification of DENV in clinical samples [37]. A fragment located at the 5'-UTR region of the virus genome was successfully used to identify and quantify distinct dengue virus strains and serotypes in clinical samples, in sera from patients infected with dengue virus, and in the mosquito *Aedes*



**Figure 2.** Immune response to virus dengue. In the infection course of dengue virus, the host's B cells produce IgM and IgG antibodies, which are released for recognize and neutralize the dengue virus and molecules such as the dengue NS1 protein with the purpose of eliminates the virus. The figure shows the viremia can detect by the amplification of the NS1 gene by RT-PCR and the antibodies titers by ELISA. Information based on Guzman MG, et al., 2010 [25] and WHO, Geneva, 2009 [10].

*aegypti*, as well as to study virus replication in different cell lines using TaqMan probes [38]. A real time RT-PCR (qRT-PCR) for DENV using TaqMan Minor Groove Binding (MGB) probe approach was development for detection and quantitation of all four serotypes using a single probe primer set targeted against the 3'UTR of DENV. In this assay, the limit of detection was DENV-1 (0.98 UFP/ml), DENV-2 (0.99 UFP/ml), DENV-3 (0.99 UFP/ml) and DENV-4 (0.99 UFP/ml) [39]. Other option to diagnosis to provide serotype specificity is a multiplex rRT-PCR assay targeting the 5' untranslated region and capsid gene of the DENV genome using molecular beacons. This assay was linear from 7.0 to 1.0  $\log_{10}$  cDNA equivalents/mL for each serotype. The limit of detection was calculated to be 0.3 cDNA equivalents/mL for DENV-1, 13.8 for DENV-2, 0.8 for DENV-3, and 12.4 for DENV-4 [40]. A technique for the multiplex detection and typing of all DENV serotypes from clinical samples is PCR-ligase detection reaction (LDR). In this technique, a serotype-specific PCR amplifies the regions of genes C and E simultaneously. Then, two amplicons are targeted in a multiplex LDR, and the resultant fluorescently labeled ligation products are detected on a universal array. The sensitivity of the assay was 98.7%, and its specificity was 98.4%, relative to the results of real-time PCR. The detection threshold was 0.017 PFU for DENV-1, 0.004 PFU for DENV-2, 0.8 PFU for DENV-3, and 0.7 PFU for DENV-4. The assay is specific; it does not cross-react with the other flaviviruses tested (West Nile virus, St. Louis encephalitis virus, Japanese encephalitis virus, Kunjin virus, Murray Valley virus, Powassan virus, and yellow fever virus). The detection limit of the assay ranged from 0.004 to 0.7 equivalent PFU/reaction. The assay was 100 times more sensitive for DENV-2 and DENV-1 (LOD, 0.004 and 0.017 equivalent PFU, respectively) than for the others serotypes [41]. Detection of DENV in urine is other diagnostic method, a study by real-time RT-PCR, reported the detection of viral genome in urine between days 6 to 16[42]. Recently, CDC development a CDC DENV-1-4 Real-Time RT-PCR Assay, the first molecular test

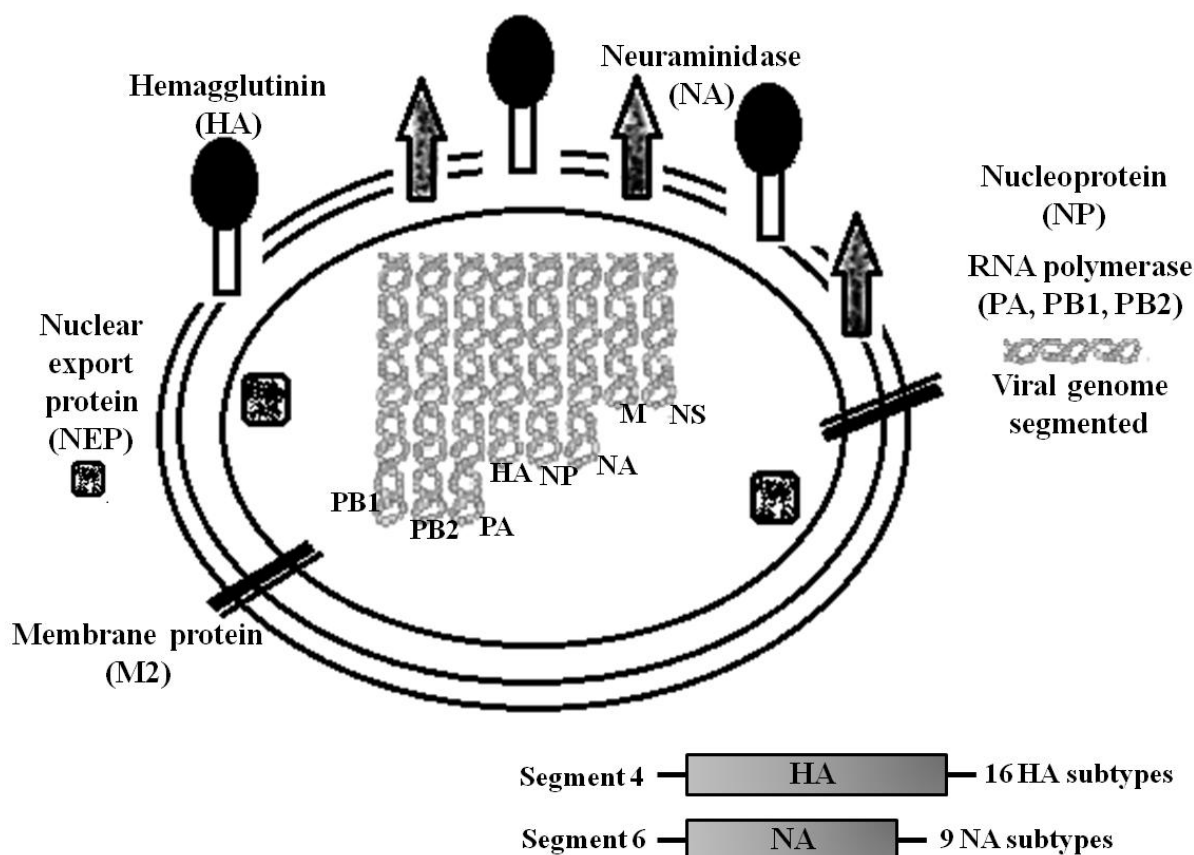
approved by the US Food and Drug Administration (FDA) for the identification of dengue virus serotypes 1, 2, 3 or 4 from viral RNA in serum or plasma (sodium citrate) collected during the acute phase. The limit of detection (LoD) of assay was determined to be between  $1 \times 10^4$  and  $1 \times 10^3$  GCE/mL for all serotypes in both formats in serum and plasma [43]. In all reactions by real-time RT-PCR, a positive PCR result is a definite proof of current infection and it usually confirms the infecting serotype. However, a negative result is interpreted as "indeterminate". Patients receiving negative results before 5 days of illness are usually asked to submit a second serum sample for serological confirmation after the 5th day of illness. Also, it was used other options to diagnostics dengue infection. The first is Virochip is a pan-viral microarray platform; this assay can detect to virus of Herpesviridae, Flaviviridae, Circoviridae, Anelloviridae, Asfarviridae, and Parvoviridae families. In other hand, for viral detection exists the option Deep sequencing and shotgun sequencing of human clinical samples by pyrosequencing using the Illumina GAII platform [44].

### 3. Influenza virus

Influenza is a highly contagious respiratory disease of humans, with propensity for seasonal epidemics and occasional pandemics. This disease constitutes a global health issue, leading to morbidity, mortality, and economic losses. During influenza season, influenza viruses circulate ubiquitously in the population. Global influenza surveillance forms the primary line of defense against the occurrence of influenza pandemics by identifying emerging influenza virus strains that pose a potential threat [45]. Influenza affects all age groups that result from its pulmonary complications. The virus initially infects the upper airways but can directly extend to the lower airways in severe cases, resulting in a viral pneumonia with significant morbidity and mortality [46, 47]. Influenza viruses belong to the Orthomyxoviridae family and are organized into types A, B and C. Influenza types A and B are responsible for epidemics of respiratory illness in humans and animals. The etiologic agent of influenza is the influenza virus with negative-strand, segmented RNA genome. Influenza type A and B viruses have 8 genes that code for 10 proteins. The virion has two surface glycoproteins (hemagglutinin (HA) and neuraminidase (NA) and the M2 protein protrude through envelop (Figure 3). Influenza virus binds to its sialic (neuraminic) acid receptor on respiratory epithelial cells by means of the HA protein [48]. The influenza type A viruses are sub classified into different subtypes according to HA and NA proteins, there are 16 HA subtypes and 9 NA subtypes, all of them have been identified and isolated from birds, humans and can affect a range of mammal species. The influenza A subtypes that circulated extensively in humans are A(H1N1); A(H1N2); A(H2N2); and seasonal influenza A (H3N2) [49]. Influenza A viruses (IAV) are the cause of pandemics, which are generated by the rearrangement (reassortment) of viral RNA segments in cells infected with two different viral strains [50].

Pandemic viruses of influenza A virus including "Spanish influenza" (H1N1 in 1918) and A/H1N1 or A(H1N1)pdm09 (H1N1 in 2009) [51] or rarely, a novel influenza A virus infection. For the diagnosis, the influenza tests that provide accurate and timely results are the most recommended. The appropriate respiratory samples for influenza testing are upper respiratory





**Figure 3.** Schematic representation of influenza virus A (not drawn to scale). On the envelope viral are anchored: HA, M2 (ion channel protein) and NA protein. Inside the virion the negative-RNA single-stranded and segmented viral genome with ARN polymerase formed for PB1, P2 and PA. Also, there is a NEP protein.

tract specimen such as deep nostrils (nasal swab), throat (throat swab) and nasopharynx (nasopharyngeal swab). Nasopharyngeal aspirate and bronchial aspirate are also useful. Samples should be collected within the first 4 days of illness [52,53]. According to WHO the diagnosis of influenza is based in enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests, egg and cell culture inoculation, and conventional reverse transcriptase PCR (RT-PCR) and Real-Time PCR (Table 2). The reference standards for laboratory confirmation of influenza virus infection are reverse transcription-polymerase chain reaction (RT-PCR) or viral culture [54]. Serologic testing is usually not recommended to detect evidence of human influenza virus infection for management of acute illness. However, a variety of serological tests, including the hemagglutination inhibition (HAI) test, complement fixation and EIA are used for testing paired acute and convalescent phase sera to detect antibody titers. The Rapid Influenza Diagnostic Tests (RIDTs) can identify the presence of influenza A and B viral nucleoprotein antigens, the result only is positive or negative. The RIDTs are immunoassays than provide results in 10–30 min but exhibit decreased sensitivity (70%–90% in children and 40% to 60% in adults), compared with RT-PCR and with viral culture. There is a potential for false negative results. Negative results of RIDTs do not exclude influenza virus infection in patients with signs and symptoms

suggestive of influenza, follow-up testing with RT-PCR and/or viral culture should be considered to confirm negative test results [55]. Besides, of its low sensitivity RIDTs may be used to help with diagnostic and treatment decisions for patients in clinical settings, such as whether to prescribe antiviral medications [45]. These tools are continuously improved, a double-sandwich ELISA (pH1N1 ELISA), based on two monoclonal antibodies against haemagglutinin (HA) of the pH1N1 virus has a sensitivity of 92.3% (84/91, 95% CI 84.8–96.9%), being significantly higher than that of the BD Directigen EZ Flu A+B test (70.3%,  $p < 0.01$ ). In addition, this assay can directly differentiate pandemic (H1N1) 2009 (pH1N1) virus from other respiratory pathogens, including seasonal influenza virus [56]. A hemagglutination inhibition assay is an extremely reliable tool for typing, subtyping and analyze the antigenic characteristics of influenza viral isolates if the reference antisera used contain antibodies to currently circulating viruses [53]. An HAI test showed that the patients with influenza A H1N1 have effective immune response [57]. The Directigen EZ Flu A+B test is a rapid chromatographic immunoassay for the qualitative detection of influenza A and B viral antigens, it has an accuracy of 95.5% and 96.8%, respectively. This kit has the ability to detect H5N1 isolates and of the A/California/07/2004 strain [BD Diagnostics, Becton Dickinson and Company, 2005]. Viral culture is considering one “gold standard” for detection of infection with human influenza viruses. Although, viral isolation is not a screening routine test in outbreaks, during periods of low influenza activity, could performed on respiratory specimens collected from persons with suspected influenza. Viral culture does not provide timely results (1-14 days) [58, 59]. In the influenza seasonal and in outbreaks, the viral culture is essential as a source of virologic data about strain characteristics, such as antigenic comparison to influenza vaccine strains and antiviral susceptibility that are important for clinicians and public health. Shell vial centrifugation cultures have been used to shorten the time to results to 1 to 5 days, to detect viral antigens [60]. Immunofluorescence using direct fluorescent antibody or indirect fluorescent antibody staining for influenza antigen detection are used as screening tests. This test exhibits slightly lower sensitivity and specificity than viral isolation in cell culture, but results are obtained within hours [45]. An example is the Respiratory Screen direct immunofluorescence antibody (DFA) (direct fluorescent antibody) staining of cells collected using nasopharyngeal (NP) swabs or NP aspirates can detects not only influenza, but RSV, parainfluenza types 1,2,3, and adenovirus. DFA is reported within 2-3 hrs [61]. However, for detection of respiratory viruses in clinical specimens, the nucleic acid tests (NATs) are fast, accurate and sensitive test. Several nucleic acid based amplification approaches have been applied for the detection of individual respiratory viruses including PCR, nucleic acid sequence-based amplification (NASBA), loop mediated isothermal amplification (LAMP), and multiplex ligation-dependent probe amplification (MLPA) [62-65]. For NATs, the nucleic acid is extracted from the samples by using commercial kits, like QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) using silica gel membrane technology. KingFisher mL (ThermoFisher Scientific Inc., Worcester, MA, USA) and easyMAG (bioMérieux, Marcy l’Etoile, France) kits using magnetic beads-based technology [66, 67]. TruTip (Akonni Biosystems, Inc., Frederick, MD, USA) use a porous nucleic acid binding matrix embedded within a pipette tip [68]. Reverse transcriptase-PCR (RT-PCR) or Real-time RT-PCR can be considered the other “gold standard” for detection of influenza viruses due to its high sensitivity and specificity for detection of influenza A and B

viruses. The results are available within 4–6 h after specimen submission. These molecular tools are used as a confirmatory test. Several different gene targets have been used for amplification such as the matrix (*m*) to detect all influenza A subtypes, *ha* to distinguish between influenza A, B and C or between influenza A subtypes and non-structural protein genes (*nsp*) [69–71]. In all eight segments of influenza A virus, the first 12 nucleotides of the 3' terminus (Uni12) and the first 13 nucleotides of 5' terminus (Uni13) are conservative and are the target to designed a primers pair. Detection of influenza A virus in human nasal swabs can be performed by RT-PCR with Uni12 and Uni13 primers. The coupling of RT-PCR with sequencing analysis provides information about viral genotype [72]. Multiplex RT-PCR assays have been widely used for detection and differentiation of a panel of respiratory viral pathogens. FluPlex is a multiplex RT-PCR enzyme hybridization assay, capable of typing influenza viruses and subtyping HA (H1, H2, H3, H5, H7, and H9) and NA (human N1, animal N1, N2, and N7) with high sensitivity (10–100 copies/reaction) [73]. As the Qiagen ResPlex II multiplex RT-PCR kit, an test with high specificity for detection of 17 viral pathogens in nasopharyngeal swab samples such as influenza A virus (FluA), FluB, FluA 2009 pandemic A(H1N1)pdm09 and others pathogens as parainfluenza virus 1 (PIV1), PIV2, PIV3, PIV4, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinoviruses (RhV), adenoviruses (AdV), four coronaviruses (229E, OC43, NL63 and HKU1) [74]. The TrueScience RespiFinder Identification Panels (Applied Biosystems) is other multiplex PCR test to detect and differentiate 15 respiratory pathogens, using the multiplex ligation-dependent probe amplification (MLPA) technology, which starts by a preamplification step, which ensures the detection of both RNA and DNA viruses.

