Molecular Virology

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L. Cheng, D.Y. Zhang, J.N. Eble (eds.), *Molecular Genetic Pathology*,
DOI 10.1007/978-1-4614-4800-6_25,

25.1 General

- Limitation and pitfalls
  - In the past decade, molecular methods for detection and quantification of virus infections have replaced many traditional viral culture and serological methods
  - The molecular tests significantly improved clinical turnaround time and reduced hands-on time in addition to increase the diagnostic sensitivity and specificity
  - Many commercial assay kits and automatic instruments are available which allow many clinical microbiology laboratories to offer molecular tests
  - However, the routine implementation of nucleic acid (both DNA and RNA) amplification and hybridization methodologies in clinical laboratories is still associated with a number of limitations
    - Increased cost/test due to expensive instrumentation and reagents
    - Amplification carryover contamination
    - Standardization of positive, negative, and quantitative controls
    - Integrated coamplified internal DNA control to demonstrate absence of polymerase chain reaction (PCR) inhibitors and amplification
    - Prevention of false-positive and false-negative reports due to antigenic and pathogen nucleic acid sequence drift and accurate interpretation of data and software analyses
- Specimens
  - Collection of adequate specimens is important for molecular diagnosis of virus. These are specimen types commonly used for molecular diagnosis
  - Whole blood: 3–5 mls collected in an EDTA (lavender top) tube. Store at 4–25 °C. Do not freeze
  - Plasma: Collect 7–10 mls of whole blood in EDTA, ACD solution A, or PPT sterile tube. Store whole blood at room temperature (18–30 °C) for no more than 4 h. Remove plasma from cells

within 4 h of collection by centrifugation at 1,000 × g for 10–15 min. Do not clarify by filtration or further centrifugation. Store plasma at –60 to –80 °C within 30 min of separation. Plasma may also be stored at –20 °C in nonfrost-free freezer for up to 72 h if colder freezer is not available. Ship on dry ice for overnight delivery. The minimum volume of specimen is 2 mls of plasma
  - Urine: first 10–20 mls of voided urine collected in a sterile urinalysis container (15 ml sterile screw cap tube preferred). Store at 4–25 °C for less than 24 h or store at –70 °C for long term
  - Bronchial lavage/tracheal aspirate: 1–4 mls, collected in a sterile tube. Store at 4–25 °C for less than 24 h
  - Bone marrow: 1–2 mls, collected in EDTA tube. Store at 4–25 °C. Do not freeze
  - Tissue: ~0.5-cm tissue block collected in a sterile screw-top container, add small amount of saline to keep it moist. Avoid the use of viral transport media to avoid potential inhibition of PCR. Fresh tissues should be stored at –72 °C immediately to preserve the nucleic acids
    - Paraffin-embedded tissue is acceptable. Usually 5–10 sections (5 μm thickness) are sufficient for PCR analysis. The tissue sections must be deparaffinized with xylene before DNA extraction
  - Fecal: sterile swab (plastic shaft only) or very small fecal sample placed in 1–2 mls sterile saline in a container with tight fitting lid. Do not use viral transport media to avoid potential inhibition of PCR
  - Swab: sterile swab (plastic shaft only) placed in 1–2 mls sterile saline. Do not use viral transport media to avoid potential inhibition of PCR
  - Cerebrospinal fluid (CSF): 1–1.5 mls fluid, submitted in a sterile, leakproof tube, store at 4–25 °C for less than 24 h or store at –70 °C for long term
- Assay performance analysis
  - Analytical performance
    - Analytical sensitivity: to determine the lowest number of targets that can be detected by the assay
    - Cross-reactivity (specificity): to determine if the assay can produce false-positive results in the presence of high concentration of other similar or unrelated pathogens (bacteria, yeast, and virus)
    - Linearity: to evaluate the log differences from the expected concentration; this difference should be within ±0.1 log (or a ratio of observed mean quantitation to expected concentration within 95%)
    - Quantitative range: the measured concentrations within the linear range with a good reproducibility
  - Clinical performance
    - Limits of detection: the lowest concentration of target nucleic acids that can be detected (at or above the detection cutoff in 95% of replicates, usually 10 replicates)
    - Detection cutoff: the point on the assay quantitation scale such that 95% of negative specimens produce results below this cutoff with 95% confidence
    - Limits of quantification: the lowest concentration of target nucleic acids that can be quantified in 95% of replicates
    - Reproducibility: The reproducibility of the test is usually established by testing three to six sample panels with known concentrations of target in triplicate or quadruplicate. A commercial panel should be used to establish this parameter, if available. Reproducibility is expressed as percent correlation coefficient. For quantitative assays, the CVs range from 10% to 50%
    - Precision: the reproducibility of a test result (e.g., inter- and intratechnologist and inter- and intra-assay)
    - Sensitivity: true positive samples, % of true positive samples above the limits of detection
    - Specificity: true negative samples, % of true negative samples below the limits of detection

- Quality controls: For quantitative assay, additional quality control procedures should be performed, including calibration and calibration verification
  - Calibration is the set of operations that establish, under specified conditions, the relationship between reagent system/instrument response and the corresponding concentration/activity values of an analyte. Calibration procedures are typically specified by a method manufacturer, but may also be established by the laboratory
  - Calibration verification denotes the process of confirming that the current calibration settings remain valid for a method
  - Recalibration or calibration verification and analytical measurement range validation must be performed at least once every 6 months
  - For each run, sensitivity controls should be included in addition to positive and negative controls. It is recommended that two levels of controls (high and low) should be included

25.2 Human Immunodeficiency Virus

25.2.1 General Characteristics

- Human immunodeficiency virus (HIV) is an RNA retrovirus belonging to the lentivirus family. HIV1 and HIV2 are genetically different; HIV2 shares 40% nucleotide homology with HIV1. HIV2 is more related to SIV than to HIV1. Both types appear to cause clinically indistinguishable AIDS. However, it seems that HIV2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV2. Worldwide, the predominant virus is HIV1, and generally, when people refer to HIV without specifying the type of virus, they will be referring to HIV1. The relatively uncommon HIV2 is concentrated in West Africa and is rarely found elsewhere
- Structure of HIV virion (Fig. 25.1)
  - HIV virus consists of a spherical viral particle encased in a lipid bilayer derived from host cell covered by protruding peg-like structures composed of gp41 and gp120 glycoproteins
  - The virus core nucleocapsid contains the major capsid protein, p24; two copies of genomic RNA; and three viral enzymes (protease, reverse transcriptase, and integrase)
- Viral replication (Fig. 25.2)
  - The first step of infection is entry into the host cell, which requires binding of the gp120 molecule on the virus to CD4 molecules on the host cell's surface, and is mediated by the gp41 molecule. Two surface molecules CCR5 and CXCR4, chemokine receptors for beta-chemokines and alpha-chemokines are also required for entry
  - Once bound, the viral envelope fuses with the cell membrane and the virus' RNA and enzymes enter the cytoplasm
  - Reverse transcriptase catalyzes, first, the synthesis of a DNA copy of the viral RNA and, second, the synthesis of a second DNA strand complementary to the first one. Therefore, a double-stranded DNA (dsDNA) is generated
  - Integrase then facilitates the integration of viral DNA into the cellular chromosome when the cell divides and provides latency enabling the virus to effectively evade host responses
  - Transcription of the DNA results in the production of RNA. This RNA can serve as the genome for new viruses and can be translated to produce viral proteins. Viral proteins are facilitated by protease and assembled into viral particles using the host cell's protein-making machinery
  - Complete HIV particles are assembled. In macrophages, HIV buds out of the cell without rupturing the cell, and the cycle begins again. In T cells, HIV exits the cell by rupturing it, effectively killing the cell
- The gag, pol, and env genes encode for structural proteins for new virus particles.