Follow is performed a reverse transcription, a PCR reaction, the hybridization probes to their target regions to the PCR product. A ligation probes and the final PCR exponentially amplifies the ligated probes with only two primers. The targets are detected through capillary electrophoresis. The pathogens potentially identified are Influenza A, Influenza B, Influenza A H5N1, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4, RSV-A, RSV-B, Corona 229E, Corona NL63, Corona OC43, Adenovirus, Rhinovirus, Bordetella pertussis, Chlamydomydia pneumoniae, Legionella pneumophila and Mycoplasma pneumoniae. MPLA shows specificities and sensitivities of 98.2% and 100%, respectively, for influenza A virus [75]. A successful test to detect a H5N1 virus was based on Loop-Mediated Isothermal Amplification (LAMP) method. For other side, detection, quantification and subtyping of influenza viruses can be performed by real-time RT-PCR (qRT-PCR). There are many papers of this technique applied to influenza A diagnosis such as TaqMan qRT-PCR method, which detects HA and NA genes of HPAI H5N1 virus [76]. A new generation qRT-PCR approach designated as Super high-speed qRT-PCR (SHRT-PCR) is a version of qRT-PCR with an extremely short reaction time (less than 20 min per run for 40 cycles) capable to detects viral RNA segments of influenza A [77]. RealTime ready Influenza A/H1N1 Detection Set (Roche) is an assay to detect the M2 gene (M2 PCR) of a generic influenza virus A and a specific PCR targeting the HA of A/H1N1-pdm09 (HA PCR, 2009 H1N1), to detect and quantify the 2009 H1N1 virus in clinical samples [78]. CDC developed the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel-Influenza A/H7 (Eurasian Lineage) assay for patients with signs and symptoms of respiratory infection. The kit contains a dual-labeled hydrolysis probe (TaqMan). The limit of detection of

METHOD	TYPE INFLUENZA VIRUS	TYPE SPECIMENS
Cell culture (Madin-Darby canine kidney (MDCK), mink lung epithelial cell line (Mv1Lu), rhesus monkey kidney (LLC MK2), and buffalo green monkey kidney (BGMK)	A and B	NP swab, throat swab, NP or bronchial wash, nasal or endotracheal aspirate, sputum
Rapid cell culture (commercial shell vials, single or mixed cell lines)	A and B	NP swab, throat swab, NP or bronchial wash, nasal or endotracheal aspirate, sputum
Immunofluorescence microscopy by direct fluorescent antibody (DFA) test or an immunofluorescent antibody (IFA) test, using commercial monoclonal antibody for influenza A and B viruses; or non commercial monoclonal antibody specific for H1, H3 H5, H7 and H9	A and B Identification of subtypes of A	NP swab, thrat swab, NP or bronchial wash, NP or endotracheal aspirate, sputum
Immunospecific assay for viral antigen detection Rapid influenza diagnostic test (RIDT's)	A and B	NP swab, nasal wash, NP aspirate, throat swab
Viral antibody detection Virus neutralization test Haemmaglutination inhibition ELISA using commercially anti-human antibody conjugates Complement fixation	A and B	Serum
Nucleic acid testing (Conventional RT-PCR singleplex and multiplex, Multiplex PCR, Degenerate PCR, Probe-based real-time PCR (TaqMan), SBRY Green I-based real-time PCR, Microarrays, NABA, LAMP, Pyrosequencing	A and B Identification of subtypes of A	NP swab, throat swab, NP or bronchial wash, NP or endotracheal aspirate, sputum
Information based on Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases (NCIRD). Guidance for Clinicians on the Use of Rapid Influenza Diagnostic.2011. Petric M, et al. 2006 [64]; Wang&Taubenberger, 2010 [65].		

**Table 2.** Methods of diagnosis of influenza virus.

this kit was 10<sup>3.4</sup> [79]. On April 1, 2013, an outbreak of human infections with a new avian influenza A (H7N9) virus was first reported in China by the World Health Organization. The diagnostics of this virus used the real time RT-PCR by TaqMan assay. Other molecular technique to diagnosis is DNA microarray such as FluChip-55 [Sakurai & Shibasaki, 2012] used



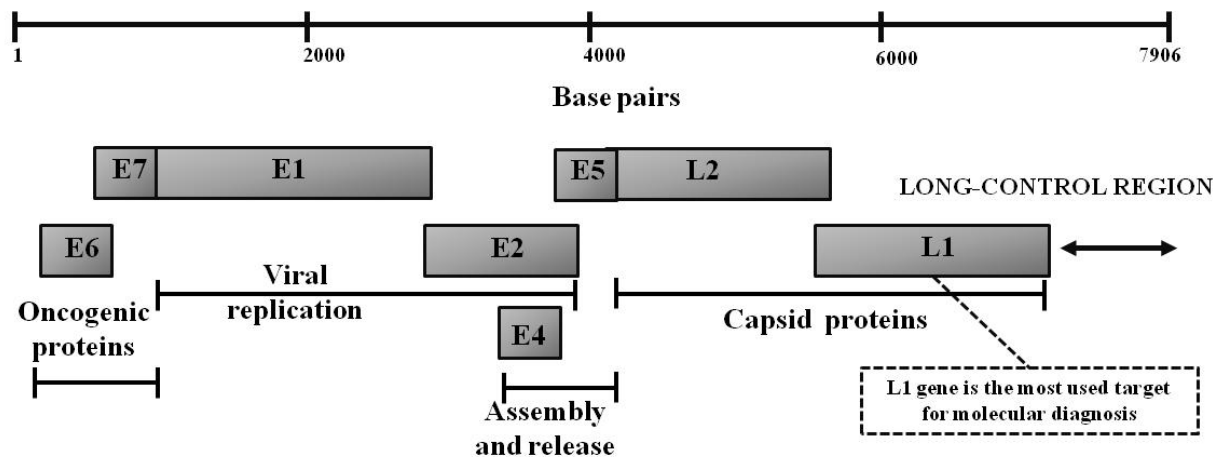
in influenza diagnosis and others viruses such as parainfluenza, respiratory syncytial virus, human metapneumovirus, adenovirus, rhinoviruses, coronaviruses causes pneumonia, which can be analyzed by multiplex PCR that assist in the discrimination of the etiologic agent [Pavia, 2011; Mahony, 2008]. The NGEN respiratory virus analyte-specific assay (Nanogen, San Diego, CA) detects influenza A, influenza B and others pathogens such as PIV type 1 (PIV-1), PIV-2, PIV-3, and RSV on a NanoChip 400 electronic microarray. The FilmArray Respiratory Panel (RP) is a PCR array test (Idaho Technology, Inc., Salt Lake City, UT) that can detect up to 21 viral and bacterial respiratory pathogens within about an hour as Adenovirus, Bocavirus, Coronavirus 229E, Coronavirus HKU1, Coronavirus NL63, Coronavirus OC43, Human Metapneumovirus, Parainfluenza virus 1, Parainfluenza virus 2, Parainfluenza virus 3, Parainfluenza virus 4, Rhinovirus/Enterovirus, Respiratory Syncytial Virus, Bordetella pertussis, Chlamydia pneumoniae, and Mycoplasma pneumonia and Influenza A, Influenza A subtype H1, Influenza A subtype H3, Influenza A subtype H1 2009, Influenza B. This assay is a feasible alternative to viral culture in an acute clinical setting [80]. Recently, it was reported the method for the detection of genetic markers associated with high pathogenicity of influenza virus [81].

#### 4. Human papillomavirus

Human papillomavirus (HPV) is a pathogen associated to development of cervical cancer, which is the second more common cancer in women worldwide. These viruses typically infect the skin and mucosal surfaces of humans. HPV is a non-enveloped virus with a circular and supercoiled double-stranded DNA genome of approximately 8,000 bp long within an icosahedral coat or capsid comprised by 72 pentameric capsomers. The open reading frames (ORFs) in the viral genome are organized in three regions: the early expression region (E), the late region (L) and the long control region (LCR) that bears the origin of viral replication and transcription. The E region codes for proteins related to replication (E1) and to activation or repression of the viral DNA (E2), and the oncogenes E6, E7, E5 [82]. The L1 and L2 genes encode the mayor and minor capsid proteins. Late genes L6 and L7 code for structural capsid proteins which encapsidate the viral genome (Figure 4) [83]. The L1 gene is the region most conserved between individual types and used to analyze phylogenetic relationship.

Those HPVs with L1 sequence divergence of 2–10% are known as subtypes and less than 2%, variants. The term “genus” is used for the higher order clusters, named using the Greek alphabet, and within genus, small clusters are referred to as species and given a number [84–86]. There are more than 200 different types, of which approximately 100 are fully sequenced and classified according to their biological niche, phylogenetic position and oncogenic potential [87]. About 40 can infect the anogenital mucosa of humans (mucosotrophic HPVs) [88–89]. According to their association with cervical cancer, there are low-risk HPV, which can cause benign or low-grade cervical cell changes, genital warts, and recurrent respiratory papillomatosis. High-risk HPV types act as carcinogens in the development of cervical cancer and other anogenital cancers [90]. Cervical cancer is a commonly-encountered malignant tumor in women. The surveys demonstrated that the increase of women with cervical cancer





**Figure 4.** Linear representation of Human Papillomavirus (HPV) genome. The circular HPV genome has approximately 7900 base pairs, in the schematic with eight overlapping reading frames. L1 is signaled as the most used genomic target for detection of HPV.

less than 35 years old is particularly significant. The possible causes that result in cervical cancer to tend to occur in young include remarkable increase of HPV infection rate, especially the high-risk HPV 16 and 18 infection closely related to cervical cancer [91]. The primary screening and diagnostic methods have been cytology and histology. Papanicolaou (Pap) staining is the gold standard for detecting abnormal cervical epithelial cells, using microscopic analyses of conventional cervical smears or cell suspensions from liquid cytology medium. The limitation of Pap smear are low specificity, is need repeat the screening at short intervals [92]. Morphological findings from a cytology analysis determine the level of risk for developing cervical malignancies. Cervical epithelial cells atypical or abnormal are known as “atypical squamous cells of undetermined significance” (ASCUS). Some ASCUS signals the presence of low-grade squamous intraepithelial lesions (LSIL). However, some ASCUS are associated with underlying high-grade disease, including cervical intraepithelial neoplasia (CIN) [93-95]. HPV serology is not used diagnostically. Detection of the humoral antibody response is type-specific and first detected 6–18 months after infection. The response is weak and only between 50–60% of patients positive to HPV DNA-positive mount a measurable antibody response [96]. Although, the role played by the humoral immune response during the HPV infection is not very well understood; it was observed that this response is generated all throughout the malignant process; 54%-69% of women with incident HPV 16, 6, or 18 infections had antibodies. Serum antibodies against many different viral products of HPV have been detected by ELISA. The best characterized and most type-specific antibodies are those directed against conformational epitopes of the L1 capsid protein assembled as VLPs (virus-like particles) such as HPV-16 L1 VLP (virus-like particle)-based ELISA [97]. There is some high-throughput single-serum-dilution enzyme-linked immunoassay (ELISA) system for determining anti-HPV antibody titers following vaccination against HPV [98].

At present there is no “gold standard” for HPV detection. Guidelines for the management of women with cervical neoplasia or abnormal cervical cancer screening tests indicates the immediate colposcopy (cervical exam), cytologic follow-up, and triage by HPV DNA testing

[99]. HPV serves as paradigm for the use of NAATs due to how difficult it is to obtain the virus via cell cultures or to develop indirect diagnosis techniques [100]. There are several molecular techniques for HPV DNA detection (Table 3). They include DNA hybridization, PCR-RFLP, reverse-line hybridization and hybrid capture assay. The method most commonly used is the polymerase chain reaction (PCR). All target amplification techniques such as PCR for HPV virus detection currently use consensus or type-specific primers group-or type-specific conventional PCR, to amplify a broad-spectrum of HPV genotypes by targeting a conserved region within the HPV genome [101, 102]. L1 and E1 regions are the most conserved parts of the genome [103]. Many assays use primers targeted to the viral capsid L1 gene, which can detect numerous HPV types [104]. There are several PCR primer sets as GP5+/6+ that amplify a 140 bp region in the L1 gene allowing the identification of 30 HPV genotypes. This method is useful in predicting high-grade cervical intraepithelial neoplasia. The MY09/11 system identifies high-risk HPV genotypes by amplifying a 450 bp sequence in the conserved L1 region. The MY09/11 primer set uses degenerate bases to reduce variability due to different genotypes. The SPF10 system primers amplify a 65 bp sequence from a highly conserved region of the viral L1 gene for the identification of 16 different genotypes of the human papillomavirus (HPV) [105]. Commonly used L1 consensus primer sets include PGMY09/11, GP5+/6+, and SPF10, having the ability to identify a large range of HPV types with 1 amplification [106, 107]. Hybridization on PCR products (Cervista HPV HR Test, INNO-LiPa HPV Genotyping, Linear Array HPV Genotyping Test, Digene HPV Genotyping RH Test) [Estrade et al., 2011; Jeney et al., 2007; Chan et al., 2012]. Linear Array HPV Genotyping Test (Roche Diagnostics, Indianapolis, IN) is able to identify 37 types of HPV, 14 are high-risk genotypes. Linear Array also includes PGMY primers and is a commonly used method for genotyping HPV using Probes for multiple HPV types are fixed on a membrane strip, and the PCR product is hybridized to the strip, followed by visual detection [108]. Other assay so used is INNO-LiPA HPV Genotyping Extra (Innogenetics, Ghent, Belgium). This kit amplifies HPV DNA with SPF10 primers at the L1 region. The probes are fixed to membrane strips in sequence-specific lines and visualized as purple/brown bands. The test can detect and distinguish 24 low-and high-risk HPV types. [INNO-LiPA HPV Genotyping Extra. Ghent, Belgium: Innogenetics; 2007. A HPV viral target to detect is the L1 gene to amplify a broad spectrum of HPV types with a single set of degenerated primers or a cocktail of primers (Amplicor HPV Test) [Sepehr et al., 2012] and HPV mRNA amplification against HPV E6/E7 mRNA (APTIMA HPV Assay, NucliSENS EasyQ HPV, PreTect HPV-Proofer). Another test is the Multiplex Genotyping Kit (Multimetrix, Heidelberg, Germany). The test is a PCR-based fluorescent bead array that can detect 24 low-and high-risk HPV types [109]. The quadruplex quantitative PCR method (AllGlo fluorescent probes) was established to simultaneously detect and differentiate HPV 6, 11, 16 and 18 [110]. Between the signal amplification methods are liquid-phase or in situ hybridization as Hybrid Capture 2 HPV DNA Test for to detect 13 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and five low-risk types [111, 112]. Microarray on PCR products is other molecular technique to detect influenza virus such as Infiniti HPV-HR QUAD Assay, PapilloCheck or HybriMax for HPV genotyping [113]. PapilloCheck (Greiner Bio-One, Monroe, NC) for HPV genotyping PapilloCheck identifies 24 types of low-and high-risk HPV with a high specificity and sensitivity [114]. Genotyping with this method is based

on PCR amplification of the E1 gene by a group of new E1-specific primers, followed by hybridization to a DNA chip with immobilized HPV oligoprobes. A novel assay for molecular diagnostics and typing application known as Sequencing Bead Array (SBA) is an alternative method to HPV diagnosis. SBA is a digital suspension array using Next-Generation Sequencing (NGS) that in the case of HPV could distinguish ten Human Papillomavirus (HPV) genotypes associated with cervical cancer progression. This is a robust system capable to identify genetic signatures or single nucleotide polymorphisms (SNPs). SBA has the potential to change the probe-based diagnostics, and allow for a transition towards the technology by genomic sequencing [115]. Some groups are studying other molecular factors as possible markers of infection by HPV as a complementary diagnostics. The overexpression of the HPV E6 and E7 genes is indicated in HPV-induced carcinogenesis, making these genes a potential measure of virulence. Monitoring the expression levels of these genes may allow for screening and monitoring of cancer progression [116].

MOLECULAR DIAGNOSIS METHOD	NAME TEST	GENOTYPES AND TARGET DETECTED
Real time	Real Time High risk HPV test (Abbott Molecular)	14 high risk HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and to partially genotype 16, 18 from other 12 high risk genotypes using L1 gene.
	Cobas 4800 HPV Test Amplification/Detection kit (Roche Molecular Diagnostics)	HPV 16 and HPV 18 and concurrently detecting the rest of the high risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), using L1 gene
Hybrid capture	Hybrid Capture 2 High-Risk HPV DNA Test	High-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 using all genome.
PCR and hybridization	AMPLICOR Human Papillomavirus Test (Roche Molecular Diagnostics)	HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 using L1 gene.
TMA	APTIMA HPV assay (Hologic)	Qualitative detection of E6/E7 viral messenger RNA (mRNA) from 14 high-risk types of HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.
Reverse hybridization	Digene HPV Genotyping RH Test (Qiagen)	Detection of HPV subtypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 using L1 gene.
	INNO-LiPA HPV Genotyping Extra (Innogenetics)	Detection of at least 54 HPV types using L1 gene.
	Linear Array HPV Genotyping test (Roche Molecular Diagnostics)	Linear Array detects thirty seven anogenital HPV DNA genotypes 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66,

MOLECULAR DIAGNOSIS METHOD	NAME TEST	GENOTYPES AND TARGET DETECTED
		67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82, 83, 84, IS39 and CP6108.
Invader chemistry	Cervista HPV HR Test (Hologic)	Detection of 14-type High Risk HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 using L1 gene.
PCR-microarray	PapilloCheck HPV-Screening Test (Greiner Bio-One)	Type-specific identification of 18 high-risk(16,18, 31,33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82) and 6 low-risk types of HPV (6, 11, 40, 42, 43, 44) using E1 gene.
	Clart HPV 2 (Genomics)	Identification of HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85 and 89 genotypes using L1 gene.
	Infiniti HPV Genotyping assay (AutoGenomics)	Genotyping of HPV 6, 11, 16, 18, 26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82 and 85 using E1 gene.
NASBA amplification and real-time detection using molecular beacon probes	NucliSENS EasyQ HPV (BioMérieux)	Discriminates the E6/E7 mRNA between genotypes 16, 18, 31, 33 and 45.
Next-Generation assay	NextGen HPV Screening Assay and Platform (Qiagen)	15 high risk subtypes (+ types 66 and 82)

Information based on Kroupis C, et al., 2011 [90]; Arney A, et al., 2010 [100].

**Table 3.** Principal methods of molecular diagnosis of HPV.

Analysis of the host factor p16INK4a (p16), a cyclin-dependent kinase inhibitor could be a molecular marker to HPV infection. The increase of p16 indicates removal of the negative feedback control supplied by the retinoblastoma gene, pRB. When oncogenic HPV E7 proteins bind to pRB, p16 is overexpressed and elevated, representing active expression of HPV oncogenes [117]. The sialylation modification observed during oncogenic transformation, tumor metastases and invasion, has been associated with enhanced sialyltransferases (STs) transcription such as ST3Gal III, ST3Gal IV and ST6Gal I in CIN [118].

5. Viral hepatitis

Viral hepatitis is a necroinflammatory liver disease of variable severity. Persistent infection by HBV is often associated with chronic liver disease that can lead to the development of cirrosis

and hepatocellular carcinoma being a global public health as chronic diseases, cause of infectious disease mortality globally, each year causing approximately 1.4 million deaths. In addition, viral hepatitis are cause of liver cancer and the most common reason for liver transplantation [119]. The viruses more common are Hepatitis A, Hepatitis B, and Hepatitis C. Approximately 400 million persons living with chronic hepatitis B virus (HBV) or hepatitis C virus infection who die from cirrhosis or liver cancer, years and decades after of their infection [120]. Hepatitis B is caused by infection with HBV, which may lead to acute or chronic hepatitis. HBV is the 9th leading cause of death worldwide. It causes cirrhosis, liver failure and hepatocellular carcinoma. HBV is a small nonenveloped DNA virus that is a member of the Hepadnaviridae family, HBV contains a 3.2-kb partially double-stranded DNA genome with 4 open reading frames encoding 7 proteins (P/viral polymerase, S/Surface antigen proteins/HBsAg, C/core protein, HBeAg y X/HBx protein) [121]. Eight genotypes of HBV (designated-H) have been identified by sequence divergence of >8% over the entire genome of HBV DNA [122]. HBV is efficiently transmitted by percutaneous or mucous membrane exposure to infectious blood or body fluids that contain blood. Acute HBV infection can be either asymptomatic or symptomatic. Symptoms in acute HBV infection are clinically indistinguishable from those in other acute viral hepatitis infections [123]. Diagnosis of hepatic viral infection is carried out by studying biochemical as liver function tests, serologic assays and histological parameters. Serological HBV diagnosis identifies virally-encoded antigens and their corresponding antibodies in serum. Three clinical useful antigen-antibody systems have been identified for hepatitis B: hepatitis B surface antigen (HBsAg) and antibody to HBsAg (anti-HBs), antibody (anti-HBc IgM and anti-HBc IgG) to hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg) and antibody to HBeAg (anti-HBe) [124]. B surface antigen (HBsAg) is the first serological marker to appear during the course of HBV infection, is present in both acute and chronic infection. The immunoassays to detect HBsAg are highly specific and show a sensitivity, allowing the detection of <0.15 ng/ml of HbsAg [125]. Detection of HBsAg allowed for the first time screening of inapparently infected blood donors for a dangerous pathogen. The simultaneous detection of antibodies against HBsAg (anti-HBs) and HBcAg (anti-HBc) can be useful to know the evolution of disease. The presence of IgM antibody to hepatitis B core antigen (IgM anti-HBc) is diagnostic of acute or recently acquired HBV infection. Antibody to HBsAg (anti-HBs) is produced after a resolved infection and is the only HBV antibody marker present after immunization. The presence of HBsAg and total anti-HBc, with a negative test for IgM anti-HBc, indicates chronic HBV infection. The presence of anti-HBc alone might indicate a false-positive result or acute, resolved, or chronic infection [126, 127]. Chronic HBV infection is defined by the persistence of serum HBsAg for more than 6 months [128]. Serologic assays for HBV are the mainstay diagnostic tools for HBV infection. The clinically silent HBV infections are a strong driving force in the development of modern virus diagnostics to analyze the HBV replication profile, HBV DNA levels and the viral protein expression [129]. When serological testing could be inconclusive for the diagnosis of a HBV infection (due to the presence of genetic variants of HBV), the molecular detection of HBV DNA may help to resolve the uncertainties. The NAT assays in plasma or serum can detect to 10 copies/mL [130]. The viral genomes can be detected and quantified by polymerase chain reaction (PCR), transcription-mediated amplification (TMA), and with signal amplification



methods such as hybrid-capture and the branched DNA assay. Quantification of genome of HBV can be used for diagnosing HBV infection and monitoring the effect of antiviral therapy. HBV DNA is the earliest detectable marker in acute HBV infection and the gold standard for the diagnosis of occult HBV infection. HBV DNA testing is particularly useful in the detection of the early phase of acute HBV infection prior to the appearance of serum HBsAg as well as in occult HBV infection [131, 132]. Several commercial molecular assays have been developed for quantitation of HBV DNA. Such as COBAS Amplicor HBV Monitor, which is based on the amplification of DNA targets by PCR with HBV-specific primers. Between the hybridizations methods are Hybrid capture Ultrasensitive hybrid capture II and Branched DNA VERSANT hepatitis B virus DNA 3.0. Recently developed real-time target amplification methods have improved viral genome detection and quantification for clinical and research purposes. Real time PCR for HBV DNA has reached an excellent level of performance with a detection limit close to the theoretical minimum of 1 DNA molecule per reaction mix and a huge dynamic range up to  $10^7$  or more. In 1991 the WHO introduced International Standard preparations and an arbitrary International Unit (IU) of HBV DNA. The number of molecules per IU depends on the assay; but typically 5 molecules correspond to one IU HBV DNA [133]. Real-Time PCR (Real Time Abbott PCR, Smart HBVTM, Real Art HBV, COBAS AmPliprep, Cobas TaqMan HBV, Aptima HBV Quantitative assay) [134-136]. Fosun real-time PCR HBV kit is a commercial assay for quantitation of serum HBV DNA based on TaqMan PCR technology, which is useful for monitoring HBV DNA levels in patients with chronic hepatitis B. The limit of the duplex real-time PCR assay was 29.5 IU/ml, whereas the specificity was 100% for the detection of HBV DNA [137]. A trial has been tested, a TaqMan locked nucleic acid (LNA) real-time polymerase chain reaction (PCR) probe for the accurate quantification and detection of hepatitis B virus (HBV) DNA in serum (plasma) [138]. The genotyping analysis of HBV can be performed by real-time PCR using (GQ-PCR) method or the direct sequencing and reverse hybridization with INNO-LiPA HBV genotyping assay [139]. Other option to diagnostics of hepatotropic viruses is Real-time PCR array, useful in the rapid detection of multiple viral pathogens, between them hepatitis B virus (HBV), hepatitis C virus (HCV) using the SYBR Green chemistry. The array detected: 10 genome equivalents (geq)/ml of HCV, 50 geq of HBV (genotype A) [140]. Micro-RNAs (miRNAs) are noncoding RNAs that regulate gene expression primarily at the post-transcriptional level by binding to mRNAs. The circulating miRNA in serum or plasma might be a very useful biomarker for the diagnosis and prognosis of HBV-related diseases, indicating a promising future in the treatment of HBV-related diseases [141]. Hepatitis C virus (HCV) is a major public health problem and a leading cause of chronic liver disease. An estimated 180 million people are infected worldwide, several of these patients go on to develop chronic HCV infection, often developing into liver cirrhosis, hepatic failure and hepatocellular carcinoma [142]. Hepatitis C virus is a single stranded RNA, enveloped virus, belongs to the Flaviviridae family and is the only member of the Hepacivirus genus [143]. The commercially-available diagnostic tests are based on enzyme immunosorbent assays (EIA) for the detection of HCV-specific antibodies and recombinant immunoblot assays (RIBA). Although, in the diagnosis of influenza is applied the algorithm showed in figure 5 [144-146]. Testing for circulating HCV by genomic sequence amplification (PCR and branched DNA assay) has been successfully utilized for confirmation of serological results and the effectiveness of antiviral therapy [134,

147]. An alternative to HCV diagnosis is Loop-mediated isothermal amplification (LAMP) assay for rapid detection of HCV genomic RNA [148]. The molecular HCV assays includes to RT-PCR (AmpliScreen 2.0, Amplicor HCV 2.0, Cobas Amplicor Monitor HCV 2.0, Versant HCV RNA, Procleix HIV/HCV assay, Procleix Ultrio assay). The methods for accurate quantification of HCV RNA levels are key tools in the clinical management of patients. The HCV RNA Assay by RT-PCR includes to Amplicor HCV Monitor 2.0, Cobas Amplicor Monitor HCV 2.0, Cobas AmliPrep/Cobas TaqMan HCV, Versant HCV RNA 3.0 Quantitative assay, LCx HCV RNA Quantitative assay, SuperQuant, Abbott RealTime [135]. To evaluate the response to antiviral therapies is possible analyze the absence or alteration of genetic material in clinical specimens from successfully treated patients. In situ hybridization (ISH) enables visualization of specific nucleic acid in morphologically preserved cells and tissue sections. The anti-sense probe detected HCV RNA, with a sensitivity and specificity of 95% and 100%, respectively. HCV genomic RNA can be variably distributed in tissue sections and was located primarily in the perinuclear regions in hepatocytes [149]. Viral hepatitis is one of the major health problems worldwide. Hepatitis delta virus (HDV) is also not uncommon world-wide. Hepatitis D virus (HDV) or delta virus is a defective virus. It requires the help of another virus that is hepatitis B virus for its multiplication. It always occur with HBV either in the form of co-infection or super-infection [150]. HCV, HBV, and HDV share parallel routes of transmission due to which dual or triple viral infection can occur in a proportion of patients at the same time. HBV and HCV are important factors in the development of liver cirrhosis (LC) and hepatocellular carcinoma [151]. In the diagnosis of co-infections, individually each infection (HBV, HCV and HDV) is confirmed by the presence of the serum surface antigen, hepatitis B, C and D envelope antigen and specific antibodies to the hepatitis B, C and D core [152]. By Deep Sequencing were readily detected at high coverage in plasma of patients with chronic viral hepatitis B and C. Although, this protocol also is adapted to other samples such as urine, bile, saliva and other body fluids by viral metagenomic survey [153]. For other side, other molecular technique as the protein micro-array gives a way to diagnosis multiple viral infections; using two viral antigens (HBsAg, HBeAg) and seven viral antibodies (HBsAb, HBcAb, HBeAb, HCVAb, HDVAb, HEVAb, HGVAb) of human hepatitis viruses [154]. Finally, the successful simultaneous detection of HAV, HBV and HCV was performed with the Magicplex HepaTrio Real-time Detection test, whose sensitivity and specificity of the Hepa-Trio test were 93.8% and 98.2%, respectively, for detecting HBV infection, and 99.1% and 100.0%, respectively, for HCV infection [155].

## 6. Mumps virus

Mumps is an acute viral infection caused by a member of the *Rubulavirus* genus in the *Paramyxoviridae* family. Mumps virus (MuV) is pleomorphic, enveloped, comprising a nucleocapsid core with helical structure composed of the 15384 nucleotide. Nonsegmented negative-sense RNA genome has the organization: 3'-NP-P-M-F-SH-HN-L-5' [156]. The molecular epidemiology of MuV is characterized by the co-existence of 10 (or more) distinct genotypes named A-J based on the nucleotide sequence of the SH gene. MuV genotypes (C,

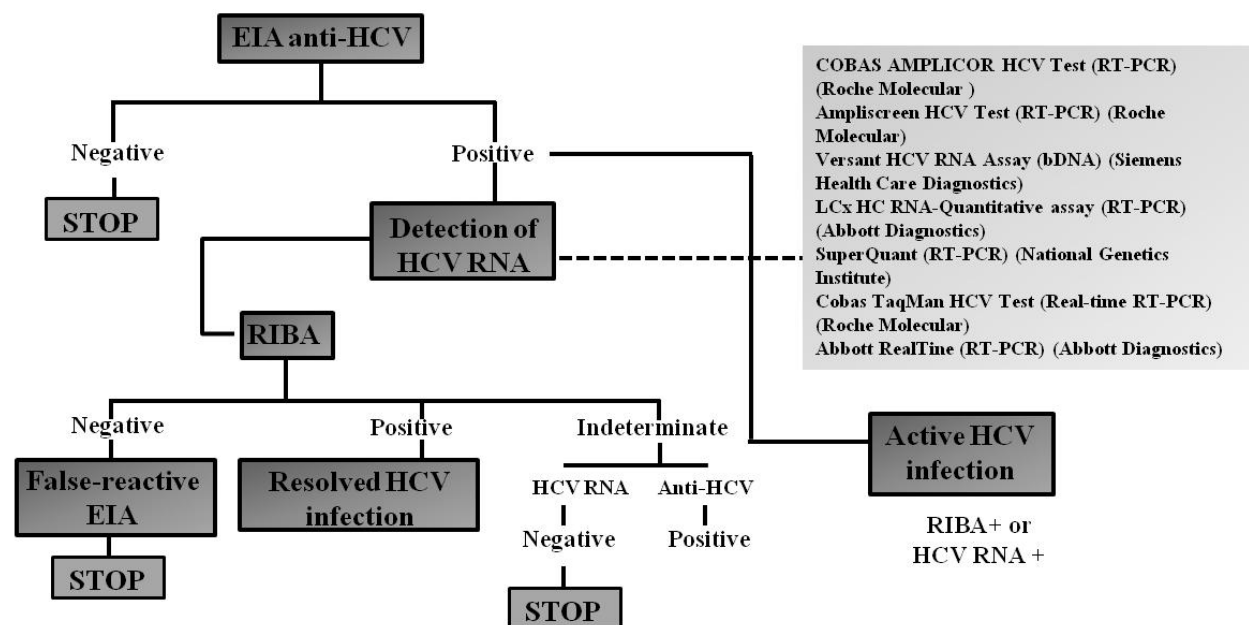


Figure 5. HCV Testing Algorithm and molecular assays.

D, H, J) and vaccine strains (Urabe Am9) have been associated with enhanced neurovirulence [157]. MuV is known to affect the salivary glands causing parotid swelling lasting at least two days, but may persist longer than ten days [158]. The mumps incubation period ranges from 12–25 days, but parotitis typically develops 16 to 18 days after exposure to mumps virus. The MuV also produce an acute systemic infection involving glandular, lymphoid and nervous tissues, leading to some important complications, the main central nervous system (CNS) complication of mumps virus infection is aseptic meningitis (in up to 15% of cases) [159, 160, 161]. Mumps epidemics are usually caused by airborne transmission of mumps virus (MuV) and have high morbidity in non-immunized children. Massive vaccination programs have decreased the incidence of MuV infection worldwide. The annual incidence of mumps in the absence of immunization was in the range of 100–1000 cases/100 000 people, outbreaks have not been completely eliminated even in populations with high vaccination coverage [162]. Laboratory confirmation of mumps infection can be made by the detection of immunoglobulin M (IgM) antibodies specific to mumps virus in acute-phase serum samples (gold standard for mumps diagnosis). Mumps virus can be detected from fluid collected from the parotid duct, other affected salivary gland ducts, the throat, from urine, and from cerebrospinal fluid (CSF). Parotid duct swabs yield the best viral sample. With previous contact with mumps virus either through vaccination or natural infection, serum mumps IgM test results may be negative; IgG test results may be positive at the initial blood draw. The serologic tests available for laboratory confirmation of mumps acute infection and confirmation of previous exposure to mumps vary among laboratories. Tests for IgM antibody includes Enzyme Immunoassay (EIA): a highly specific test for diagnosing acute mumps infection and Immunofluorescence assay (IFA) a test that is relatively inexpensive and simple, but the test is particularly susceptible to interference by high levels of mumps-specific IgG. A significant rise in IgG antibody titer, in acute-and convalescent-phase serum specimens is a positive result of infection. The presence of mumps-

specific IgG, detected using a serologic assay (EIA or IFA), is considered evidence of mumps immunity but does not predict the presence of neutralizing antibodies or protection from mumps disease [163]. A near patient test (NPT) for the detection of mumps-specific IgM in oral fluid specimens was developed and evaluated using 196 oral fluid specimens from suspected cases of mumps and measles. Compared to EIA, the sensitivity, specificity, positive and negative predictive value of the mumps IgM NPT were 79.5%, 100%, 100%, and 72.6%, respectively. Mumps IgM NPT is rapid and simple to perform for confirmation of a clinical diagnosis. The NPT strip is also a suitable matrix for preserving nucleic acid, enabling virus-specific RT-PCR to be performed [164]. Standard diagnostics that detect virus or virus-specific antibody are dependable for confirming primary mumps infection in immunologically naïve persons, but these methods perform inconsistently for individuals with prior immune exposure. Detection of activated mumps-specific antibody-secreting B cells (ASCs) by an enzyme-linked immunospot (ELISPOT) assay has the potential for use as an alternative method of diagnosis when suspect cases cannot be confirmed by detection of IgM or virus. The mumps-specific memory B cells are detected at a much lower frequency than measles-or rubella-specific cells, suggesting that mumps infection may not generate robust B-cell memory [165]. Successful virus isolation by cell culture should always be confirmed by immunofluorescence with a mumps-specific monoclonal antibody or by molecular techniques such as RT-PCR, nested-PCR or real-time RT-PCR [166-168]. In patients vaccinated, pre-existing vaccine-induced antibodies. Moreover, acute infection has to be demonstrated by direct detection of the virus by viral isolation or genomic amplification. RT-PCR allows a diagnosis and also forms the basis for genotype characterization by sequencing the SH gene, useful tool for mumps surveillance, management and control, according to WHO recommendations. Virus genotyping allows the building of a sequence database that will help to know transmission pathways of mumps strains circulating in the world and to distinguish wild-type mumps virus from vaccine virus [169]. Standard RT-PCR to detect the SH gene of mumps virus can be used to detect mumps RNA in clinical samples or in infected cell culture [170]. The sequence of the PCR product containing the coding region of the SH gene can be used to determine the viral genotype [171]. A multiplex real-time RT-PCR test for the detection of RNA from mumps virus in patient specimens, using primers and probes that target the mumps SH gene [172]. Several test of real-time RT-PCR are performed with TaqMan Assay [173]. A multiplex real-time RT-PCR assay, for rapid mumps diagnosis in a clinical setting. The assay used oligonucleotide primers and a TaqMan probe targeting the mumps SH gene. This test showed 100% correlation with results from viral culture. [172]. Other qRT-PCR assay is directed to the mumps virus F gene [174]. Recently, by RT-PCR it was detect a case of corneal endothelitis following the mumps parotitis [175].