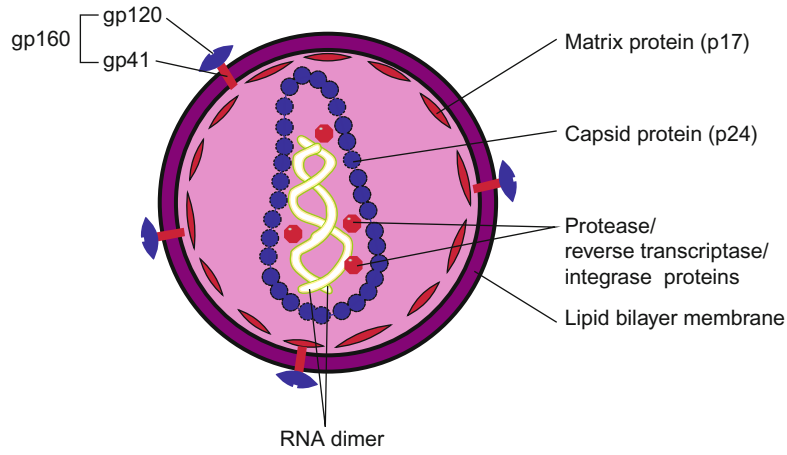


Fig. 25.1 HIV viral particle (www.mcld.co.uk/hiv)

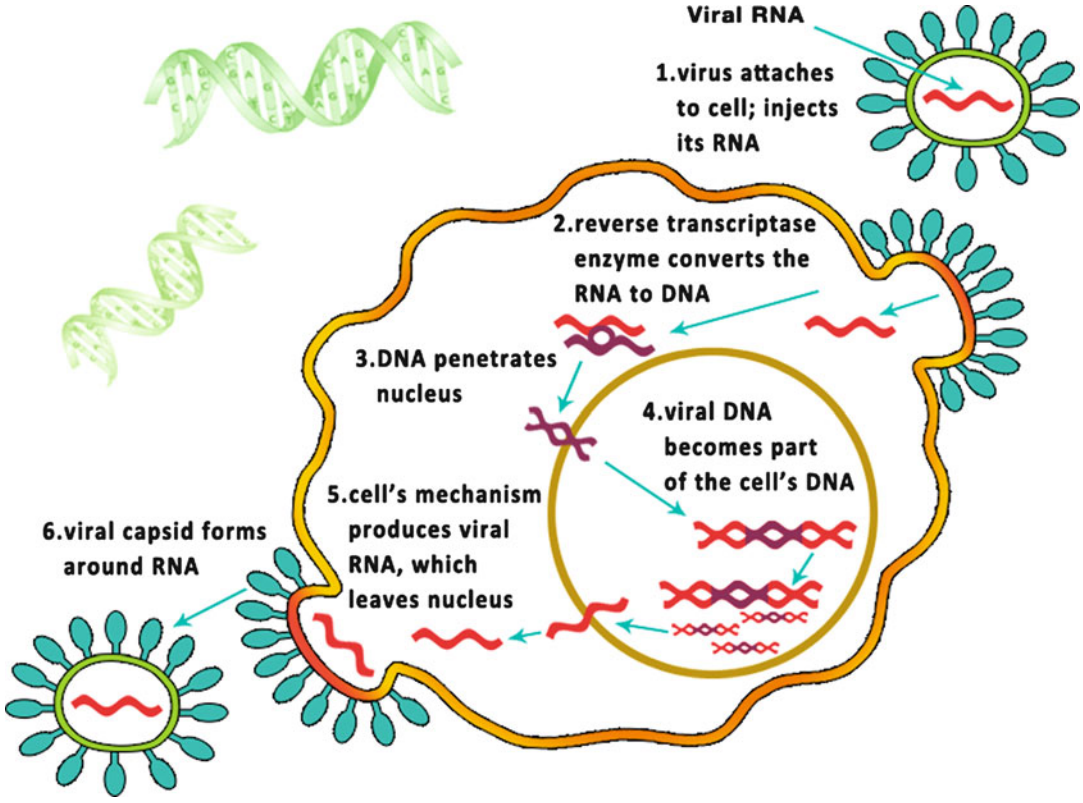


Fig. 25.2 The replication cycle of HIV

The other six genes, tat, rev, nef, vif, vpr, and vpu, regulate the synthesis and assembly of viral particles

- The phylogenic analysis of the nucleotide sequences of the env gene has enabled classification of HIV1 into three groups: M (major), N (non-M), and O (outlier). The group M of HIV1 infection has been classified into nine different genetic subtypes A–K. More than 90% of HIV1 infections belong to HIV1 group M. Subtype/class B is the most prevalent in the developed world

- HIV is transmitted via sexual contact, blood (via transfusion, blood products, or contaminated needles), or passage from mother to child (in utero, during birth, or ingestion of breast milk). Although saliva can contain small quantities of the virus, the virus cannot be spread by kissing. HIV is not spread by the fecal–oral route, aerosols, insects, or casual contact

25.2.2 Clinical Presentation

- HIV is the causative agent of acquired immunodeficiency syndrome (AIDS), the leading cause of death in humans between the ages of 25–44 years
- Two main targets of HIV: immune system and central nervous system. HIV targets CD4+ T cells, monocytes/macrophages, and Langerhans cells/dendritic cells causing severe immunosuppression and neuropathologic symptoms such as dementia, meningitis, and encephalopathy in the host
- Common opportunistic infections: *Pneumocystis carinii*, candidiasis, tuberculosis, *Cryptococcus*, cytomegaloretinitis
- Common malignancies: Kaposi sarcoma, lymphoma (non-Hodgkin and brain primary), and uterine carcinoma
- Individuals who have HIV face a long challenging road. The disease has a steady natural history, starting with an asymptomatic state and progressing toward AIDS. Natural history includes three phases
  - Early-stage HIV infection is defined as the presence of HIV with a CD4 count greater than 500. Early stage develops 3–6 weeks after initial exposure with self-limited flu-like symptoms resolving 2–4 weeks later in 50–60% of patients by high level of viral production, viremia, and widespread seeding of lymphoid tissues
  - Chronic phase HIV infection occurs when the CD4 count is between 200 and 500. Chronic phase is associated with a period of latency in which the immune system is intact, but there is continuous HIV replication that may last for years. Patients are either asymptomatic or develop persistent lymphadenopathy with minor opportunistic infections, such as candidiasis or herpes zoster
  - When the CD4 count drops below 200, the HIV infection has entered the crisis phase. This is when certain infections that are easily handled by an intact immune system take advantage of this immunocompromised state (opportunistic infections). Certain cancers may also appear for the same reason. When a patient has a CD4 count less than 200 and at least one opportunistic infection or cancer specifically seen in crisis phase HIV, he or she is officially designated as having AIDS

25.2.3 Diagnostic Methods

- Specimens – whole blood, serum, and plasma (Table 25.1)
- Conventional tests and problems
  - Lymphocyte count
    - D4 cells (also called T cells or T-helper cells) are the primary targets of the HIV virus. Quantitation of CD4 cells was the first effective predictor of HIV progression. The CD4 count is one of many factors (including clinical status, HIV viral load, and medication adherence) that should be assessed before starting or changing antiretroviral (ARV) treatment
    - The CD4 cell count (<200 cells/mm^3) is important in determining the staging of HIV disease and for indicating the need for prophylaxis against opportunistic pathogens
    - Most laboratories report the CD4 count as part of a list of several types of lymphocytes, as both an absolute count and a relative percentage. Measurement and trending of CD4 percentage in addition to absolute count must be performed prior to initiation or adjustment of ARV treatment management decisions
    - The CD4 percentage sometimes is used in coordination with the absolute value

Table 25.1 Specimens handling in different HIV assays

Assay	Collection	Transport	Storage	Comments
Antibody screening assay	Serum (including serum collected in serum separator tubes) or plasma containing heparin, EDTA, citrate, or CPDA1 anticoagulants		For long-term storage, specimens should be stored frozen. Specimens can be stored at 2–8 °C for a maximum of 14 days	Samples may be tested up to three freeze–thaw cycles
HIV monitoring assay	Plasma specimens anticoagulated with EDTA or ACD only. Specimens must **not** be anticoagulated with heparin	Whole blood should be stored at 2–25 °C for no longer than 6 h. Plasma must be separated within 6 h of collection by centrifugation at 800–1,600x g for 20 min at room temperature and transferred to a polypropylene tube to prevent viral degradation	Plasma may be stored at 2–8 °C for up to 5 days or frozen at –70 °C	Specimens should be stored in 600–700 ul aliquots in sterile, 2 ml polypropylene tubes. Freeze–thaw studies have shown that specimens may be tested for up to three freeze–thaw cycles without loss of viral RNA
HIV genotyping	Plasma specimens anticoagulated with EDTA. Specimens must not be anticoagulated with heparin	Whole blood should be stored at 2–25 °C for no longer than 2 h. Plasma should be separated within 30 mins, but no later than 120 min by centrifugation at 1,000–2,000x g for 15 min at 15–25 °C and transferred to a polypropylene tube	Plasma may be stored frozen at –65–80 °C for up to 6 months	Samples may be tested up to two freeze–thaw cycles. Plasma specimens containing the following have been shown to interfere with results: lipids up to 30 mg/ml bilirubin up to 0.6 mg/ml hemoglobin up to 5 mg/ml

to assess the significance of changes in the absolute CD4 count. The absolute CD4 count can fluctuate as overall lymphocyte counts vary, but the CD4 percentage often remains stable during insignificant CD4 fluctuations. CD8 cell (or cytotoxic T cell) counts do not appear to predict clinical outcomes