## 7. Measles virus

Measles produces a highly contagious respiratory infection and may cause extensive epidemics. Measles is one of the most important causes of child morbidity and mortality. [176]. A safe and efficacious live attenuated virus vaccine is available toward global measles control [177,



178]. Measles vaccination is highly effective, safe and relatively cost-effective and has interrupted measles transmission in most parts of the world [179]. Despite tremendous achievements towards global measles mortality reduction and measles elimination goals, globally, in 2010, there were 327305 measles cases reported and an estimated 139300 measles deaths) [180, 181]. Mortality is highest in children and most acute measles deaths are due to secondary infections that result from a poorly understood measles-induced suppression of immune responses [182]. In addition to the risks of acute infection, children under the age of 2 years are also vulnerable to development of subacute sclerosing panencephalitis (SSPE), a progressive, uniformly fatal neurologic disease associated with persistent measles virus infection of the nervous system, have documented high levels of antibody to measles virus [183]. Other diseases related to measles are systemic lupus erythematosus [184] and multiple sclerosis [185]. The measles virus is a member of the Morbillivirus genus of the family Paramyxoviridae. The virions are pleomorphic and range in size from 100 to 300 nm. The measles virus is antigenically stable and genetic differences are few among vaccine strains. Wild-type viruses are more variable. Several different genotypes of wild measles virus are currently circulating worldwide and this genetic variation provides the basis for the application of molecular epidemiological techniques to study the transmission of measles virus [186]. Laboratory confirmation of clinically diagnosed measles was traditionally based on methods such as immunofluorescence antibody for detection of viral antigen and haemagglutination inhibition (HI), haemolysin inhibition, complement fixation, and plaque-reduction neutralization (PRN) for detection of measles antibody in serum (Table 4) [187]. Currently, detection of measles-specific IgM antibody and measles RNA by real-time RT-PCR are the most common methods for confirmation of measles infection [168]. Initially laboratory confirmation of cases of measles infection is performed by detection of measles-specific immunoglobulin M (IgM) antibodies, the test of choice for rapid diagnosis of measles cases in a single serum specimen collected within the first few days of rash onset, usually become detectable in serum after four days post onset of rash (90-100% sensitivity) and decline rapidly after one month. The virus can be detected in serum samples, blood spots, throat swab (or nasopharyngeal swab), urine and or collection of both respiratory and urine samples that can increase the likelihood of detecting virus. [188]. Oral fluids can also be used to detect viral ribonucleic acid (RNA) [189]. The enzyme immunoassay is the most commonly used method for detecting measles-specific IgM or IgG antibodies. Both capture and indirect formats for IgM detection are available commercially. Some tests available are LIAISON IgM measles (DiaSorin, Saluggia, Italy) a new automated chemiluminescence immunoassay and the enzyme immunoassay (EIA) Enzygnost (Siemens, Marburg, Germany), which have a sensitivity of 93.7% and 98.8%, whereas the specificity was 96.8% and 97.9%, respectively [190]. DiaSorin Liaison (Saluggia, Italy) is other option for measles diagnosis, with a sensitivity and specificity for measles IgM of 92% and 100% respectively [191]. Commercially, there are some test directs to simultaneous detection of measles and rubella as Enzygnost (Siemens) and Platelia (Bio-Rad), useful for detecting IgM against measles and rubella [192].

In acute, uncomplicated measles, there is a significant rise in measles-specific IgG antibodies between acute-and convalescent-phase serum specimens. A positive test result for specific IgG antibodies in a serum indicates past infection with measles virus or measles vaccination, but



does not ensure protection from infection or re-infection. Screening the young adult population about to enter college or the military, pregnant women, and other individuals at risk, for seropositivity, is a valuable tool for determining their immune status.

LABORATORY TEST
Serological assays
Using serum sample
Detection of IgM antibody by indirect IgM ELISA, IgM-capture ELISA or EIA for IgM
Detection of IgG antibody by IgG ELISA
Virus neutralization test
HI
Cell culture and immunofluorescence microscopy
Using urine, nasopharyngeal swabs or blood lymphocytes and detect to virus using specific measles antibody
NAT analysis
measles RNA can be detected from nasopharyngeal swab, urine or peripheral blood lymphocytes (oral fluid/ throat swabs) up to 5 days post disease manifestation.
RT-PCR, RT-nested PCR, Real-Time RT-PCR and sequencing

**Table 4.** Laboratory diagnosis for measles in clinical materials.

Enzyme-linked immunosorbent assay (ELISA) is normally used to quantify the amount of serum IgG antibodies against measles (measles in addition to mumps, rubella, and varicella-zoster virus, MMRV). However, a multiplex immunoassay for the simultaneous detection of antibodies against MMRV showed be a good alternative to conventional ELISAs and suitable for use in serosurveillance and vaccine studies [193]. An enzyme linked Immunosorbent commercial assay is Captia Measles IgG based (Trinity, Biotech, USA). In countries where disease prevalence is low, intensified surveillance typically implemented during and after an importation will result in some false positive IgM results since no assay is 100% specific. So, it is necessary to introduce other techniques of diagnosis related to ARN genome. In the Fifth Hands-on Training on the Laboratory Diagnosis of Measles and Rubella focusing on Molecular Detection and Sequence Analysis, in Hong Kong, China. It was exposed the need of introduce the molecular detection of measles and rubella viruses using new real-time polymerase chain reaction (PCR) as well as conventional PCR, quantitative real-time PCR and others molecular techniques such as sequencing. Dr Paul Rota (Chief, Measles Virus Section, Centers for Disease Control and Prevention USA), presented on the use of real-time and conventional RT-PCR for case classification and molecular surveillance of measles virus. These tests can detect 10-100 copies of RNA/sample in a high throughput format and produce results within two hours. It can help to confirm a case when serologic results are inconclusive but negative results do not

rule out a case. These molecular tests are more sensitive than conventional (endpoint) RT-PCR. Sequence information from the conventional PCR is required for genotype assignment and confirmation of vaccine reactions [194].

Measles RNA can be detected (oral fluid/ throat swabs) up to 5 days post disease manifestation. Molecular assays to measles virus includes measles H (haemagglutinin) gene real-time PCR and hybridization [195] and nucleic acid sequence analysis of the nested N-gene PCR amplicons (nucleocapsid) [196, 197]. A nested reverse transcriptase PCR (RT-PCR) that detected measles virus (MV) from dried filter papers was set up using MV infected cells diluted in sterile phosphate-buffered saline. Although, the nested RT-PCR results of low titer viruses dried onto filter papers are not reproducible and reliable [198, 199]. Detection of RNA in PBMC by RT-PCR is the most effective method for diagnosis of measles. A study performed in 2010, analyzed sixty-three throat swabs, 84 peripheral blood mononuclear cell (PBMC) samples, and 85 plasma samples were collected from 85 cases of suspected measles. The percentage of positive results from PBMC by RT-PCR and virus isolation was 100 and 91.7%, respectively. The percentage of positive results from throat swabs by RT-PCR and virus isolation was 91.2 and 52.8%, respectively [200]. Measles can produce congenital infections with a risk of neurological complications in the newborn. Mother-to-child transmission of the measles virus, it has been widely documented in the newborns either by RT-PCR in saliva or by IgM detection in blood. An early viral RT-PCR detection allows successful immunoglobulin prophylaxis in one newborn avoiding the development classical or neurological clinical signs of measles infection [201]. Molecular detection of measles virus has been optimized by amplification of nucleocapsid (N) and human RNase P mRNA for a one-step quantitative reverse transcription (qRT)-PCR [202]. The qRT-PCR for measles diagnosis can use SYBR Green or TaqMan (ABI) in real-time reverse transcription-polymerase chain reaction (RT-PCR) assays. For the real-time RT-PCR, primer sets are design from a region of the MV H gene of the Edmonston strain (genotype A) and a TaqMan probe specific for the H gene of genotype D MV [203]. Currently, is possible estimate the titer of measles, mumps and rubella (MMR) viruses by a TaqMan-based real-time reverse transcription-polymerase chain reaction (qPCR-RT) assay optimized in infected cell culture supernatants [204]. For genetic typing of measles virus in clinical samples is xMAP technology that employs specific oligonucleotide probes of genotypes D4, D6 and D7 of virus [205]. Other alternative employed for the genotype analysis of measles virus is sequencing of the 450 nucleotide of nucleoprotein gene (N450) that contributes to the genetic characterization of wild-type measles viruses and offers data in the study of viral transmission pathways. N450 is amplifying with the primer pair, MeV216/MeV214. It is clear that the molecular tools improve the molecular characterization of circulating measles viruses globally and provides enhanced quality control measures [206]. Demonstration of the usefulness of molecular tests in the diagnosis of measles during outbreaks or epidemic peaks was reported in an outbreak in Paris (France) in 2011. 171 oral fluid samples and 235 serum samples collected from 270 patients were tested using a novel one-step real-time RT-PCR assay. This study showed that the detection rate of MV-RNA by RT-PCR was 98% (100/102) for oral fluid and 95% (97/102) for serum samples. The detection rate of MV-IgM was 85% (87/102). In addition, it was found than during the early stage of infection, the MV-RNA viral load in serum was lower in patient's positive than in those negative for MV-IgG [207]. The detection and identification of the virus in cell culture may take

several weeks. The samples should undergo virus isolation on sensitive cell lines, such as B95a cells. Measles virus can be isolated from clinical specimens, including; throat swab, conjunctival swabs, nasopharyngeal aspirates or urine [208].

## 8. HIV

Acquired Immunodeficiency Syndrome (AIDS) is one of the most critically acclaimed endemic diseases, caused by two lentiviruses HIV-1 and 2. Human immunodeficiency virus (HIV) is the pathogen causing the acquired immunodeficiency syndrome (AIDS) [209, 210]. HIV is an enveloped virus with tropism for CD4+lymphocytes and monocytes. HIV is classified in the family *Retroviridae*, subfamily *Lentivirinae*, and genus *Lentivirus*. HIV comprising a single stranded, positive-sense ribonucleic acid (RNA) genome of about 9.7 kilobases. From the 5' end of genome are located the three genes that characterize retroviruses: *gag-pol-env*. The *gag* gene codes for the internal structural proteins, the *pol* gene for the three viral enzymes, and the *env* gene for the envelope glycoproteins. LTR (Long Terminal Repeat) sequences are found at each extremity of the genome, containing the signals for the regulation of expression of the viral genes. The genome also has six additional genes called "accessory" genes: *vif*, *nef*, *vpr*, *tat*, *rev* and *vpu* (HIV-1) or *vpx* (HIV-2) [211]. There are two strands of HIV RNA and each strand has a copy with nine genes, which encode 15 proteins. The RNA is surrounding by a cone-shaped capsid which consists of approximately 2000 copies of the p24 viral protein. Surrounding the capsid is the viral envelope. Each envelope subunit consists of two non-covalently linked membrane proteins: glycoprotein (gp) 120, the outer envelope protein, and gp41, the transmembrane protein that anchors the glycoprotein complex to the surface of the virion. The envelope protein is the most variable component of HIV, although gp120 itself is structurally divided into highly variable (V) and more constant (C) regions. The variability of the HIV envelope also confers a uniquely complex antigenic diversity. The virion contains three enzymes necessary for multiplication: reverse transcriptase (enables the viral RNA to be transcribed into DNA), and endonuclease (enables the DNA to be integrated into the host cell, the viral genome then becomes proviral DNA) and the protease (enables the virus to mature at a late stage in the cycle of intracellular multiplication). The presence of two copies of the retroviral genome in each particle promotes genetic recombination between the RNAs. This and the high error rate of the viral reverse transcriptase leads to considerable genetic variation in the viral progeny. AIDS is characterized by the selective targeting of the CD4+/CD8+T cells by HIV which fatally impairs the immune system. The window period for this retrovirus is from several weeks to few months altogether before detection of earliest antibodies in blood serum raised against HIV [210]. HIV infection is one of the major threats to human health due to the lack of relevant vaccine and drugs to cure AIDS. Its early diagnosis is thus important in controlling HIV transmission. With acute HIV infection, high levels of infectious virus are detectable in serum and genital secretions. The rate of transmission during acute HIV infection is higher than the established HIV infection, for this reason, new HIV testing strategies need to focus on sensitivity, especially for this highly contagious phase immediately after infection. There are two types of virus, HIV-1 and HIV-2, which are further divided into groups and/or

subtypes. The pandemic is caused by HIV-1 group M. HIV-1 and HIV-2 have differences as clinical progression of the disease is slower and mother-to-child transmission is less likely with HIV-2 than with HIV-1 (maternal-fetal transmission < 2% in the absence of treatment). Recombinant HIV strains are known as CRF (Circulating Recombinant Forms). Although HIV-2 is also associated with AIDS, it is not transmitted as readily and, generally speaking, progression toward immunodeficiency is much slower in individuals with an HIV-2 infection. HIV-1 is responsible for a chronic infection that gradually develops and causes the destruction of the body's CD4<sup>+</sup>T lymphocytes. HIV-1 is responsible for a chronic infection that gradually causes the destruction of CD4<sup>+</sup>T lymphocytes [212]. To detect a HIV infection several tests are used to analyze the HIV infection status of a patient, evaluate the progression of disease, and monitor the effectiveness of antiretroviral therapy (ART). HIV infection can be diagnosed by direct visualization of virions or electron microscopy; cultivation by lymphocyte culture; measurement of HIV-specific serologic responses; detection of viral antigens; and detection of viral nucleic acids [213]. For many years, laboratory diagnosis of HIV is based on the identification of HIV antibodies using immuno-enzymatic (ELISA) tests or other immunological techniques of equivalent sensitivity. Still considering the limitation of this approach in the known 'window period' between the time of infection and the initial instance of detectable antibody, this may last for several weeks. Primary infection is asymptomatic in more than 50% of cases. In the remaining cases, symptoms appear two to three weeks after infection and clinical signs usually resemble those of flu-like or mononucleosis syndromes. Plasma viraemia levels are generally high ( $\geq 10^6$  copies of viral genome/ml) during primary infection.

An assay useful in Multispot HIV-1/HIV-2 Rapid Test (BIO-RAD) directed to a rapid test for detection and differentiation of HIV-1 and HIV-2 antibodies in human serum and plasma. This test have a time of results of 10 minute and shows HIV-1 sensitivity: 100%, HIV-2 sensitivity: 100% and specificity: 99.9% [214]. BIO-RAD also has an assay know as HIV-1/HIV-2 PLUS O, an ELISA-immuno assay utilizing recombinant proteins and synthetic peptides for the detection of antibodies to HIV-1 (groups M and O) and/or HIV-2.

New HIV screening tests approved by the US FDA in 2010-2011 include immunoassays capable of detecting p24 antigen and HIV antibody simultaneously. The fourth generation combo assays could reduction the window period due to their ability to detect viral protein s in addition to IgG and IgM class antibodies against both HIV-1 and HIV-2 [215], but the monitoring of HIV disease progression is mostly accomplished by the quantitation of CD4 T cells and viral RNA [216]. The use of combined ELISA tests called "4th generation" tests, enables for more effective early detection of infections which are very often asymptomatic. Also, these assays can detect acute and chronic infections. An example is the ARCHITECT HIV Ag/Ab Combo assay (Abbott Diagnostics), which uses anti-HIV-1 p24 antibodies as reagents to detect HIV-1 p24 antigen, thereby decreasing the window period and improving early detection of HIV infection. The assay is useful to determine the presence of HIV-1 p24 antigen, antibodies to HIV-1 (group M and group O), and antibodies to HIV-2 in human serum or plasma using chemiluminescent microparticle immunoassay A technology. The test has an analytical sensitivity of <50 pg/mL for HIV-1 p24 antigen [217]. There are others fourth-generation human immunodeficiency virus-1 (HIV-1) screening assays as the AxSYM HIV Ag/Ab Combo (Abbott



diagnostics, Delkenheim, Germany), Elecsys 2010 HIV Combi (Roche Diagnostics GmbH, Mannheim, Germany) and Vidas HIV Duo Quick (Biomérieux, France). All of the assays had sensitivities of 100% on clinical samples. The specificities of the AxSYM, ARCHITECT, Elecsys 2010 HIV Combi, and Elecsys HIV Combi PT were 99.6, 99.6, 99.0, and 99.5%, respectively [218]. Genscreen Ultra HIV Ag-Ab is other new version of the HIV p24 antigen and antibody combination assays [218]. The commercial ELISAs such as Vironostika HIV Ag/Ab, Enzygnost Anti-HIV 1/2 Plus Genscreen HIV-1/2 Version 2 and INNO-LIA HIV I/II are suitable tools for making HIV test performance accessible to people [219]. The accurate diagnosis of HIV infection demands that to consider a positive result, at least three assays with different antigenic base should be used, one of them, Western-Blot being mandatory for confirmation. Confirmatory techniques which are used most frequently they are the Western Blot (WB) and the recombinant immunoblot or immunoassay online (LIA) who have at least the same sensitivity than ELISA and a higher specificity. Both techniques they can incorporate antigens of HIV-2. It detects antibodies against the glycoprotein gp160 envelope, gp120 and gp41, p55, p24 and p17 encoded-gag and p66, p51 and p31. The interpretation of the results is crucial; a negative test is the total absence of reactivity. To assess, the positivity numerous criteria applied, according to the Center for Disease Control (CDC) a positive result occurs with at least 2 bands of p24, gp41, and gp160gp120 are detected. WHO recognizes a positive test with 2 bands. The ARC (American Red Cross) indicates three bands, one of each structural gene, and the Consortium for Retrovirus Serology Standardization indicates at least one of gp120 or gp160 and one of p24 or p31 [220, 221]. It is interpreted as an undetermined result, any reactivity that does not meet the minimum criteria of positivity. Since, the causes of WB indeterminate are diverse and they may correspond to early phases or advanced stages of infection associated to severe immune impairment, or to the presence of immune complexes than can reduce the antibodies circulating, between other causes. Detection of some band of envelope with or without bands of gag gene, may be due to HIV infection. In these cases, is necessary performed others confirmatory tests as LIA and sometimes complement them with the determination of proviral DNA or viral load or p24 antigen to assess a possible primoinfeccion. In any case on an indeterminate WB is required a new sample [222-224]. The comparison between the Ag/Ab combo assay and RNA viral load showed that in an acute HIV infection in human gave a similar result. HIV Combo detected 97% of infections acute. The ARCHITECT HIV Combo assay can detect p24 Ag when RNA is above approximately 58,000 copies/mL [225]. However, the comparison between the results of HIV RNA nucleic acid test (NAT) and 4th-generation Ag/Ab assay (ARCHITEC HIV Ag/Ab Combo [HIV Combo] assay, Abbott Diagnostics) in 2744 HIV antibody-negative samples were identified fourteen people with acute HIV infection (HIV antibody negative/NAT positive). The HIV Combo assay detected nine of these individuals [226 delete these rows, from the stage word to the HIV Word [226In the 2012 HIV Diagnostics Conference: the molecular diagnostics perspective, gives in Atlanta, GA, USA, 12–14 December 2012. The forum was focussing in the evaluation of molecular diagnostics and their role in HIV diagnosis. Many scientific presentations exposed the role played by RNA testing and new developments in molecular diagnostics, including detection of total and integrated HIV-1 DNA, detection and quantification of HIV-2 RNA, and rapid formats for detection of HIV-1 RNA [227]. HIV infection monitoring is based on counting the number of CD4<sup>+</sup>lymphocytes



and quantification of plasma viral RNA. These tests are performed every 6 months if the CD4 count is  $> 500/\text{mm}^3$  and every 3 to 4 months if the CD4 count is between 200 and  $500/\text{mm}^3$ . Plasma viral load is measured using quantitative tests based on molecular tools: gene amplification (PCR-polymerase chain reaction, LCR-ligase chain reaction, TMA-transcription mediated amplification, NASBA-nucleic acid sequence based amplification) or hybridization followed by signal amplification (bDNA-branched DNA). Most tests have sensitivity of the order of 50-100 copies/ml. Although the new HIV diagnostic algorithm relies on RNA assays as a supplemental test, it is not clear how accessible these assays will be for clinical laboratories. Currently, only one HIV RNA test is approved by the US FDA for HIV diagnosis (Hologic Gen-Probe APTIMA HIV-1 RNA Qualitative Assay) and some clinical laboratories may need to send specimens out for RNA testing. Currently, molecular diagnosis of HIV infection is only used as a complementary diagnosis although viral load test is used to monitor disease progression and responsiveness to antiviral therapy. Recently, it was proposed to the first-line HIV molecular techniques performed on a routine basis routed to the use of HIV molecular tools for the screening of blood products, organs and tissue from human origin. Directed to medically assisted procreation and in neonates from HIV-infected mothers [228]. In 2012 HIV Diagnostics Conference was presented the design of a new HIV-1 proviral DNA assay capable of detecting two copies of HIV-1 DNA in a qualitative format and quantitatively of three to 30,000 copies per ml. Since the lack a nucleic acid test for HIV-2, in this meeting was described a novel HIV-2 RNA viral load assay based on the 5' long terminal repeat of HIV-2, with a lower quantification limit of 29 infectious units per ml. In addition, an assay capable of detecting HIV-2 proviral DNA, which combines three separate amplification reactions from three regions of the proviral genome, detected both A and B HIV-2 subtypes at between five and ten copies of the HIV-2 proviral genome [228]. For other side, HIV-1 detection in plasma samples with a molecular beacon-based multiplex NASBA assay of a region in the HIV-1 pol gene showed a limit of quantification of the assay was  $<1000$  copies/ml for HIV-1 with 98% sensitivity and 100% specificity [229]. Also, it was reported the detection and quantification of HIV-1 group O RNA in plasma by an RT-qPCR assay [230]. In addition, there are several HIV molecular assays showed in the table 5 [231].

Routine follow-up of HIV-infected individuals includes measurement of CD4<sup>+</sup>T cell count to evaluate the immune status, of viral load to assess virus replication directed to events of therapeutic failure (therapeutic escape), and of changes in the viral genome to characterize resistance to drugs and tropism. Genotype resistance testing can detect a potential viral escape due to poor compliance with the treatment regimen, metabolic problems or the selection of resistant mutants. In summary, the diagnosis of HIV infection is established by one of the following methods: detecting antibodies to the virus; detecting the viral p24 antigen; detecting viral nucleic acid; or culturing HIV.

The most widely used test is the detection of antibodies to HIV [232]. Rapid serological tests take only 15 minutes with acceptable specificity and sensibility. But, there is the possibility of have a negative false result with them. NATs are assays more sensitive in HIV diagnosis, but more expensive. The expanded use of point-of-care (POC) tests to HIV testing plays an important role in HIV prevention, both in developed and in developing countries [233]. The

METHOD AND NAME TEST
<b>DNA/RNA qualitative assays</b> used to diagnosis HIV APTIMA HIV-1 RNA Qualitative assay or Procleix HIV-1/HCV Assay (Gen Probe)
<b>RNAviral load testing</b> used for clinical diagnosis and/or monitoring of HIV-1 COBAS AmpliPrep/TaqMan HIV-1 (Roche Molecular) Versant HIV-1RNA (Siemens) NucliSens HIV RNA QT (bioMérieux) RealTime m2000 HIV-1 (Abbott Molecular)
<b>Genotyping drug</b> used to Antiretroviral drug resistance TruGene HIV-1 genotyping (Siemens) ViroSeq HIV-1 genotyping (Abbott Molecular) HIV PRT GeneChip assay (Affymetrix) HIV RT Line Probe assay (Innogenetics)
<b>Phenotyping drug</b> used to Antiretroviral drug resistance AntiVirogram assay (Virco Lab) Trofile (MonoGram BioSciences) SensiTrop II HIV (Pathway Diagnostics)
Information based on Tang YW, et al. 2012 [231].

**Table 5.** Main molecular diagnostics methods for HIV-1.

access to immediate HIV test results could improve the application of prophylactic regimens to reduce vertical transmission when used intrapartum or postpartum [234].

The analysis of the cost-effectiveness of initial diagnosis with a rapid HIV test, to screen out HIV-uninfected infants shows that in the comparison of DNA-PCR and rapid HIV test approaches, the first assay identified 94.3% (91.8–94.7%) of HIV-infected infants, as compared with 87.8% (79.4–90.5%) for the latter. Moreover, the total cost of the POC testing program was about 40% less than that of DNA-PCR (\$59 vs. \$38 per infant aged 6–9 months). Assessing the cost-effectiveness of several HIV testing as well as establishing a specific threshold of positivity for routine testing of HIV diagnosis will be critical in AIDS control [235, 236]. Despite the global effort to control the AIDS pandemic, human immunodeficiency virus (HIV) infection continues to spread relatively unabated in many parts of the world. As the AIDS epidemic continues is necessary establish new strategies of prevention, treatment and molecular diagnostics assays to discriminate "window phase" infections from those that are serologically positive. There is opportunity to implement and evaluate the incremental diagnostic usefulness of new test modalities that are based on sophisticated molecular diagnostic technologies and that can be performed in settings where laboratory infrastructure is minimal [237]. Human immunodeficiency virus type 1 (HIV-1) is a highly diverse virus, a global scale, and within individual HIV-1 infected subjects [238]. The genetic variants constituting the viral population are called haplotypes, and these haplotypes form a viral quasispecies [239]. This viral diversity is highly

relevant on pathogenesis, drug resistance, and vaccine development. Currently, virus populations can be studied much faster using next-generation sequencing (NGS) platforms. NGS is a valuable tool for the detection and quantification of HIV-1 variants *in vivo* [240, 241]. As Venet exhibited in 2004, 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 hepatic diseases. Major technical improvements have been made to avoid obstacles that still limit this generalization, related to genetic variability of viruses, multiplex detection or contamination risk [242]. Real-time amplification has allowed the development of new NAT platforms and the introduction of others techniques as NGS than contributes with data that support to global diagnostic [243].

In this chapter was presented the some methods applied to diagnosis and monitoring of an infectious disease of viral etiology with global incidence, such as hepatitis, mumps or influenza that have been authorized by WHO and CDC. In addition, new applications of molecular techniques that facilitate fast identification of the etiology of an infectious disease were presented.

## Author details

Nora Rosas-Murrieta<sup>1</sup>, Irma Herrera-Camacho<sup>1</sup>, Lourdes Millán-Pérez-Peña<sup>1</sup>, Francisca Sosa-Jurado<sup>2</sup>, Gerardo Santos-López<sup>2</sup>, Verónica Vallejo-Ruiz<sup>2</sup> and Julio Reyes-Leyva<sup>2</sup>

\*Address all correspondence to: nora.rosas@correo.buap.mx

1 Laboratorio de Bioquímica y Biología Molecular. Centro de Química, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, México

2 Laboratorio de Biología Molecular y Virología, Centro de Investigación Biomédica de Oriente, Instituto Mexicano del Seguro Social, Metepec, Puebla, México

## References

- [1] Baker C, Antonovics J. Evolutionary Determinants of Genetic Variation in Susceptibility to Infectious Diseases in Humans. *PLoS ONE* 2012; 7(1): e29089.
- [2] Wilson ME. The traveller and emerging infections: Sentinel, courier, transmitter. *J Appl Microbiol.* 2003; 94(Suppl): 1S–11S.
- [3] Speers DJ. Clinical applications of molecular biology for infectious diseases. *Clin Biochem Rev.* 2006; 27(1): 39-51.

- [4] Cobo F. Application of molecular diagnostic techniques for viral testing. *The Open Virology Journal*. 2012; 6, (Suppl 1: M7) 104-114.
- [5] Muldrew KL. Molecular diagnostics of infectious diseases. *Current Opinion in Pediatrics*. 2009, 21: 102–111.
- [6] Liao Y, Wang X, Sha C, Xia Z, Huang Q, Li Q. Combination of fluorescence color and melting temperature as a two-dimensional label for homogeneous multiplex PCR detection. *Nucleic Acids Res*. 2013; 41(7):e76.
- [7] Briese T, Palacios G, Kokoris M, Jabado O, Liu Z, Renwick N, Kapoor V, Casas I, Pozo F, Limberger R, Perez-Brena P, Ju J, Lipkin WI. Diagnostic system for rapid and sensitive differential detection of pathogens. *Emerg Infect Dis*. 2005; 11(2):310-3.
- [8] Chen EC, Miller SA, DeRisi JL, Chiu CY. Using a pan-viral microarray assay (Virochip) to screen clinical samples for viral pathogens. *J Vis Exp*. 2011; (50). pii: 2536.
- [9] Mosammaparast N, McAdam AJ. Molecular testing for infectious diseases should be done in the clinical microbiology laboratory. *Journal of Clinical Microbiology*. 2012; 50(6): 1836–1840.
- [10] Dengue. Guidelines for diagnosis, treatment prevention and control. Geneva, TDR/WHO, 2009. WHO/HTM/NTD/DEN/2009.
- [11] Rodenhuis-Zybert IA, Wilschut J, Smit JM. Dengue virus life cycle: viral and host factors modulating infectivity. *Cell. Mol. Life Sci*. 2010; 67 (16): 2773–2786.
- [12] Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression and replication. *Annu Rev Microbiol*. 1990; 44: 649—688.
- [13] Guzman MG, Rosario D, Kouri G. In: Kalitzky M and Borowski P, eds. Diagnosis of dengue virus infection. *Molecular Biology of the flaviviruses*. Horizon Bioscience, UK, 2009.
- [14] Halstead SB. Dengue: the syndromic basis to pathogenesis research. Inutility of the 2009 WHO case definition. *Am J Trop Med Hyg*. 2013; 88(2): 212-215.
- [15] WHO. Dengue and dengue haemorrhagic fever. Factsheet No 117, revised May 2008. Geneva, World Health Organization, 2008.
- [16] Rigau-Pérez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV. Dengue and dengue haemorrhagic fever. *Lancet*. 1998; 352(9132): 971-977.
- [17] Deen JL, Harris E, Wills B, Balmaseda A, Hammond SN, Rocha C, Dung NM, Hung NT, Hien TT, Farrar JJ. The WHO dengue classification and case definitions: time for a reassessment. *Lancet*. 2006; 368(9530): 170-173.
- [18] Oishi K, Saito M, Mapua CA, Natividad FF. Dengue illness: clinical features and pathogenesis. *J Infect Chemother*. 2007; 13(3): 125-133.