- For monitoring purposes, the CD4 count should be repeated approximately every 3–4 months both in stable untreated patients and in patients on stable ART. The CD4 count should be checked more frequently according to the clinical situation

– Viral culture
  - Although very specific, single positive culture must be confirmed with a second specimen
  - Rarely used due to high cost, labor-intensive, and less sensitivity than antibody testing
  - Negative culture may be caused by technical problems, a defective virus, or the inability of the virus to replicate in culture
– Serological studies
  - p24 antigen
    – Early developed assay to detect HIV infection and screen donated blood for HIV
    – Advantage is to detect HIV infection prior to development of antibodies
    – Disadvantage is limited utility due to the short window of time and should only be used when other tests are unavailable

- Antibody screening assays (qualitative)
  - Detection of antibodies to HIV is the most common way to diagnose HIV infection in adults and children >18 months old
  - These antibodies are usually detectable within 3–6 weeks after infection
  - Most individuals seroconvert by 12 weeks, although may not be detectable for months or years
  - The window period is the time between infection and the development of antibodies. When a person is infected with HIV, it takes a few weeks for the body to make antibodies to the virus. Most people develop antibodies within a month of infection, although some people can take up to 3 months. In very rare cases, it can take 6 months of the test to be positive. In general, repeat testing for HIV should occur at 3 months if there is a significant concern of recent HIV infection. Additionally, because the level of virus in blood is high during the window period, people can more easily transmit HIV
  - Serologic HIV antibody screening testing is highly sensitive (ELISA, rapid test, or home test), but requires followup of preliminary positive specimens with a highly specific HIV antibody confirmatory assay (Western blot) (Fig. 25.3)
  - ELISA method is most common and earliest developed antibody screening assay
  - Home Access HIV1 test system analyzes a dried-blood spot from finger stick collected on filter paper at home and sent to a testing facility
  - Rapid tests for HIV are assays that detect antibodies to HIV within minutes. The rapid test is highly specific: negative means negative except during window period; and the test is also highly sensitive: positive means most likely has HIV, but must be confirmed using Western blot for HIV diagnosis
- Confirmatory antibody assays: Western blot
  - Gold standard for HIV diagnostic testing
  - The virus is disrupted, and the individual proteins are separated by molecular weight via differential migration on a polyacrylamide gel and blotted onto a membrane support. HIV serum antibodies from the patient are allowed to bind to the proteins in the membrane support, and patterns of reactivity can be visibly read
  - Detects three major proteins/viral bands: p24 core protein and two envelope proteins, gp41 and gp120/160
  - Reactive WB demonstrates antibody to two of the three major bands; nonreactive WB will have no detectable viral bands (Fig. 25.4)
  - Repeated reactivity by ELISA and reactivity by the confirmatory assay are reported as positive for antibody to HIV1
  - Nonreactive specimens by ELISA or repeatedly reactive by ELISA and nonreactive by the confirmatory assay are negative for antibody to HIV1
  - WB in which serum antibodies bind to any other combination of viral bands is considered indeterminate; followup blood specimen should be obtained 1 month later for repeat HIV antibody testing
  - Individuals with repeat indeterminate results should undergo further testing using molecular assays, such as PCR
  - At least as sensitive as and more specific than screening assays, although they are not as sensitive in the detection of early seroconversion
  - Disadvantages: more labor-intensive, more prone to subjective interpretation, and more costly than screening assays

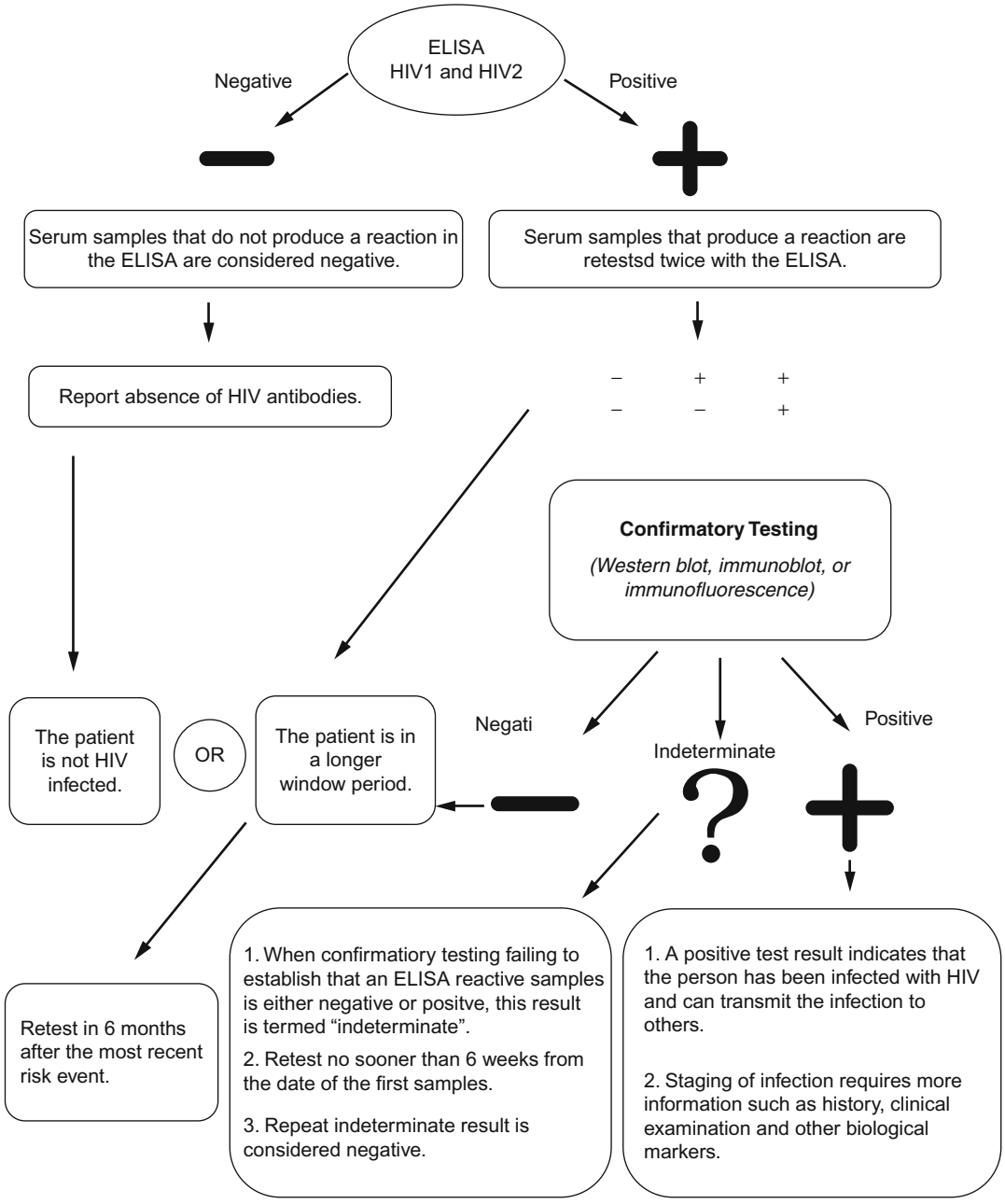
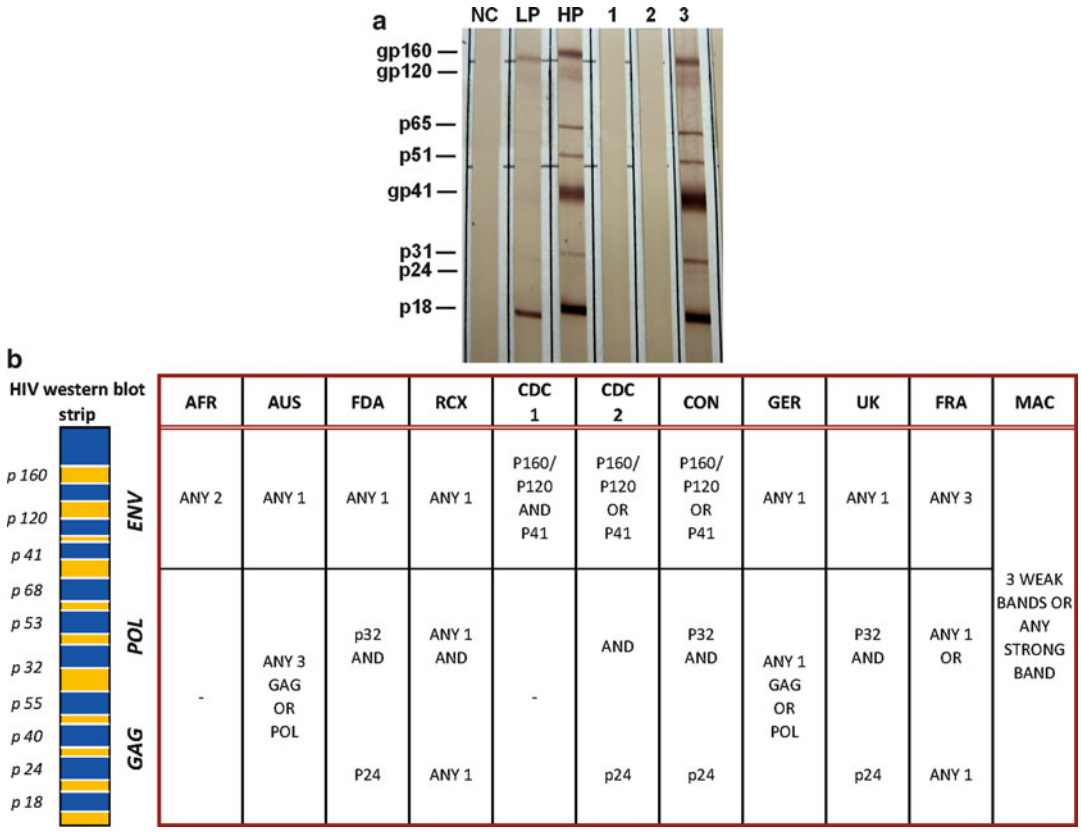