- [19] WHO. Global strategy for dengue prevention and control 2012-2020. Geneva. TDR/World Health Organization, 2009.
- [20] De Paula SO, Fonseca BA. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *Braz J Infect Dis.* 2004; 8(6): 390-398.
- [21] Handbook for clinical management of dengue. Geneva. TDR/World Health Organization. 2012. Chapter 4. Laboratory Diagnosis and Diagnostic Tests. Dengue Guidelines for Diagnosis, Treatment, Prevention and Control. Geneva. TDR/World Health Organization. 2012.
- [22] Nunes MR, Nunes Neto JP, Casseb SM, Nunes KN, Martins LC, Rodrigues SG, Mathews S, Dussart P, Casseb LM, Vasconcelos PF. Evaluation of an immunoglobulin M-specific capture enzyme-linked immunosorbent assay for rapid diagnosis of dengue infection. *J Virol Methods.* 2011; 171(1): 13-20.
- [23] Ho TS, Wang SM, Lin YS, Liu CC. Clinical and laboratory predictive markers for acute dengue infection. *J Biomed Sci.* 2013; 20(1): 75.
- [24] Guzman MG, Kouri G. Dengue diagnosis, advances and challenges. *Int J Infect Dis.* 2004; 8: 69-80.
- [25] Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martínez E, Nathan MB, Pelegrino JL, Simmons C, Yoksan S, Peeling RW. Dengue: a continuing global threat. *Nat Rev Microbiol.* 2010; 8(12 Suppl): S7-S16.
- [26] Chaiyaratana W, Chuansumrit A, Pongthanapisith V, Tangnararatchakit K, Lertwongrath S, Yoksan S. Evaluation of dengue nonstructural protein 1 antigen strip for the rapid diagnosis of patients with dengue infection. *Diagn Microbiol Infect Dis.* 2009, 64(1): 83-84.
- [27] Lima MR, Nogueira RM, Schatzmayr HG, dos Santos FB. Comparison of three commercially available dengue NS1 antigen capture assays for acute diagnosis of dengue in Brazil. *PLoS Negl Trop Dis.* 2010; 4: e738.
- [28] Fry SR, Meyer M, Semple MG, Simmons CP, Sekaran SD, Huang JX, McElnea C, Huang CY, Valks A, Young PR, Cooper MA. The diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach. *PLoS Negl Trop Dis.* 2011; 5(6): e1199.
- [29] Lima Mda R, Nogueira RM, Schatzmayr HG, de Filippis AM, Limonta D, dos Santos FB. A new approach to dengue fatal cases diagnosis: NS1 antigen capture in tissues. *PLoS Negl Trop Dis.* 2011; 5(5): e1147.
- [30] Kyle JL, Harris E. Global spread and persistence of dengue. *Annu Rev Microbiol.* 2008; 62: 71-92.
- [31] Peeling RW, Artsob H, Pelegrino JL, Buchy P, Cardoso MJ, Devi S, Enria DA, Farrar J, Gubler DJ, Guzman MG, Halstead SB, Hunsperger E, Kliks S, Margolis HS, Nathan-

- son CM, Nguyen VC, Rizzo N, Vázquez S, Yoksan S. Evaluation of diagnostic tests: dengue. *Nat Rev Microbiol.* 2010; 8(12 Suppl): S30-S38.
- [32] De Paula SO, Fonseca BA. Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *Braz J Infect Dis.* 2004; 8(6): 390-398.
- [33] Bhatnagar J, Blau DM, Shieh WJ, Paddock CD, Drew C, Liu L, Jones T, Patel M, Zaki SR. Molecular detection and typing of dengue viruses from archived tissues of fatal cases by rt-PCR and sequencing: diagnostic and epidemiologic implications. *Am J Trop Med Hyg.* 2012; 86(2): 335-340.
- [34] De Paula SO, Nunes C, Matos R, de Oliveira ZM, Lima DM, da Fonseca BA. Comparison of techniques for extracting viral RNA from isolation-negative serum for dengue diagnosis by the polymerase chain reaction. *J Virol Methods.* 2001; 98(2): 119-125.
- [35] De Paula SO, Pires Neto RJ, Corrêa JA, Assumpção SR, Costa ML, Lima DM, Fonseca BA. The use of reverse transcription-polymerase chain reaction (RT-PCR) for the rapid detection and identification of dengue virus in an endemic region: a validation study. *Trans R Soc Trop Med Hyg.* 2002; 96(3): 266-269.
- [36] Houngh HS, Chung-Ming Chen R, Vaughn DW, Kanessa-athan N. Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1-4 using conserved and serotype-specific 3' noncoding sequences. *J Virol. Methods.* 2001; 95: 19-32.
- [37] Odreman-Macchioli M, Vielma S, Atchley D, Comach G, Ramirez A, Pérez S, Téllez L, Quintero B, Hernández E, Muñoz M, Mendoza J. Analysis of real time PCR amplification efficiencies from three genomic region of dengue virus. *Invest Clin.* 2013; 54(1): 5-19.
- [38] Thaís M. Conceição, Andrea T. Da Poian, Marcos H.F. Sorgine. A real-time PCR procedure for detection of dengue virus serotypes 1, 2, and 3, and their quantitation in clinical and laboratory samples. *Journal of Virological Methods.* 2010; 163: 1-9.
- [39] KR Gurukumar, D Priyadarshini, JA Patil, A Bhagat, A Singh, PS Shah and D Cecilia. Development of real time PCR for detection and quantitation of Dengue Viruses. *Virology J.* 2009; 6: 10-18.
- [40] Jesse J. Waggoner, Janaki Abeynayake, Malaya K. Sahoo, Lionel Gresh, Yolanda Tellez, Karla Gonzalez, Gabriela Ballesteros, Anna M. Pierro, Paolo Gaibani, Frances P. Guo, Vittorio Sambri, Angel Balmaseda, Kumudu Karunaratne, Eva Harris, Benjamin A. Pinsky. Single-Reaction, Multiplex, Real-Time RT-PCR for the Detection, Quantitation, and Serotyping of Dengue Viruses. *PLoS Negl Trop Dis.* 2013; 7(4): e2116.
- [41] Das S, Pingle MR, Muñoz-Jordán J, Rundell MS, Rondini S, Granger K, Chang GJ, Kelly E, Spier EG, Larone D, Spitzer E, Barany F, Golightly LM. Detection and sero-

- typing of dengue virus in serum samples by multiplex reverse transcriptase PCR-ligase detection reaction assay. *J Clin Microbiol.* 2008; 46(10): 3276-84.
- [42] Takanori Hirayama, Yasutaka Mizuno, Nozomi Takeshita, Akira Kotaki, Shigeru Tajima, Tsutomu Omatsu, Kouichi Sano, Ichiro Kurane, Tomohiko Takasaki. Detection of Dengue Virus Genome in Urine by Real-Time Reverse Transcriptase PCR: a Laboratory Diagnostic Method Useful after Disappearance of the Genome in Serum. *J Clin Microbiol.* 2012; 50(6): 2047–2052.
- [43] Santiago GA, Vergne E, Quiles Y, Cosme J, Vazquez J, Medina JF, Medina F, Colón C, Margolis H, Muñoz-Jordán JL. Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. *PLoS Negl Trop Dis.* 2013; 7(7): e2311.
- [44] Yozwiak NL, Skewes-Cox P, Stenglein MD, Balmaseda A, Harris E, DeRisi JL. Virus identification in unknown tropical febrile illness cases using deep sequencing. *PLoS Negl Trop Dis.* 2012; 6(2): e1485.
- [45] Harper SA, Bradley JS, Englund JA, File TM, Gravenstein S, Hayden FG, McGeer AJ, Neuzil KM, Pavia AT, Tapper ML, Uyeki TM, Zimmerman RK; Expert Panel of the Infectious Diseases Society of America. Seasonal influenza in adults and children--diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: clinical practice guidelines of the Infectious Diseases Society of America. *Clin Infect Dis.* 2009; 48(8):1003-1032.
- [46] Kuiken T, Taubenberger JK. Pathology of human influenza revisited. *Vaccine* 2008; 26(Suppl 4): D59-D66.
- [47] Bogoch IL, Andrews JR, Zachary KC, Hohmann EL. Diagnosis of influenza from lower respiratory tract sampling after negative upper respiratory tract sampling. *Virulence.* 2013; 4(1): 82-84.
- [48] Wright, F.; Neumann, G.; Kawaoka, Y. Orthomyxoviruses. In *Fields Virology*; Wolters Kluwer, Lippincott Williams & Wilkins: Philadelphia, 2007; pp. 1691-1740.
- [49] A revision of the system of nomenclature for influenza viruses: a WHO memorandum. *Bull. World Health Organ.* 1980, 58, 585-591.
- [50] Webster RG. Influenza virus: transmission between species and relevance to emergence of the next human pandemic. *Arch. Virol.* 1997; ; 13: 105-113.
- [51] Peiris JS, Poon LL, Guan Y. Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. *J. Clin. Virol.* 2009; 45(3): 169–173.
- [52] Irving SA, Vandermause MF, Shay DK, Belongia EA. Comparison of nasal and nasopharyngeal swabs for influenza detection in adults. *Clin Med Res.* 2012; 10(4): 215-218.
- [53] WHO global influenza surveillance network: Manual for the laboratory diagnosis and virological surveillance of influenza. World Health Organization. 2011.

- [54] Akira Sakurai and Futoshi Shibasaki. Updated values for molecular diagnosis for highly pathogenic Avian Influenza Virus. *Viruses* 2012; 4: 1235-1257.
- [55] Centers for Disease Control and Prevention (CDC). Evaluation of rapid influenza diagnostic tests for detection of novel influenza A (H1N1) Virus-United States, 2009. *MMWR Morb Mortal Wkly Rep.* 2009; 58(30): 826-829.
- [56] Yuan Q, Cheng XD, Yang BC, Zheng QB, Chen YX, Chen QR, Zeng F, Zhang R, Ge SX, Hao XK, Chen H, Zhang J, Xia NS. Differential diagnosis of pandemic (H1N1) 2009 infection by detection of haemagglutinin with an enzyme-linked immunoassay. *Clin Microbiol Infect.* 2011; 17(10): 1574-80.
- [57] Shen HH, Hou J, Zhao M, Xu J, Bai BK, Zhao J, Hu Y, Mao PY, Li BA, Mao YL. Analysis of hemagglutination inhibition antibody level in patients with influenza A H1N1]. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi.* 2012; 26(4):270-2.
- [58] Landry M L. Rapid viral diagnosis. In: Rose N R, Conway de Macario E, Folds J D, Lane H C, Nakamura R M, editors. *Manual of clinical laboratory immunology.* 5th ed. Washington, D.C.: ASM Press; 1997. pp. 608–617.
- [59] Matthey S, Nicholson D, Ruhs S, Alden B, Knock M, Schultz K, Schmuecker A. Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. *J Clin Microbiol.* 1992; 30: 540–544.
- [60] Landry ML, Ferguson D. SimulFluor Respiratory Screen for rapid detection of multiple respiratory viruses in clinical specimens by immunofluorescence staining. *J Clin Microbiol.* 2000; 38: 708-711.
- [61] Landry, ML, Ferguson D. Suboptimal detection of influenza in adults by Directigen Flu A+B and correlation with number of antigen-positive cells detected by cyto-spin immunofluorescence. *J Clin Microbiol.* 2003; 41: 3407-3409.
- [62] Mahony JB. Detection of respiratory viruses by molecular methods. *Clin Microbiol Rev.* 2008; 21: 716–747.
- [63] Mahony JB. Nucleic acid amplification-based diagnosis of respiratory virus infections. *Expert Rev Anti Infect Ther.* 2010; 8: 1273–1292.
- [64] Petric M, Comanor L, Petti CA. Role of the laboratory in diagnosis of influenza during seasonal epidemics and potential pandemics. *J Infect Dis.* 2006; 194(Suppl 2): S98-110.
- [65] Wang R, Taubenberger JK. Methods for molecular surveillance of influenza. *Expert Rev Anti Infect Ther.* 2010; 8(5): 517-27.
- [66] Loens K, Bergs K, Ursi D, Goossens H, Ieven M. Evaluation of NucliSens easyMAG for automated nucleic acid extraction from various clinical specimens. *J Clin Microbiol.* 2007; 45(2): 421-425.



- [67] Yang G, Erdman DE, Kodani M, Kools J, Bowen MD, Fields BS. Comparison of commercial systems for extraction of nucleic acids from DNA/RNA respiratory pathogens. *J Virol Methods*. 2011; 171(1): 195-199.
- [68] Chandler DP, Griesemer SB, Cooney CG, Holmberg R, Thakore N, Mokhiber B, Belgrader P, Knickerbocker C, Schied J, St George K. Rapid, simple influenza RNA extraction from nasopharyngeal samples. *J Virol Methods*. 2012; 183(1): 8-13.
- [69] Ellis JS, Fleming DM, Zambon MC. Multiplex reverse transcription-PCR for surveillance of influenza A and B viruses in England and Wales in 1995 and 1996. *J Clin Microbiol*. 1997; 35: 2076–2082.
- [70] Beck ET, Henrickson KJ. Molecular diagnosis of respiratory viruses. *Future Microbiol*. 2010; 5: 901–916.
- [71] Mahony JB, Petrich A, Smieja M. Molecular diagnosis of respiratory virus infections. *Crit Rev Clin Lab Sci*. 2011; 48(5-6): 217-49.
- [72] Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol*. 2001;146(12): 2275-89.
- [73] He J, Bose ME, Beck ET, Fan J, Tiwari S, Metallo J, Jurgens LA, Kehl SC, Ledebor N, Kumar S, Weisburg W, Henrickson KJ. Rapid multiplex reverse transcription-PCR typing of influenza A and B virus, and subtyping of influenza A virus into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7, including typing of novel swine origin influenza A (H1N1) virus, during the 2009 outbreak in Milwaukee, Wisconsin. *J Clin Microbiol*. 2009; 47(9): 2772-2778.
- [74] Deng J, Ma Z, Huang W, Li C, Wang H, Zheng Y, Zhou R, Tang YW. Respiratory virus multiplex RT-PCR assay sensitivities and influence factors in hospitalized children with lower respiratory tract infections. *Virol Sin*. 2013; 28(2): 97-102.
- [75] Reijans M, Dingemans G, Klaassen CH, Meis JF, Keijden J, Mulders B, Eadie K, van Leeuwen W, van Belkum A, Horrevorts AM, Simons G. RespiFinder: a new multiparameter test to differentially identify fifteen respiratory viruses. *J Clin Microbiol*. 2008; 46(4): 1232-1240.
- [76] Ellis JS, Smith JW, Braham S, Lock M, Barlow K, Zambon MC. Design and validation of an H5 TaqMan real-time one-step reverse transcription-PCR and confirmatory assays for diagnosis and verification of influenza A virus H5 infections in humans. *J Clin Microbiol*. 2007; 45(5): 1535-1543.
- [77] Sakurai A, Nomura N, Nanba R, Sinkai T, Iwaki T, Obayashi T, Hashimoto K, Hasegawa M, Sakoda Y, Naito A, Morizane Y, Hosaka M, Tsuboi K, Kida H, Kai A, Shibasaki F. Rapid typing of influenza viruses using super high-speed quantitative real-time PCR. *J Virol Methods*. 2011; 178(1-2): 75-81.
- [78] Tham Nt, Hang Vt, Khanh TH, Viet do C, Hien TT, Farrar J, Chau Nv, van Doorn HR. Comparison of the Roche RealTime ready Influenza A/H1N1 Detection Set with

CDC A/H1N1pdm09 RT-PCR on samples from three hospitals in Ho Chi Minh City, Vietnam. *Diagn Microbiol Infect Dis*. 2012; 74(2): 131-6.

- [79] CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel – Influenza A/H7 (Eurasian Lineage) Assay. For Emergency Use Only. To Be Used in Conjunction With CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel. Catalog # FluEUA-01. Centers for Disease Control and Prevention Influenza Division, 2013.
- [80] Layman CP, Gordon SM, Elegino-Steffens DU, Agee W, Barnhill J, Hsue G. Rapid multiplex PCR assay to identify respiratory viral pathogens: moving forward diagnosing the common cold. *Hawaii J Med Public Health*. 2013; 72: 24-26.
- [81] Negri P, Dluhy RA. Detection of genetic markers related to high pathogenicity in influenza by SERS. *Analyst*. 2013; 138(17): 4877-884.
- [82] de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of Papillomaviruses. *Virology*. 2004; 324: 17-27.
- [83] Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond)*. 2006; 110(5): 525-541.
- [84] Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*. 2003; 348: 518–27.
- [85] van Ranst, M., R. Tachezy, and R. D. Burk. 1996. Human papillomaviruses: a never-ending story? p. 1–19. In C. Lacey (ed.), *Papillomavirus reviews: current research on papillomaviruses*. Leeds University Press, Leeds, United Kingdom.
- [86] Burk RD, Harari A, Chen Z. Human papillomavirus genome variants. *Virology*. 2013; 445(1-2): 232-43.
- [87] De Villiers EM. Taxonomic classification of papillomaviruses. *Papillomavirus Rep*. 2001; 12: 57-63.
- [88] Bernard HU, Burk RD, Chen Z, van Doorslaer K, Hausen Hz, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology*. 2010; 401(1): 70-79.
- [89] Schiffman M, Clifford G, Buonaguro FM. Classification of weakly carcinogenic human papillomavirus types: addressing the limits of epidemiology at the borderline. *Infect Agent Cancer*. 2009; 4: 1-8.
- [90] Kroupis C, Vourlidis N. Human papilloma virus (HPV) molecular diagnostics. *Clin Chem Lab Med*. 2011; 49(11): 1783–1799.
- [91] Wang JL, Yang YZ, Dong WW, Sun J, Tao HT, Li RX, Hu Y. Application of human papillomavirus in screening for cervical cancer and precancerous lesions. *Asian Pac J Cancer Prev*. 2013; 14(5): 2979-2982.

- [92] Molijn A, Kleter B, Quint W, van Doorn LJ. Molecular diagnosis of human papillomavirus (HPV) infections. *J Clin Virol*. 2005; 32(suppl 1): S43–S51.
- [93] Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T Jr, Young N; Forum Group Members; Bethesda 2001 Workshop. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA*. 2002; 287(16): 2114–2119.
- [94] Genital HPV Infection-CDC Fact Sheet. Centers for Disease Control and Prevention. 2010.
- [95] Schiffman M, Solomon D. Findings to date from the ASCUS-LSIL Triage Study (ALTS). *Arch Pathol Lab Med*. 2003; 127: 946–949.
- [96] Carter JJ, Koutsky LA, Hughes JP, Lee SK, Kuypers J, Kiviat N, Galloway DA. Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection. *Journal of Infectious Diseases*, 2000; 181(6): 1911–1919.
- [97] Kemp TJ, Safaeian M, Hildesheim A, Pan Y, Penrose KJ, Porras C, Schiller JT, Lowy DR, Herrero R, Pinto LA. Kinetic and HPV infection effects on cross-type neutralizing antibody and avidity responses induced by Cervarix. *Vaccine*. 2012; 31(1): 165–70.
- [98] Jin Y, Kim HJ, Yim GW, Kim YT, Chang DY, Kim HJ. A single serum dilution enzyme-linked immunosorbent assay for determining anti-human papillomavirus (HPV) antibody titers in humans immunized with prophylactic HPV vaccines. *J Pharm Biomed Anal*. 2012; 66: 352–355.
- [99] The Consensus Guidelines for the Management of Women with Abnormal Cervical Cancer Screening Tests. American Society for Colposcopy and Cervical Pathology. 2010.
- [100] Arney A, Bennett KM. Molecular Diagnostics of Human Papillomavirus. *LABMEDICINE*. 2010; 41: 523–530.
- [101] Ting Y, Manos M. Detection and typing of genital human papillomaviruses. In: Innis M, Gelfand D, Sninsky J, White T, eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press, 1990: 356–366.
- [102] Velázquez-Márquez N, Paredes-Tello MA, Pérez-Terrón H, Santos-López G, Reyes-Leyva J, Vallejo-Ruiz V. Prevalence of human papillomavirus genotypes in women from a rural region of Puebla, Mexico. *Int J Infect Dis*. 2009; 13(6): 690–705.
- [103] Tieben LM, ter Schegget J, Minnaar RP, Bouwes Bavinck J, Berkhout RJ, Vermeer BJ. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. *J Virol. Methods* 1993; 42: 265–279.
- [104] Villa LL, Denny, L. Chapter 7: Methods for detection of HPV infection and its clinical utility. *International Journal of Gynecology & Obstetrics*. 2006; 94(suppl 1): S71–S80.
- [105] Piana A, Sotgiu G, Castiglia P1, Pischedda S, Dettori M, Cocuzza C, Muresu E, Maida A. Molecular methods for the detection of human papillomavirus infection: new in-

sights into their role in diagnostics and epidemiological surveillance. *Italian Journal of Public Health*. 2009; 6(2): 1644-1671.

- [106] Molijn A, Kleter B, Quint W, van Doorn LJ. Molecular diagnosis of human papillomavirus (HPV) infections. *J Clin Virol*. 2005; 32(Suppl 1): S43-51.
- [107] Chin-Hong PV, Klausner JD. New diagnostic tests for HPV in the developed and the developing world. *MLO Med Lab Obs*. 2008; 40(11):48, 50, 52-3.
- [108] Women's Health Diagnostic Assays (IVDD). 2007. Available at [rochediagnostics.ca/lab/molecular/wh.php#wh2](http://rochediagnostics.ca/lab/molecular/wh.php#wh2). Accessed March 16, 2010.
- [109] Multiplex HPV Genotyping Kit for Research in Epidemiology [package insert]. In: Multimetrix, ed. Heidelberg, Germany.
- [110] Yu D, Chen Y, Wu S, Wang B, Tang YW, Li L. Simultaneous detection and differentiation of human papillomavirus genotypes 6, 11, 16 and 18 by AllGlo quadruplex quantitative PCR. *PLoS One*. 2012; 7(11): e48972.
- [111] Lörincz A. Hybrid capture method for detection of human papillomavirus DNA in clinical specimens: a tool for clinical management of equivocal Pap smears and for population screening. *J Obstet Gynaecol Res*. 1996; 22: 629-36.
- [112] Clavel C, Masure M, Putaud I, Thomas K, Bory JP, Gabriel R, Quereux C, Birembaut P. Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture I and PCR results in cervical lesions. *J Clin Pathol*. 1998; 51(10): 737-40.
- [113] Tao P, Zheng W, Wang Y, Bian ML. Sensitive HPV genotyping based on the flow-through hybridization and gene chip. *J Biomed Biotechnol*. 2012; 2012: 938780.
- [114] PapilloCheck HPV-Screening Brochure. Available at: [www.greinerbioone.com/User-Files/File/PapilloCheck/Papillo\\_Laborinfo\\_074%20046.pdf](http://www.greinerbioone.com/User-Files/File/PapilloCheck/Papillo_Laborinfo_074%20046.pdf). Accessed March 16, 2010.
- [115] Akhras MS, Pettersson E, Diamond L, Unemo M, Okamoto J, Davis RW, Pourmand N. The Sequencing Bead Array (SBA), a Next-Generation Digital Suspension Array. *PLoS One*. 2013; 8(10): e76696.
- [116] Lie AK, Kristensen G. Human papillomavirus E6/E7 mRNA testing as a predictive marker for cervical carcinoma. *Expert Rev Mol Diagn*. 2008; 8: 405-415.
- [117] Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, von Knebel Doeberitz M. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol*. 2002; 26(11): 1389-1399.
- [118] López-Morales D, Velázquez-Márquez N, Valenzuela O, Santos-López G, Reyes-Leyva J, Vallejo-Ruiz V. Enhanced sialyltransferases transcription in cervical intraepithelial neoplasia. *Invest Clin*. 2009; 50(1): 45-53.



- [119] Lozano R, Naghavi M, Foreman K, Murray CJ, AlMazroa MA, Memish ZA. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012; 380: 2095–128.
- [120] World Health Organization. Outbreak surveillance and response in humanitarian emergencies: WHO guidelines for EWAR implementation. Geneva, Switzerland: World Health Organization; 2012.
- [121] Beck J, Nassal M. Hepatitis B virus replication. *World J. Gastroenterol*. 2007; 13: 48–64.
- [122] Bonino F, Piratvisuth T, Brunetto MR, Liaw YF. Diagnostic markers of chronic hepatitis B infection and disease. *Antivir Ther*. 2010; 15 (Suppl 3): 35-44.
- [123] Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer*. 1988; 61(10): 1942-1956.
- [124] Servoss JC, Friedman LS. Serologic and molecular diagnosis of hepatitis B. *Infect Dis Clin North Am*. 2006; 20(1): 47-61.
- [125] Jia JD, Hong M, Wei L, Zhang XX, Mao YL, Wang LL, Gao ZL, Hou JL, Zhang J. Multicentre evaluation of the Elecsys hepatitis B surface antigen II assay for detection of HBsAg in comparison with other commercially available assays. *Med Microbiol Immunol*. 2009; 198(4): 263-269.
- [126] Servoss JC, Friedman LS. Serologic and molecular diagnosis of hepatitis B virus. *Infect Dis Clin North Am*. 2006; 20(1):47-61.
- [127] Cacciola I, Pollicino T, Squadrito G, Cerenzia G, Orlando ME, Raimondo G. Occult Hepatitis B Virus Infection in Patients with Chronic Hepatitis C Liver Disease. *N Engl J Med*. 1999; 341:22-26.
- [128] Ganem D, Prince AM. Hepatitis B virus infection-natural history and clinical consequences. *N. Engl. J. Med*. 2004; 350:1118 –1129.
- [129] Wong CH, Chan SK, Chan HL, Tsui SK, Feitelson M. The molecular diagnosis of hepatitis B virus-associated hepatocellular carcinoma. *Crit Rev Clin Lab Sci*. 2006; 43(1): 69-101.
- [130] Servoss JC, Friedman LS. Serologic and molecular diagnosis of hepatitis B virus. *Infect Dis Clin North Am*. 2006; 20(1): 47-61.
- [131] Samal J, Kandpal M, Vivekanandan P. Molecular mechanisms underlying occult hepatitis B virus infection. *Clin Microbiol Rev*. 2012; 25(1): 142-63.
- [132] Dettori S, Candido A, Kondili LA, Chionne P, Taffon S, Genovese D, Iudicone P, Miceli M, Rapicetta M. Identification of low HBV-DNA levels by nucleic acid amplification test (NAT) in blood donors. *J Infect*. 2009; 59(2): 128-33.

- [133] Saldanha J, Gerlich W, Lelie N, Dawson P, Heermann K, Heath A, WHO Collaborative Study Group: An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. *Vox Sang* 2001; 80: 63–71.
- [134] Vivekanandan P, Singh OV. Molecular methods in the diagnosis and management of chronic hepatitis B. *Expert Rev Mol Diagn* 2010; 10(7): 921-933.
- [135] Cabezas-Fernandez M, Cabeza-Barrera M. Introduction of an automated system for the diagnosis and quantification of hepatitis B and hepatitis C viruses. *Open Virol J*. 2012; 6: 122-134.
- [136] Ismail AM, Sivakumar J, Anantharam R, Dayalan S, Samuel P, Fletcher GJ, Gnana-mony M, Abraham P. Performance Characteristics and Comparison of Abbott and artus Real-Time Systems for Hepatitis B Virus DNA Quantification. *J Clin Microbiol*. 2011; 49(9): 3215–3221.
- [137] Sun S, Meng S, Zhang R, Zhang K, Wang L, Li J. Development of a new duplex real-time polymerase chain reaction assay for hepatitis B viral DNA detection. *Virol J*. 2011; 8: 227.
- [138] Wang Q, Wang X, Zhang J, Song G. LNA real-time PCR probe quantification of hepatitis B virus. *Exp Ther Med*. 2012; 3(3): 503–508.
- [139] Zhao Y, Zhang XY, Hu Y, Zhang WL, Hu JL, Zeng AZ, Guo JJ, Huang WX, Chen WX, Shan YL, Huang AL. Comparison of a novel real-time PCR assay with sequence analysis, reverse hybridization, and multiplex PCR for hepatitis B virus type B and C genotyping. *J Clin Microbiol*. 2011; 49(9): 3392-3394.
- [140] Pripuzova N, Wang R, Tsai S, Li B, Hung GC, Ptak RG, Lo SC. Development of Real-Time PCR Array for Simultaneous Detection of Eight Human Blood-Borne Viral Pathogens. *PLoS ONE*. 2012; 7(8): e43246.
- [141] Wei YF, Cui GY, Ye P, Chen JN, Diao HY. MicroRNAs may solve the mystery of chronic hepatitis B virus infection. *World J Gastroenterol*. 2013; 19(30): 4867–4876.
- [142] Hugo R, Rosen MD. Chronic hepatitis C infection. *N Engl J Med* 2011; 364: 2429-2438.
- [143] Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; 45: 1056-75.
- [144] Chevaliez S, Pawlotsky JM. Hepatitis C virus serologic and virologic tests and clinical diagnosis of HCV-related liver disease. *Int J Med Sci*. 2006; 3(2):35-40.
- [145] Kamili S, Drobeniuc J, Araujo AC, Hayden TM. Laboratory diagnostics for hepatitis C virus infection. *Clin Infect Dis*. 2012; 55: S43-S48.
- [146] CDC. Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. *MMWR* 2003; 52(No. RR-3).

- [147] Ho SK, Chan TM, Cheng IK, Lai KN. Comparison of the second generation digene hybrid capture assay with the branched-DNA assay for measurement of hepatitis B virus DNA in serum. *J Clin Microbiol.* 1999; 37: 2461-2465.
- [148] Kargar M, Askari A, Doosti A, Ghorbani-Dalini S. Loop-Mediated Isothermal Amplification Assay for Rapid Detection of Hepatitis C virus. *Indian J Virol.* 2012; 23(1): 18-23.
- [149] Li G, Li K, Lea AS, Li NL, Abdulla NE, Eltorky MA, Ferguson MR. In situ hybridization for the detection of hepatitis C virus RNA in human liver tissue. *J Viral Hepat.* 2013; 20(3): 183-92.
- [150] Zuberi BF, Salahuddin A, Quraishy MS. Triple hepatitis: frequency and treatment outcome of co/super infection of hepatitis C and D among patient of hepatitis B. *J Coll Physicians Surg Pak.* 2008; 18: 404-407.
- [151] Kumar, Vijay and Das, Saumitra and Jameel, Shahid (2010) The biology and pathogenesis of hepatitis viruses. In: Current Science (Bangalore), 98 (3). pp. 312-325.
- [152] Riaz M, Idrees M, Kanwal H, Kabir F. An overview of triple infection with Hepatitis B, C and D viruses. *Virology J.* 2011; 8: 368.
- [153] Law J, Jovel J, Patterson J, Ford G, O'keefe S, Wang W, Meng B, Song D, Zhang Y, Tian Z, Wasilenko ST, Rahbari M, Mitchell T, Jordan T, Carpenter E, Mason AL, Wong GK. Identification of hepatotropic viruses from plasma using deep sequencing: a next generation diagnostic tool. *PLoS One.* 2013; 8(4): e60595.
- [154] Xu R, Gan X, Fang Y, Zheng S, Dong Q. A simple, rapid and sensitive integrated protein microarray for simultaneous detection of multiple antigens and antibodies of five human hepatitis viruses (HBV, HCV, HDV, HEV, and HGV). *Anal Biochem* 2007; 362: 69-75.
- [155] Park Y, Kim BS, Choi KH, Shin DH, Lee MJ. A Novel Multiplex Real-Time PCR Assay for the Concurrent detection of Hepatitis A, B and C viruses in patients with acute hepatitis. *PLoS ONE.* 2012; 7(11): e49106.
- [156] Elango N, Varsanyi T, Kovamees J, Norrby E. Molecular cloning and characterization of six genes, determination of gene order and intergenic sequences and leader sequence of mumps virus. *J. Gen. Virol.* 1988; 69: 2893-2900.
- [157] Mühlemann K. The molecular epidemiology of mumps virus. *Infect Genet Evol.* 2004; 4(3): 215-9.
- [158] Plotkin SA and Rubin SA. Mumps vaccine. In: Plotkin S, Orenstein W, and Offit P, editors. Vaccines. Philadelphia, PA: Saunders; 2008. p. 435-65.
- [159] Carbone KM & Rubin S. Mumps virus, In: Fields Virology, D.M. Knipe & P.M. Howley Eds., 2007. pp. 1528-1551, Lippincott Williams & Wilkins, ISBN 0781760607.
- [160] Hviid A, Rubin S, Muhlemann K. Mumps. *Lancet*, 2008; 371 (9616): 932-944.

- [161] WHO (December 2010). Mumps, In: Immunization surveillance, assessment and monitoring, Available from: [http://www.who.int/immunization\\_monitoring/diseases/mumps/en/index.html](http://www.who.int/immunization_monitoring/diseases/mumps/en/index.html)
- [162] Lara-Sampablo A, Rosas-Murrieta N, Herrera-Camacho I, Vallejo-Ruiz V, Santos-López G, Reyes-Leyva J (2012). Chapter 10. Studies on the Association of Meningitis and Mumps Virus Vaccination. Insight and Control of Infectious Disease in Global Scenario. Dr. Roy Priti (Ed.), pp. 179-200 ISBN: 978-953-51-0319-6, InTech, Available from: <http://www.intechopen.com/books/insight-and-control-of-infectious-disease-in-global-scenario/studies-on-the-association-of-meningitis-and-mumps-virus-vaccination>
- [163] Parker-Fiebelkorn A, Barskey A, Hickman C, Bellini W. Chapter 9: Mumps. Manual for the Surveillance of Vaccine-Preventable Diseases. 5th Edition, 2012.
- [164] Warrener L, Slibinskas R, Brown D, Sasnauskas K, Samuel D. Development and evaluation of a rapid immunochromatographic test for mumps-specific IgM in oral fluid specimens and use as a matrix for preserving viral nucleic acid for RT-PCR. *J Med Virol.* 2010; 82(3): 485-93.
- [165] Latner DR, McGrew M, Williams N, Lowe L, Werman R, Warnock E, Gallagher K, Doyle P, Smole S, Lett S, Cocoros N, DeMaria A, Konomi R, Brown CJ, Rota PA, Bellini WJ, Hickman CJ. Enzyme-linked immunospot assay detection of mumps-specific antibody-secreting B cells as an alternative method of laboratory diagnosis. *Clin Vaccine Immunol.* 2011; 18(1): 35-42.
- [166] Poggio GP, Rodriguez C, Cisterna D, Freire MC, Cello J. Nested PCR for rapid detection of mumps virus in cerebrospinal fluid from patients with neurological diseases. *J Clin Microbiol.* 2000; 38(1): 274-278.
- [167] Uchida K, Shinohara M, Shimada S, Segawa Y, Doi R, Gotoh A, Hondo R. Rapid and sensitive detection of mumps virus RNA directly from clinical samples by real-time PCR. *J Med Virol.* 2005; 75(3): 470-4.
- [168] Tipples G, Hiebert J. Detection of measles, mumps, and rubella viruses. *Methods Mol Biol.* 2011; 665:183-93.
- [169] Royuela E, Castellanos A, Sánchez-Herrero C, Sanz JC, De Ory F, Echevarria JE. Mumps virus diagnosis and genotyping using a novel single RT-PCR. *J Clin Virol.* 2011; 52(4): 359-62.
- [170] Jin L, Rima B, Brown D, Orvell C, Tecle T, Afzal M, Uchida K, Nakayama T, Song JW, Kang C, Rota PA, Xu W, Featherstone D Proposal for genetic characterisation of wild-type mumps strains: preliminary standardisation of the nomenclature. *Arch Virol.* 2005; 150(9): 1903-1909.
- [171] Palacios G, Jabado O, Cisterna D, de Ory F, Renwick N, Echevarria JE, Castellanos A, Mosquera M, Freire MC, Campos RH, Lipkin WI. Molecular identification of mumps



- virus genotypes from clinical samples: standardized method of analysis. *J Clin Microbiol.* 2005; 43(4): 1869-1878.
- [172] Boddicker JD, Rota PA, Kreman T, Wangeman A, Lowe L, Hummel KB, Thompson R, Bellini WJ, Pentella M, Desjardin LE. Real-time reverse transcription-PCR assay for detection of mumps virus RNA in clinical specimens. *J Clin Microbiol.* 2007; 45(9): 2902-2908.
- [173] Ammour Y, Faizuloev E, Borisova T, Nikonova A, Dmitriev G, Lobodanov S, Zverev V. Quantification of measles, mumps and rubella viruses using real-time quantitative TaqMan-based RT-PCR assay. *J Virol Methods.* 2013; 187(1): 57-64.
- [174] Leblanc JJ, Pettipas J, Davidson RJ, Tipples GA, Hiebert J, Hatchette TF. Detection of mumps virus RNA by real-time one-step reverse transcriptase PCR using the Light-Cycler platform. *J Clin Microbiol.* 2008; 46(12): 4049-4051.
- [175] Ando K, Ishihara M, Kusumoto Y, Shibuya E, Nakamura S, Mizuki N. A case of corneal endotheliitis with mumps virus RNA in aqueous humor detected by rt-PCR. *Ocul Immunol Inflamm.* 2013; 21(2): 150-152.
- [176] Wolfson LJ, Grais RF, Luquero FJ, Birmingham ME, Strebel PM. Estimates of measles case fatality ratios: a comprehensive review of community-based studies. *Int J Epidemiol.* 2009; 38: 192-205.
- [177] Bellini WJ, Rota JS, Lowe LE, Katz RS, Dyken PR, Zaki SR, Shieh WJ, Rota PA. Subacute sclerosing panencephalitis: more cases of this fatal disease are prevented by measles immunization than was previously recognized. *J Infect Dis.* 2005; 192: 1686-1693.
- [178] Griffin DE, Lin WH, Pan CH. Measles virus, immune control, and persistence. *FEMS Microbiol Rev.* 2012; 36(3): 649-662.
- [179] Cairns KL, Perry RT, Ryman TK, Nandy RK, Grais RF. Should outbreak response immunization be recommended for measles outbreaks in middle-and low-income countries? An update. *J Infect Dis.* 2011; 204: S35-S46.
- [180] Simons E, Ferrari M, Fricks J, Wannemuehler K, Anand A, Burton A, Strebel P. Assessment of the 2010 global measles mortality reduction goal: results from a model of surveillance data. *Lancet.* 2012; 379(9832): 2173-2178.
- [181] World Health Organization. Measles fact sheet No.286 External Web Site Icon. Accessed Feb 2013.
- [182] Shanks GD, Lee S-H, Howard A, Brunden KR. Extreme mortality after first introduction of measles virus to the Polynesian Island of Rotuma, 1911. *Am J Epidemiol.* 2011; 173:1211-1222.
- [183] Anlar B. Subacute sclerosing panencephalitis and chronic viral encephalitis. *Handb Clin Neurol.* 2013; 112: 1183-1189.