Fig. 25.3 HIV antibody screening algorithm

- Alternative antibody screening assay (qualitative)
  - US Food and Drug Administration (FDA) has approved assays that test body fluids other than blood to detect HIV1 antibodies, although sensitivity and specificity are less reliable
  - Utilizes same testing algorithm as serum (ELISA followed by WB)



	AFR	AUS	FDA	RCX	CDC 1	CDC 2	CON	GER	UK	FRA	MAC
ENV	ANY 2	ANY 1	ANY 1	ANY 1	P160/ P120 AND P41	P160/ P120 OR P41	P160/ P120 OR P41	ANY 1	ANY 1	ANY 3	3 WEAK BANDS OR ANY STRONG BAND
POL	-	ANY 3 GAG OR POL	p32 AND	ANY 1 AND	-	AND	P32 AND	ANY 1 GAG OR POL	P32 AND	ANY 1 OR	
GAG			P24	ANY 1		p24	p24		p24	ANY 1	

Fig. 25.4 (**a**) Western blot analysis and band pattern interpretation: *NR* negative control, *LR* low reactive control, *HR* high reactive control, *1–2* nonreactive, *3* reactive. (**b**) Criteria used to define a positive HIV Western blot. *AFR* Africa, 1 *AUS* Australia, 2 *FDA* US Food and Drug Administration, 3 *RCX* US Red Cross, 3 *CDC* US Center for Disease Control, 3 *CON* US Consortium for Retrovirus Serology Standardization, 3 *GER* Germany, *UK* United Kingdom, *FRA* France, *MACS* US Multicenter AIDS Cohort Study 1983–1992. *Bands not in electrophoretic order

  - Advantages are noninvasive sample collection, increased safety due to lack of needles, and disposal of infectious waste minimized
  - Oral fluid (oral mucosal transudate): antibodies detectable, but significantly lower (800– 1,000-fold) than those of serum
  - Urine: Interpretative criteria for a reactive WB requires only presence of visible band at gp160 region
- Molecular methods
  - Qualitative assay: viral identification assays – recommended for resolving indeterminate Western blot results
    - DNA PCR (Roche)
    - The HIV DNA PCR test is a new technology used for HIV early detection testing. The new HIV PCR test looks for the DNA copy of the HIV virus itself in peripheral blood. It is extremely accurate, sensitive, and the cutting edge of HIV early detection testing
    - The average window period with HIV1 antibody tests is 25 days. Antigen testing cuts the window period to approximately 16 days, and HIV DNA test further reduces this period to 12 days
    - Detection of HIV DNA in peripheral blood mononuclear cells by PCR is recommended for children <18 months old born to HIV1-infected mothers

Table 25.2 Comparison of commonly used HIV viral load assays

Characteristic	1) Amplicor HIV-1 Monitor; 2) COBAS® AmpliPrep/ COBAS® TaqMan® HIV-1 Test,v2.0 [*Roche*]	Branched Chain DNA (bDNA) [*Versant*]	Nucleic Acid Sequence-based Assay (NASBA) [bioMérieux]
Amplification method	Target amplification	Signal amplification	Target amplification
Specimen type	Plasma in ACD or EDTA tube	Plasma in EDTA tube	Plasma in ACD, EDTA, or heparin tube
Specimen volume	Standard 1.5:0.2 mL Ultrasensitive 1.5:0.5 mL V2.0:0.5 mL	1.0–2.0 mL	1.0 mL
Specimen transport	Prepare plasma within 6 h of collection; store specimens at –20°C or –70°C	Prepare plasma within 4 h of collection; store specimens at –20°C or –70°C	Prepare plasma within 4 h of collection; store specimen at –20°C or –70°C
Sensitivity (copies/mL)	Standard 1.5 (400) Ultrasensitive 1.5 (50) v2.0 (20)	Version 3.0 (75)	NucliSens QT (176)
Dynamic range (copies/mL)	Standard 1.5 (400–750,000) Ultrasensitive 1.5 (50–100,000) V2.0: (20–10,000,000)	Version 3.0 (75–500,000)	NucliSens QT (80–3,470,000)
Area of HIV genome selected for amplification	Gag or Gag/LTR	Pol	Gag

- False-positive reactions common due to small amounts of background "noise" or contamination
- All initial positive DNA PCR reactions must be confirmed with a second PCR test on a separate specimen
- Currently, recommended only for detection in infants born to mothers infected with HIV1. However, potential for false-positive result must still be recognized
- Plasma HIV RNA
- Surrogate marker of HIV disease progression
- During acute infection, viral load levels are very high (ranging from 100,000 to over 10 million copies/mL) and detectable before seroconversion
- Important to use both a plasma HIV RNA assay and antibody/Western blot testing to establish diagnosis in acute and primary infections
- Low levels of virus (<5,000 copies/mL) may be indicative of a false-positive result and should not be considered diagnostic of primary HIV infection. Standard antibody testing should be repeated

– Quantitative
  - Viral monitoring (Table 25.2 and Fig. 25.5)
  - Amplicor HIV1 Monitor and COBAS® AmpliPrep/COBAS® TaqMan® HIV1 Test,v2.0 (Roche) (FDA approved)
    – Quantitation of HIV1 RNA in plasma
    – Standard assay limit of detection for Amplicor HIV1 Monitor: >400 copies/ml and used for monitoring patient not on ARV therapy
    – Ultrasensitive assay and v2.0: as low as 50 or 20 copies/ml and used for monitoring viral loads <400 copies/ml. Viral load results of <50 or 20 copies/mL do not necessarily indicate absence of HIV1 viral replication. Inhibitory substances may be present in the plasma specimen, leading to negative or falsely low HIV1 RNA results. Improper specimen collection

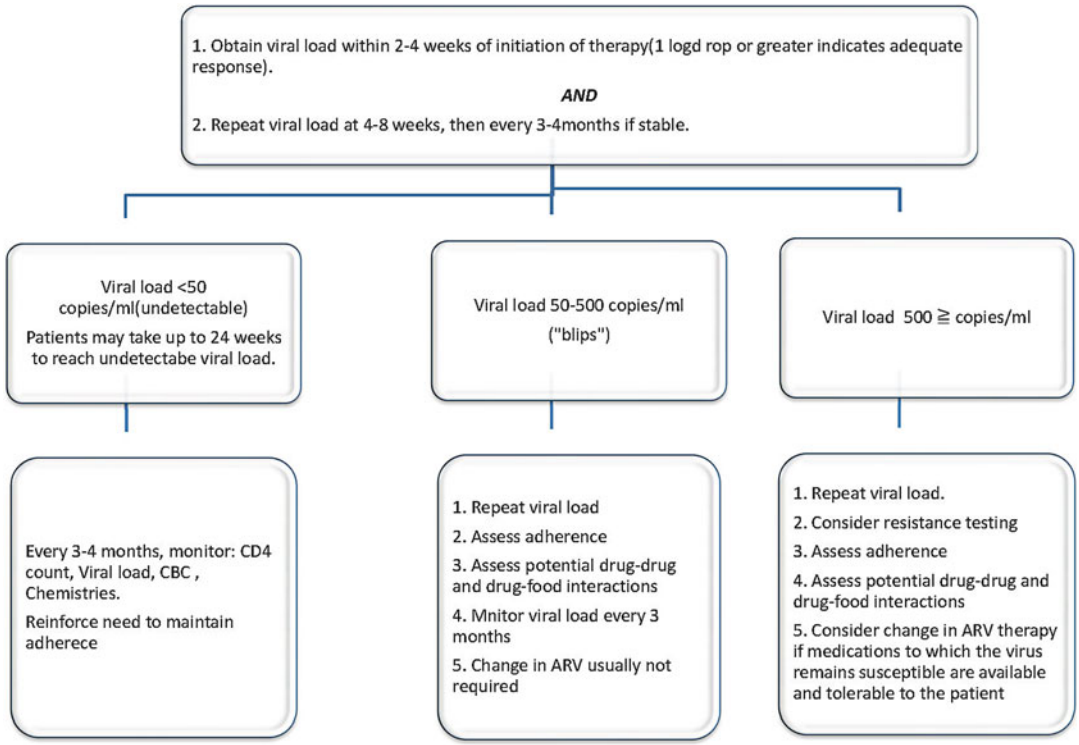


Fig. 25.5 HIV viral monitoring algorithm

or storage may falsely lower the plasma viral load results

- Plasma specimens collected using ACD anticoagulant show quantitative HIV1 RNA levels that are ~15% lower than those collected in tubes containing EDTA
- Acute concurrent illness and/or recent vaccination may cause transient rise in viral load
- Calculation of HIV viremia for Amplicor HIV1 Monitor and COBAS® AmpliPrep test is based on optical density reading. Input QS copies are lot specific and provided with each kit. Standard sample volume factor = 40; ultrasensitive sample volume factor = 4. OD_{450}, optical density at 450nm; QS, quantitation standard. The well with the lowest OD_{450} reading between 0.2 and 2.0 is selected for calculation. (Figs. 25.6 and 25.7)

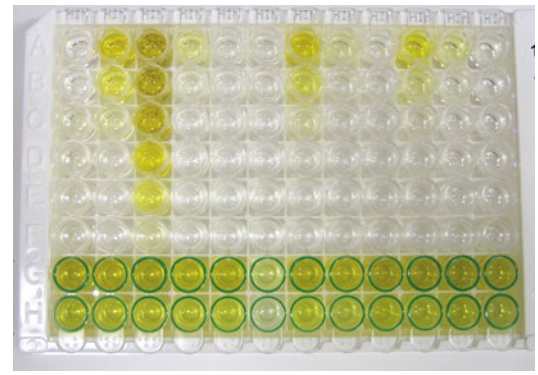


Fig. 25.6 HIV assay plate layout. *N* Negative, *L* low positive control, *H* high positive control, *S1–S9* samples, *DF* dilution factor, *QS* quantitation standard

- The HIV TaqMan test v2.0 uses three LTR primers with one FAM-labeled LTR probe in conjunction with four GAG primers and one FAM-labeled GAG probe. Therefore, the v2.0 offers primers and probes that are

$$\left[\frac{\text{HIV OD}_{450} \times \text{DF}}{\text{QS OD}_{450} \times \text{DF}}\right] \times \text{Input QS copies} \times \text{Sample volume factor} = \text{HIV -1 RNA copies/ml}$$

Calculation Example: Low Positive Control

$$\left[\frac{0.166 \times 25}{0.931 \times 5}\right] \times 51 \times 4 = \text{HIV -1 RNA copies/ml (ULTRA)} = 182 \text{ HIV RNA copies/ml}$$

	N	L	H	S1	S2	S3	S4	S5	S6	S7	S8	S9
A	0.015	2.808	-	**0.288**	0.009	0.011	2.301	**0.156**	0.013	1.343	**0.257**	0.017
B	0.010	0.703	-	0.055	0.008	0.009	**0.530**	0.031	0.006	**0.239**	0.043	0.008
C	0.085	**0.166**	-	0.022	0.010	0.013	0.117	0.011	0.008	0.056	0.014	0.011
D	0.060	0.046	2.076	0.015	0.013	0.009	0.031	0.008	0.007	0.018	0.008	0.012
E	0.044	0.018	**0.726**	0.012	0.011	0.031	0.008	0.004	0.004	0.005	0.005	0.006
F	0.046	0.013	0.120	0.009	0.010	0,009	0.003	0.003	0.002	0.004	0.003	0.003
G	2.833	2.729	-	2.628	2.700	**0.313**	2.618	2.183	2.775	2.579	2.865	2.228
H	**1.003**	**0.931**	**0.972**	**0.850**	**0.795**	0.112	**0.864**	**0.581**	**1.130**	**0.892**	**1.116**	**0.593**
HIV	**UND**	**182**	**19,046**	**< 50**	**UND**	**UND**	**125**	**<50**	**UND**	**55**	**<50**	**UND**

Fig. 25.7 HIV viral load (ultrasensitive) calculation

used to amplify the gag and LTR regions; it also provides diagnostic accuracy of test results even if mutations occur in one of the two regions and compensates for the possibility of mismatch occurring with a primer/probe region. It also ensures enhanced reliability of test results and more confidence in assessing viral loads (Fig. 25.8)

- A single HIV1 viral load test result should not be used as the only criteria to guide therapeutic decision and intervention in the clinical care of HIV1-infected patients. Viral load results should be correlated with patient symptoms, clinical presentation, and CD4 cell count
- Due to the inherent variability in the assay, physiologic variation, and concurrent illnesses in the infected patients, <100-fold (<2 log) change in plasma HIV1 viral load should not be considered as significant change

- Branched DNA (bDNA) (Bayer)
  - The VERSANT@ HIV1 RNA 3.0 Assay (bDNA) is a signal amplification nucleic acid probe assay for the direct quantitation of HIV1 RNA in plasma of HIV1-infected individuals using the Bayer@ System 340 bDNA Analyzer. The test can quantitate HIV1 RNA over the range of 75–500,000 HIV1 RNA copies/ml
  - bDNA is based on a series of hybridization procedure followed by an enzyme substrate reaction. The basic principles and procedures for bDNA 3.0 include viral lysis, overnight hybridization of viral nucleic acid, and target capture in a 96-well plate, followed by wash steps and the serial addition of probes that allow for signal amplification, detection, and quantification
  - bDNA methods have progressed from first generation assays, which were accurate and reproducible but

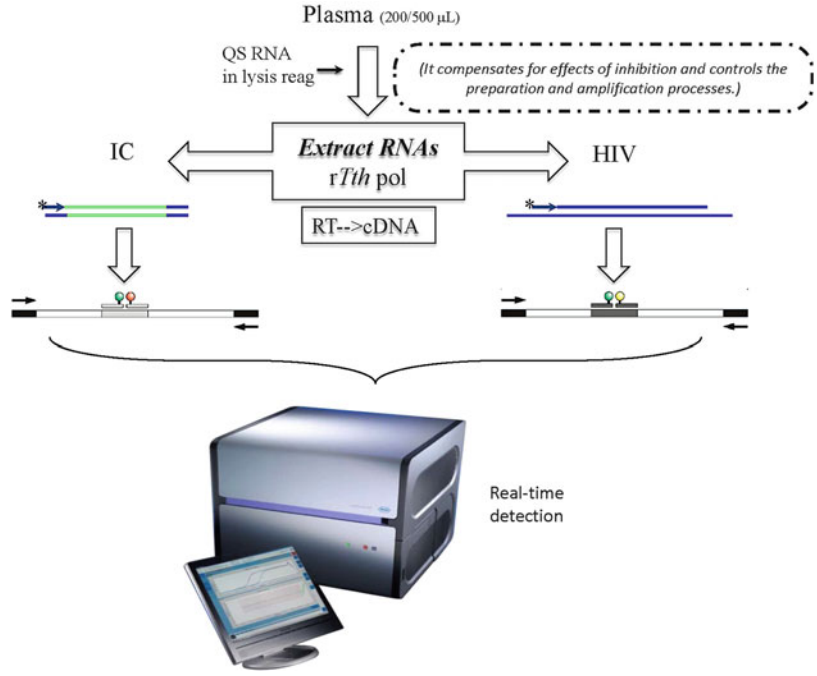


Fig. 25.8 HIV detection: quantitative RT-PCR (Roche TaqMan)