- [184] Rima BK, Duprex WP. Morbilliviruses and human disease. *J Pathol.* 2006; 208(2): 199-214.
- [185] Sips GJ, Chesik D, Glazenburg L, Wilschut J, De Keyser J, Wilczak N. Involvement of morbilliviruses in the pathogenesis of demyelinating disease. *Rev Med Virol.* 2007; 17(4): 223-244.
- [186] Kutty P, Rota J, Bellini W, Redd SB, Barskey A, Wallace G. Chapter 7: Measles. *Manual for the Surveillance of Vaccine-Preventable Diseases.* 6th Edition, 2013.
- [187] Bellini WJ, Helfand RF. The challenges and strategies for laboratory diagnosis of measles in an international setting. *J Infect Dis.* 2003; 187 Suppl 1: S283-90.
- [188] Centers for Disease Control and Prevention. Recommendations from an ad hoc meeting of the WHO measles and Rubella Laboratory Network (LabNet) on use of alternative diagnostic samples for measles and rubella surveillance. *MMWR Morb Mortal Wkly Rep* 2008; 57: 657-660.
- [189] Hutse V, Van Hecke K, De Bruyn R, Samu O, Lernout T, Muyembe JJ, Brochier B. Oral fluid for the serological and molecular diagnosis of measles. *Int J Infect Dis.* 2010; 14(11): e991.
- [190] Sampedro A, Rodríguez-Granger J, Gómez C, Lara A, Gutierrez J, Otero A. Comparative evaluation of a new chemiluminiscent assay and an ELISA for the detection of IgM against measles. *J Clin Lab Anal.* 2013; 27(6): 477-480.
- [191] Haywood B, Patel M, Hurday S, Copping R, Webster D, Irish D, Haque T. Comparison of automated chemiluminescence immunoassays with capture enzyme immunoassays for the detection of measles and mumps IgM antibodies in serum. *J Virol Methods.* 2013; 196C: 15-17.
- [192] Sanz JC, Mosquera M, Ramos B, Ramírez R, de Ory F, Echevarria JE. Assessment of RNA amplification by multiplex RT-PCR and IgM detection by indirect and capture ELISAs for the diagnosis of measles and rubella. *APMIS.* 2010; 118(3): 203-209.
- [193] Smits GP, van Gageldonk PG, Schouls LM, van der Klis FR, Berbers GA. Development of a bead-based multiplex immunoassay for simultaneous quantitative detection of IgG serum antibodies against measles, mumps, rubella, and varicella-zoster virus. *Clin Vaccine Immunol.* 2012; 19(3): 396-400.
- [194] The Fifth Regional Hands-On Training Workshop. On The Laboratory Diagnosis of Measles And Rubella. World Health Organization. Regional Office for The Western Pacific. Hong Kong (China). 29 October to 3 November 2012.
- [195] Thomas B, Beard S, Jin L, Brown KE, Brown DWG. Development and evaluation of a real-time PCR assay for rapid identification and semiquantitation of measles virus. *J Med Virol.* 2007; 79: 1587-92.

- [196] Kühne M, Brown DW, Jin L. Genetic variability of measles virus in acute and persistent infections. *Infect Genet Evol.* 2006; 6: 269–276.
- [197] Lenisha Warrenner, Rimantas Slibinskas, Kaw Bing Chua, Wondatir Nigatu, Kevin E Brown, Kestutis Sasnauskas, Dhanraj Samuel, David Brown. A point-of-care test for measles diagnosis: detection of measles-specific IgM antibodies and viral nucleic acid. *Bull World Health Organ.* 2011; 89(9): 675–682.
- [198] Sheikhabari S, Mokhtari-Azad T, Salimi V, Norouzbabaei Z, Abbasi S, Zahraei SM, Shahmahmoodi S. The use of oral fluid samples spotted on filter paper for the detection of measles virus using nested rt-PCR. *J Clin Lab Anal.* 2012; 26(3): 215–222.
- [199] Uzicanin A, Lubega I, Nanuynja M, Mercader S, Rota P, Bellini W, Helfand R. Dried blood spots on filter paper as an alternative specimen for measles diagnostics: detection of measles immunoglobulin M antibody by a commercial enzyme immunoassay. *J Infect Dis.* 2011; 204 Suppl 1: S564–S569.
- [200] Akiyoshi K, Suga T, Nukuzuma S, Kon-no M, Shibata M, Itoh M, Ito M, Ihara T. Reevaluation of laboratory methods for diagnosis of measles. *Jpn J Infect Dis.* 2010; 63(4): 225–8.
- [201] Giusti D, Burette J, Nguyen Y, Lévêque N, Graesslin O, Andreoletti L. Virological diagnosis and management of two cases of congenital measles. *J Med Virol.* 2013; 85(12): 2136–2138.
- [202] Ikeno S, Suzuki MO, Muhsen M, Ishige M, Kobayashi-Ishihara M, Ohno S, Takeda M, Nakayama T, Morikawa Y, Terahara K, Okada S, Takeyama H, Tsunetsugu-Yokota Y. Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR. *Front Microbiol.* 2013; 4: 298.
- [203] Ito M, Suga T, Akiyoshi K, Nukuzuma S, Kon-no M, Umegaki Y, Kohdera U, Ihara T. Detection of measles virus RNA on SYBR green real-time reverse transcription-polymerase chain reaction. *Pediatr Int.* 2010; 52(4): 611–615.
- [204] Ammour Y, Faizuloev E, Borisova T, Nikonova A, Dmitriev G, Lobodanov S, Zverev V. Quantification of measles, mumps and rubella viruses using real-time quantitative TaqMan-based RT-PCR assay. *J Virol Methods.* 2013; 187(1): 57–64.
- [205] Maksimov NL, Agafonov AP, Drozdov IG. The application of xMAP technology in genetic typing of measles virus. *Klin Lab Diagn.* 2011; (8): 46–58.
- [206] Bankamp B, Byrd-Leotis LA, Lopareva EN, Woo GK, Liu C, Jee Y, Ahmed H, Lim WW, Ramamurty N, Mulders MN, Featherstone D, Bellini WJ, Rota PA. Improving molecular tools for global surveillance of measles virus. *J Clin Virol.* 2013; 58(1): 176–82.
- [207] Michel Y, Saloum K, Tournier C, Quinet B, Lassel L, Pérignon A, Grimpel E, Carbal R, Vabret A, Freymuth F, Garbarg-Chenon A, Schnuriger A. Rapid molecular di-

agnosis of measles virus infection in an epidemic setting. *J Med Virol.* 2013; 85(4): 723-30.

- [208] Measles Lab Manual from the World Health Organization. Manual for the Laboratory Diagnosis of Measles and Rubella Virus Infection, 2nd edition, August 2007 from the World Health Organization (WHO).
- [209] Amadi K, Sabo AM, Ogunkeye OO, Oluwole FS. Thyroid hormone: a "prime suspect" in human immunodeficiency virus (HIV/AIDS) patients?. *Nigerian Journal of Physiological Sciences: official publication of the Physiological Society of Nigeria.* 2008; 23: 61– 66.
- [210] McCutchan FE. Global epidemiology of HIV. *J Med Virol.* 2006; 78: S7-S12.
- [211] HIV and its transmission. Centers for Disease Control and Prevention HIV/AIDS Fact Sheets Web site. <http://www.cdc.gov/hiv/resources/factsheets/transmission.htm>. July 1999.
- [212] Centers for Disease Control and Prevention. HIV-2 Infection Surveillance-United States, 1987-2009. *MMWR.* 2011; 60(29): 985-988.
- [213] Branson BM, Stekler JD. Detection of acute HIV infection: we can't close the window. *J Infect Dis* 2012; 205: 521–524.
- [214] O'Connell RJ, Peel SA. Multispot HIV-1/HIV-2 Rapid Test: advantages over other rapid HIV tests. *Expert Rev Mol Diagn.* 2007; 7(5): 499-505.
- [215] Bentsen C, McLaughlin L, Mitchell E, Ferrera C, Liska S, Myers R, Peel S, Swenson P, Gadelle S, Shriver MK. Performance evaluation of the Bio-Rad Laboratories GS HIV Combo Ag/Ab EIA, a 4th generation HIV assay for the simultaneous detection of HIV p24 antigen and antibodies to HIV-1 (groups M and O) and HIV-2 in human serum or plasma. *J Clin Virol.* 2011; 52 (Suppl 1): S57-S61.
- [216] Branson BM. The future of HIV testing. *J. Acquir. Immune Defic. Syndr.* 2010; 55 (Suppl. 2): S102–S105.
- [217] Song EY, Hur M, Roh EY, Park MH, Moon HW, Yun YM. Performances of four fourth-generation human immunodeficiency virus-1 screening assays. *J Med Virol.* 2012; 84(12): 1884-8.
- [218] Sano T, Kondo M, Yoshimura Y, Tachikawa N, Sagara H, Itoda I, Yamanaka K, Sudo K, Kato S, Imai M. Evaluation of a new version of the human immunodeficiency virus antigen and antibody combination assay with improved sensitivity in HIV-1 p24 antigen detection. *Kansenshogaku Zasshi.* 2013; 87(4): 415-23.
- [219] Fransen K, Vermoesen T, Beelaert G, Menten J, Hutse V, Wouters K, Platteau T, Florence E. Using conventional HIV tests on oral fluid. *J Virol Methods.* 2013; 194(1-2): 46-51.



- [220] O’Gorman MR, Weber D, Landis SE, Schoenbach VJ, Mittal M, Folds JD. Interpretive criteria of the Western blot assay for serodiagnosis of human immunodeficiency virus type 1 infection. *Arch Pathol Lab Med.* 1991; 115: 26–30.
- [221] The Consortium for Retrovirus Serology Standardization Serological diagnosis of human immunodeficiency virus infection by Western blot testing. *JAMA.* 1988; 260: 674–679.
- [222] Tebourski F, Slim A, Elgaaied A. The significance of combining World Health Organization and Center for Disease Control criteria to resolve indeterminate human immunodeficiency virus tipe-1 Western blot results. *Diagn Microbiol Infect Dis.* 2004; 48: 59–61.
- [223] García F, Álvarez M, Bernal C, Chueca N, Guillot V. Laboratory diagnosis of HIV infection, viral tropism and resistance to antiretrovirals. *Enferm Infecc Microbiol Clin.* 2011; 29(4): 297–307.
- [224] Grossman Z, Kra-Oz Z. HIV testing: the medical laboratory role in HIV diagnosis and monitoring. *Harefuah.* 2013; 152(8): 477–80, 498.
- [225] Brennan CA, Yamaguchi J, Vallari A, Swanson P, Hackett JR Jr. ARCHITECT HIV Ag/Ab Combo assay: correlation of HIV-1 p24 antigen sensitivity and RNA viral load using genetically diverse virus isolates. *J Clin Virol.* 2013; 57(2): 169–172.
- [226] Karris MY, Anderson CM, Morris SR, Smith DM, Little SJ. Cost savings associated with testing of antibodies, antigens, and nucleic acids for diagnosis of acute HIV infection. *J Clin Microbiol.* 2012; 50(6): 1874–1878.
- [227] Branson BM, Mark Pandori M. 2012 HIV Diagnostics Conference: the molecular diagnostics perspective. *Expert Rev. Mol. Diagn.* 2013; 3(3): 243–245.
- [228] Bourlet T, Memmi M, Saoudin H, Pozzetto B. Molecular HIV screening. *Expert Rev Mol Diagn.* 2013; 13(7): 693–705.
- [229] Mohammadi-Yeganeh S, Paryan M, Mirab Samiee S, Kia V, Rezvan H. Molecular beacon probes-base multiplex NASBA Real-time for detection of HIV-1 and HCV. *Iran J Microbiol.* 2012; 4(2): 47–54.
- [230] Gueudin M, Leoz M, Lemée V, De Oliveira F, Vessière A, Kfutwah A, Plantier JC. A new real-time quantitative PCR for diagnosis and monitoring of HIV-1 group O infection. *J Clin Microbiol.* 2012; 50(3):831–6.
- [231] Tang YW, Ou CY. Past, present and future molecular diagnosis and characterization of human immunodeficiency virus Infections. *Emerg Microbes Infect.* 2012; 1(8): e19.
- [232] Yoshimura Y, Techikawa N. HIV tests. *Rinsho Byori.* 2008; 56(11): 1019–25.
- [233] Vickerman P, Watts C, Peeling RW, Mabey D, Alary M. Modelling the cost effectiveness of rapid point of care diagnostic tests for the control of HIV and other sexually

- transmitted infections among female sex workers. *Sex Transm Infect.* 2006; 82: 403–412.
- [234] De Cock KM, Fowler MG, Mercier E et al. Prevention of mother-to child HIV transmission in resource-poor countries: translating research into policy and practice. *JA-MA* 2000; 283: 1175–1182.
- [235] Menzies NA, Homsy J, Chang Pitter JY, Pitter C, Mermin J, Downing R, Finkbeiner T, Obonyo J, Kekitiinwa A, Tappero J, Blandford JM. Cost-effectiveness of routine rapid human immunodeficiency virus antibody testing before DNA-PCR testing for early diagnosis of infants in resource-limited settings. *Pediatr Infect Dis J.* 2009; 28: 819–825.
- [236] Loubiere S, Moatti JP. Economic evaluation of point-of-care diagnostic technologies for infectious diseases. *Clin Microbiol Infect.* 2010 Aug; 16(8):1070-6.
- [237] Schito ML, D'Souza MP, Owen SM, Busch MP. Challenges for rapid molecular HIV diagnostics. *J Infect Dis.* 2010; 201 (Suppl 1): S1-S6.
- [238] Korber B, Gaschen B, Yusim K, Thakallapally R, Kesmir C, Detours V. Evolutionary and immunological implications of contemporary HIV-1 variation. *Br Med Bull.* 2001; 58: 19–42.
- [239] Lauring AS, Andino R. Quasispecies theory and the behavior of RNA viruses. *PLoS Pathog.* 2010; 6: e1001005.
- [240] Di Giallonardo F, Zagordi O, Duport Y, Leemann C, Joos B, Künzli-Gontarczyk M, Bruggmann R, Beerenwinkel N, Günthard HF, Metzner KJ. Next-Generation Sequencing of HIV-1 RNA Genomes: Determination of Error Rates and Minimizing Artificial Recombination. *PLoS One.* 2013; 8(9):e74249.
- [241] Mild M, Hedskog C, Jernberg J, Albert J. Performance of ultra-deep pyrosequencing in analysis of HIV-1 pol gene variation. *PLoS One.* 2011; 6: e22741.
- [242] Guy Vernet. Molecular diagnostics in virology. *Journal of Clinical Virology.* 2004; 31-8(4): 239-247.
- [243] Gorzer I, Guelly C, Trajanoski S, Puchhammer-Stockl E. The impact of PCR-generated recombination on diversity estimation of mixed viral populations by deep sequencing. *J Virol Methods.* 2010; 169: 248–252.