relatively insensitive, to third generation bDNA tests that are accurate, reproducible, highly sensitive, and automated laboratory tests for more optimal patient management. The results of this evolution in bDNA technology are FDA-approved methods for the detection and quantification of HIV1 (VERSANT HIV1 RNA 3.0 Assay)
  - HIV1 present in patient blood is disrupted to release viral RNA
- Nucleic acid sequence-based assay (NASBA), NucliSens HIV1 QT assay, NucliSens EasyQ HIV1 assay
  - NASBA is a sensitive, isothermal, transcription-based amplification system designed specifically for the detection of RNA targets. NucliSens HIV1 QT is both FDA approved and CE marked. The NucliSens HIV1 QT is a NASBA with endpoint electrochemiluminescence (ECL) detection. Further refinement has led to the development of the CE marked NucliSens EasyQ HIV1 – a combination of NASBA amplification and real-time detection using molecular beacons utilizing the NucliSens EasyQ analyzer
  - The NucliSens EasyQ HIV1 assay (bioMérieux, Boxtel, The Netherlands) is a quantitative, next generation amplification assay designed to overcome the labor and throughput obstacles in the earlier assays
  - Real-time detection of amplicons occurs during the NASBA reaction following primer exhaustion and continued production of antisense RNA from the T7 promoter. During the exponential phase of production, amplicons are detected using molecular beacon oligonucleotide probes that fluoresce upon binding to the specific target sequence
  - The published performance characteristics of the NucliSens EasyQ

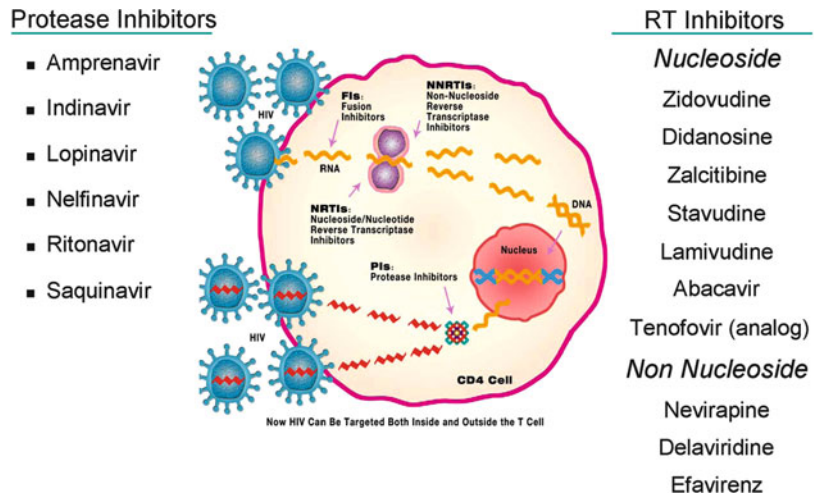


Fig. 25.9 Sixteen drugs approved for HIV treatment

HIV1 v1.1 assay show that it is comparable to the NucliSens HIV1 QT, the Versant HIV1 RNA, and the COBAS Amplicor HIV1 Monitor v1.5 assays in linearity, specificity, and reproducibility
  - Although only plasma is FDA approved, can use CSF, lymph tissue, genital secretions, and cells
  - Purified nucleic acid may be used for other molecular testing, such as sequencing
- Genotyping
  - Genotypic resistance testing uses RT-PCR and DNA sequencing techniques to identify the presence or absence of resistance related mutations in the viral genome
    - 16 ARV approved for the treatment of HIV (Fig. 25.9). ARV therapy slows the replication of HIV in the body. If patients are infected with HIV and are currently on active ARV therapy but the patients' viral load continues to increase, then the patients may undergo either genotypic or phenotypic resistance testing
    - Phenotypic testing measures the ability of the HIV1 virus to grow in different concentrations of drug under artificial conditions in the laboratory. Although phenotyping is a direct measure of resistance, it is more complex than genotyping and therefore slower and more costly to perform
    - Genotyping testing detects viral sequence change and sequence entire HIV protease (codon 1–99) and HIV-RT (codon 40–335) to cover all known drug resistance mutations
    - No consensus on genotyping versus phenotyping; however, it is anticipated that genotyping will be used more often because of its greater accessibility, lower cost, and faster turnaround time
- Mechanism of resistance
  - HIV is a highly polymorphic G virus (quasispecies) which during replication converts RNA to DNA by the action of the viral reverse transcriptase enzyme (RT)
  - The RT enzyme has very little proofreading (correction) capacity, and therefore errors are incorporated into the proviral DNA during replication. Over time, these errors, at concise drug binding sites, can provide a selection advantage for the virus in the presence of ARV drugs

  - The resistant virus predominates with a subsequent increase in viral load. However, the extent of such resistance and the implications for choice of therapy can be determined by reading the sequence of the genes encoding the protease and the RT enzymes
  - Taking medication exactly as prescribed is a very important part of avoiding resistance. Missing doses or not taking them on time lowers the amount of ARV chemicals in the body, which means the virus is not properly suppressed. The virus is then able to replicate faster, increasing the chance of it becoming resistant
  - Resistance to some ARVs can limit future treatment options. If HIV is resistant to one drug, it will sometimes be resistant to similar drugs in the same group. This is called cross-resistance and it means that some ARV drugs will not work even if they have not been used before
- Indications for drug-resistant testing
  - Drug-naïve patients with acute or recent infection
  - Therapy failure, including suboptimal treatment response, when treatment change is considered
  - Pregnant HIV1-infected women and pediatric patients with detectable viral load when treatment initiation or change is considered
  - Transmitted drug-resistant virus is common in some areas and is more likely to be detected earlier in the course of HIV infection; consider resistance testing earlier in the course of infection
- One of the FDA-approved assay for DNA sequencing: TRUGENE™ HIV1 Genotyping and OpenGene DNA Sequencing System, Bayer HealthCare, Berkeley, CA
  - It is a two-step procedure which first amplifies the protease and reverse transcriptase regions of the HIV1 genome using RT-PCR
  - The amplified DNA is then sequenced to yield to the nucleotide profile of the virus using a sequencing gel
  - Once the sequence has been generated, it is compared to the wild-type HIV1 sequence and any differences that confer drug resistance are highlighted
- Another FDA-approved assay for DNA sequencing is ViroSeq™ HIV1 genotyping system, Celera Diagnostics, Alameda, CA (distributed by Abbott Laboratories, Abbott Park, Il)
  - It is a two-step procedure which first amplifies the protease and reverse transcriptase regions of the HIV1 genome using RT-PCR and cycling sequencing
  - The amplified DNA is then sequenced to yield to the nucleotide profile of the virus using a capillary electrophoresis
  - The minimum input of viral RNA to the assay should be 1,000 copies/ml when using 1 ml of plasma to be successful in genotyping
- Pitfalls of genotyping
  - Genotypic variants comprising less than 20–30% of the sample may not be detected as genotyping results reflect the predominate subtype
  - Interpretation of genotyping results is based on the HIV1 clade B, the most prevalent clade in the developed world. However, other subtypes and recombinants of HIV1 may be undetected
  - Assessing HIV1 resistance is complicated by the replication kinetics of resistant mutants. Resistant mutants are often less fit than wild-type virus and may become undetectable with selective drugs. Nevertheless, these mutants persist in the patient and when the selective drug pressure is reapplied, the mutants replicate and a resistant population quickly predominates

25.2.4 Clinical Utility

- Plasma HIV RNA is a surrogate marker of HIV disease progression that is used to guide and monitor therapy and management
- ARV therapy should be implemented in patients with any of the following clinical findings: symptomatic HIV infection or AIDS-defining condition, CD4 count $\leq$350 cells/mm3 or viral load $\geq$100,000 copies/ml (pregnant mothers: $\geq$1,000 copies/ml)
- The initial highly active antiretroviral therapy (HAART) goal in the ARV therapy-naïve patient should be to attain a viral load of <50 copies/mL and should include the rational sequencing of ARV agents to achieve the maximum possible viral replication suppression
- In ARV treatment-naïve patients or patients who are on a successful treatment regimen, monitoring of viral loads should be measured at baseline, every 2–4 weeks after initiation and every 3–4 months once maximal suppression is attained, although patients with CD4 counts >500 cells/mm^3 may require less frequent viral load monitoring
- Typically, in patients beginning therapy or in those changing therapy as a result of virologic failure, viral load measured 2–4 weeks after therapy initiation. A decrease by at least 1 log (10-fold) indicates effective therapy. Most patients reach the goal of <50 copies/mL within 6 months. An absent or incomplete response of the viral load to ARV therapy should raise concerns about poor patient adherence to therapy and/or viral resistance
- If significant increase (3-fold increase or more) in viral load without clear explanation, viral load should be repeated to confirm virologic failure
- Genotypic resistance testing should be performed prior to initiating treatment in ARV therapy-naïve patients and in patients with >1,000 copies/ml, or nonresponsive to ARV
- Genotypic resistance testing is not recommended in patients with 500–1,000 copies/mL or less and has discontinued ARV therapy for more than 1 year

25.3 Hepatitis C Virus

25.3.1 General Characteristics

- Hepatitis C virus (HCV) was first recognized in 1974 as a non-A, non-B hepatitis virus (NANBH) and first identified in 1989 using molecular methods. HCV is the major cause of non-A, non-B hepatitis (91%) affecting about 3% of the world's population
- The most common route of transmission is via blood and blood products, i.e., immune globulin, surgery, and intravenous drug abuse which has significantly reduced with the advent of routine blood screenings. Sexual transmission as well as vertically from mother to infant occurs; the rate of vertical transmission of HCV is 6%
- HCV is a positive sense, single-stranded RNA virus that represents the third genus of the family *Flaviviridae*. The genome encodes for a single open reading frame coding structural (one core and two envelopes) proteins as well as a series of nonstructural proteins (Fig. 25.10)
  - 5′ untranslated region: most constant, used for HCV RNA assays and genotyping
  - Core region: constant, used in some genotype assays, core protein assay, PCR–RFLP, and RIBA tests
  - Envelope region: hypervariable region, associated with high rate of mutation in quasispecies
  - NS2 region: codes for protease
  - NS3 region: codes for protease/helicase, RIBA tests found in this region
  - NS4 region: c100p antigen used in anti-HCV, RIBA tests targeted this region
  - NS5a region: codes for interferon response element
  - NS5b region: codes for RNA polymerase, NS5 antigen used in anti-HCV, RIBA tests target this area
- HCV consists of a heterogeneous group of genotypes based on the sequence homology of 5′ untranslated region. Currently, there are 6 types and over 90 subtypes. Types 1, 2, and 3

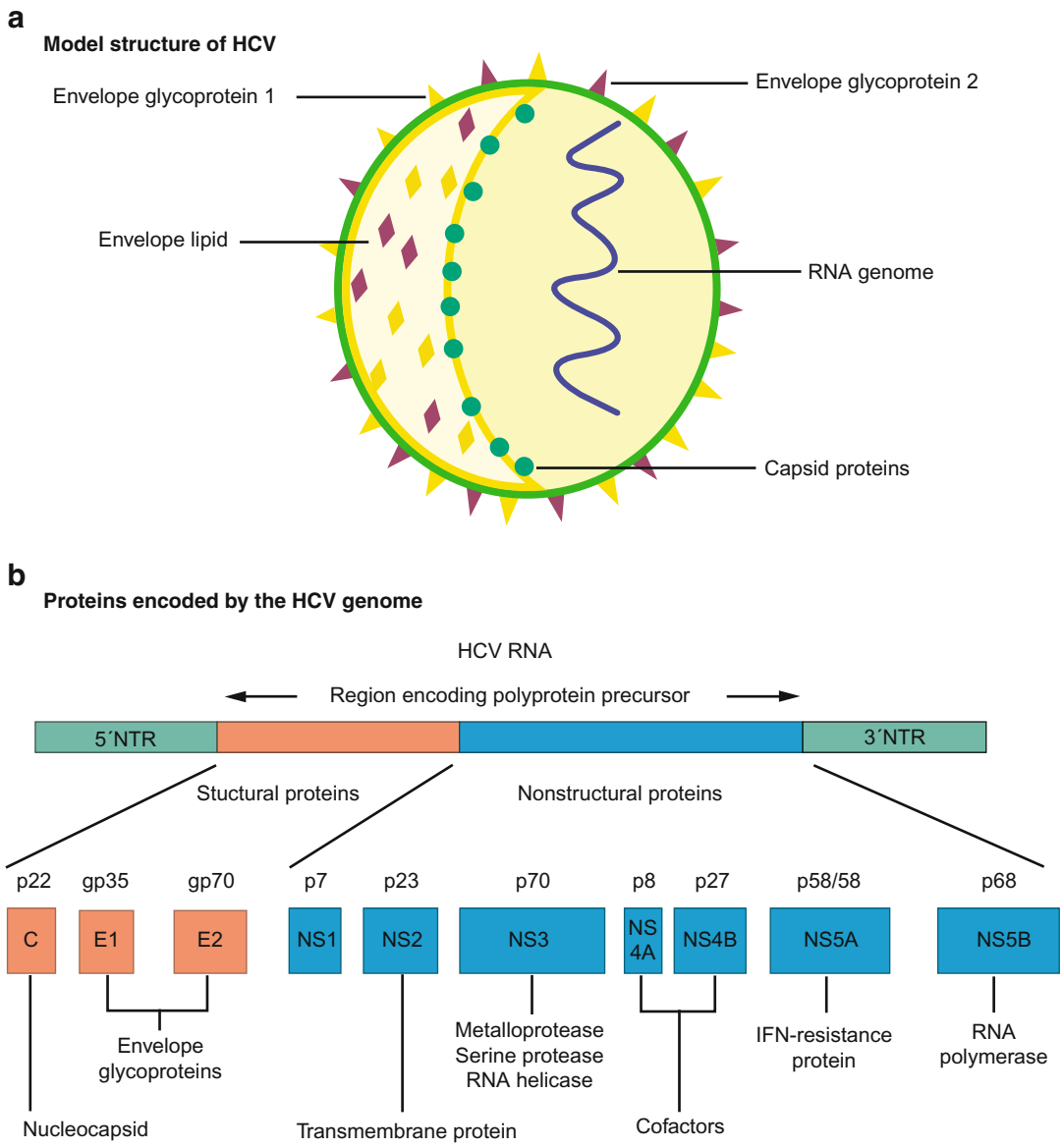


Fig. 25.10 Hepatitis C virus (*HCV*) genome structure (Adapted from Anzola et al. Expert Rev Mol Med 2003)

distributed worldwide, with types 1a and 1b responsible for approximately 60% of infections. Type 4 occurs primarily in the Middle East, type 5 in South Africa, and type 6 in Hong Kong. In the United States (US), approximately 72% of people infected with HCV have genotype 1, and most others are types 2 or 3 (genotypes 4, 5, and 6 are not common in the US)

- There is little difference in the mode of transmission or natural history of infection among the different genotypes
- Cure rates with antiviral therapy are notably higher with genotypes 2 and 3, and the duration of HCV therapy is shorter for these genotypes
- Infection with HCV is curable by therapy, with the current standard treatment based on

the combination of pegylated interferon alpha (IFNa) and ribavirin. Virus eradication, characterized by the sustained virological response, i.e., an undetectable HCV RNA 24 weeks after treatment completion, is achieved in 40–50% of patients infected with HCV genotype 1 and approximately 80% of patients infected with genotypes 2 and 3. The outcome of therapy is influenced by several parameters, including the treatment schedule, disease characteristics, viral factors, and recently identified genetic factors that include single nucleotide polymorphisms located upstream of the gene encoding IFNλ3 (IL28B)

- MicroRNA-targeted therapy has been suggested as a potential means of combatting the virus but is not currently in use

25.3.2 Clinical Presentation

- Prior to the isolation of the virus in 1989, hepatic infection with HCV was previously known as non-A, non-B hepatitis
- In the US, approximately four million have been exposed to the virus; three million are chronic carriers
  - Acute infection is usually asymptomatic. 25% of patients develop acute hepatitis with jaundice and abnormal liver function (Fig. 25.11)
    - HCV RNA can be detected in blood within 1–3 weeks and is present at the onset of symptoms
    - Antibodies to HCV are detected by enzyme immunoassay (EIA) in only 50–70% of patients at the onset of symptoms, increasing to more than 90% after 3 months RNA
- Chronic infection: 60–85% patients eventually develop chronic infection and/or chronic hepatitis (Fig. 25.11)
  - Persistence of HCV infection is diagnosed by the detection of HCV RNA in the blood for at least 6 months
  - The most important sequelae of chronic HCV infection are progressive liver fibrosis leading to cirrhosis, end-stage liver disease, and hepatocellular carcinoma (HCC)
  - Patients are often asymptomatic or have nonspecific symptoms such as fatigue, malaise, and abdominal discomfort
    - Mild to moderate elevations of ALT (SGPT) or AST (SGOT)
  - Some asymptomatic patients have normal liver enzymes
    - As many as 44% have normal levels at initial evaluation
    - May have positive HCV antibody test despite normal liver enzymes
      - 20% patients eventually develop cirrhosis which takes decades to occur. The severity of liver cirrhosis does not correlate with liver enzymes and can only be evaluated by liver biopsy
      - All etiologies of cirrhosis increase the risk, and patients with cirrhosis have an annual risk of 1–6% of developing HCC
      - Approximately 250,000 patients die each year from HCV-related liver disease

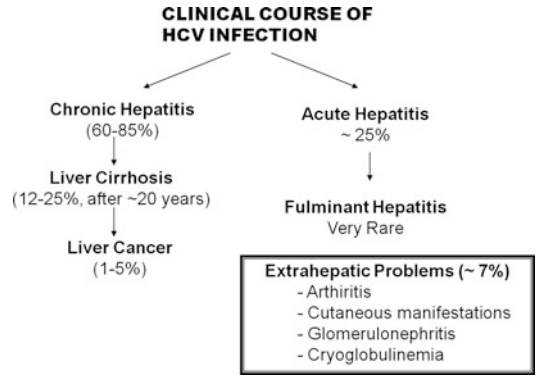


Fig. 25.11 Natural history of hepatitis C virus infection (Adapted from National Institutes of Health 2002)

25.3.3 Diagnostic Methods

- Specimens: blood plasma or serum
  - Collection of samples in EDTA plasma

  - Rapid separation of serum or plasma from cells is recommended by centrifugation within 1 h of collection
  - Unseparated EDTA plasma is stable at room temperature up to 24 h after collection
  - Separated serum or plasma is stable at room temperature for up to 3 days, at refrigerator temperatures for up to 1 week, and frozen at –70 °C for years
- Conventional tests and problems
  - Serological studies
    - Enzyme immunoassay (EIA)
      - The detection of HCV antibodies is recommended as the initial test for the identification of HCV and is useful for screening at risk populations
      - EIA is comparatively inexpensive, reproducible, and carries a high sensitivity (99%) and specificity (99%)
      - EIA can detect antibodies average 2–10 weeks after infection. However, during this "window period," a patient will have detectable viral RNA, but have undetectable levels of antibodies
      - A negative enzyme immunoassay is usually sufficient to exclude the diagnosis of HCV infection in immunocompetent patients
      - However, the test can be falsely negative in those with immunodeficiencies or end-stage renal disease
      - Conversely, false-positive EIAs may occur in patients with autoimmune disorders. In these patients, an assay for HCV RNA is necessary for diagnosis of chronic infection
      - Once patients seroconvert, they usually remain positive for HCV antibody. Thus, the presence of HCV antibody may reflect remote or recent infection
      - The immunoblot assay is still useful as a supplemental assay for persons screened in nonclinical settings and in persons with a positive EIA who test negative for HCV RNA
      - Other HCV antibody assays include anti-HCV IgM assays and avidity tests. The significance of the presence of anti-HCV IgM during HCV infection is unclear. Anti-HCV IgMs have been reported in 50–93% of patients with acute HCV and 50–70% of patients with chronic HCV
      - Therefore, anti-HCV IgM cannot be used as a reliable marker of acute HCV infection and, so far, IgM assays have not been used in clinical practice. However, increasing serial measurements of anti-HCV IgM titers early after the onset of the symptoms may help to identify patients with acute HCV. Alternatively, an increase in the anti-HCV IgG avidity index within a week after the onset of clinical symptoms has also been reported to indicate acute HCV infection. Both parameters could be used together before anti-HCV seroconversion occurs, or when no baseline sample is available to confirm the diagnosis of acute infection
    - A total HCV core antigen enzyme-linked immunosorbent assay (ELISA HCV 3.0) and ORTHO® trak-C™ assay (Ortho Diagnostics) for detection and quantification of total core antigen in blood
      - The HCV core protein is highly antigenic, induces specific cellular and humoral responses, and probably plays a pivotal role in the pathogenesis of HCV infection. The availability of an anticore monoclonal antibody allowed the development of an ELISA to detect HCV core Ag in peripheral blood of patients with HCV
      - It tests positive for anti-HCV antibodies and for prospective low-risk population screening
      - Total HCV core antigen ELISA (quantitative, Ortho Clinical Diagnostics) has sensitivity close to PCR assays in diagnosing acute HCV infection in windows period (before HCV antibodies developed)

  - It is also used in monitoring response to antiviral treatment
  - The ELISA HCV 3.0 uses three recombinant antigens (c22–3, c200, and NS5) originating from four regions of the viral genome (core, NS3, NS4, and NS5)
  - ORTHO® trak-C™ assay is a quantitative immunoassay for total (both free and antibody bound) HCV nucleocapsid core antigen. The assay uses a capture ELISA format. The difference between the two assays (ELISA HCV 3.0 and ORTHO® trak-C™ assay) is a step in this assay that disrupts immune complexes present in the sample. The assay's intended use is testing for HCV core antigen either during preseroconversion (acute) or postseroconversion (chronic) phases of HCV infection
  - Less expensive and less prone to carryover than PCR testing and can be used as a rough screen as it correlates with HCV RNA load. However, it is less sensitive than PCR-based assays and individual variation is higher
  - Similar technology is used by the Architect HCV assay by Abbot Laboratories. The analytical sensitivity of the recently developed Architect HCV assay (Abbott Laboratories, Abbott Park, IL) varies according to the HCV genotype from 500 to 3,000 international units of HCV RNA per milliliter (IU/mL)
  - A drawback of the total HCV core Ag ELISA is its lower limit of detection, and HCV-positive patients on dialysis often have low HCV RNA levels
- Recombinant immunoblot assay (RIBA)
  - RIBA has been developed for the simultaneous detection of anti-HCV antibodies and HCV core antigen
  - RIBA was used to confirm EIA results since the early generation. It had a high rate of false positives
  - Third generation of RIBA (RIBA 3.0) was developed to test HCV (which includes NS5 protein) after earlier generations. It has high specificity
  - A new combination assay has been developed and licensed in Europe (Monolisa HCV Ag/Ab ULTRA; Bio-Rad, Marnes la Coquette, France). Monolisa HCV Ag/Ab ULTRA is based on the combination of an indirect test for the antibodies and a sandwich test for Ag detection
- Molecular methods
  - Qualitative (Table 25.3)
    - The qualitative HCV RNA test is used to confirm HCV diagnosis following a positive or indeterminate antibody test result. It differentiates between resolved and active infection and may be useful for detecting acute infection prior to seroconversion. It is especially useful for confirming diagnosis in people with indeterminate HCV immunoblot (RIBA) results, as well as in immunosuppressed or immunoincompetent individuals
    - Recommended sensitivity for testing is 50 IU/ml
    - APTIMA® HCV RNA Qualitative Assay (Gen-Probe)
      - Target amplification based on sequences of the 5′ NC region of the HCV genome
      - Amplification of HCV RNA via transcription-mediated amplification method (TMA)
      - The detection limit of TMA is 10 IU/ml
    - Amplicor HCV test and COBAS Amplicor HCV test, v2.0 (Roche)
      - Use the primers KY78 and KY80 to amplify a 244-bp sequence of within the highly conserved 5′ UTR of the HCV genome
      - Limit of detection (200 ul): 25–50 IU/ml depending genotypes (i.e., 1b = 25, 1a = 50)

Table 25.3 Characteristics of current HCV RNA assays

Assay	Manufacturer	Technique	Lower limit of detection (qualitative assay)	Dynamic range of quantification (quantitative assay)
Amplicor® HCV v2.0	Roche Molecular Systems	Manual RT-PCR	50 IU/ml	NA
COBAS® Amplicor® HCV v2.0	Roche Molecular Systems	Semiautomated RT-PCR	50 IU/ml	NA
Versant® HCV RNA qualitative assay	Bayer HealthCare	Manual TMA	10 IU/ml	NA
Amplicor HCV Monitor® v2.0	Roche Molecular Systems	Manual RT-PCR	600 IU/ml	600–500,000 IU/ml
COBAS® Amplicor HCV Monitor v2.0	Roche Molecular Systems	Semiautomated RT-PCR	600 IU/ml	600–500,000 IU/ml
LCx HCV RNA Quantitative assay	Abbott Diagnostic	Semiautomated RT-PCR	25 IU/ml	25–2,630,000 IU/ml
Versant® HCV RNA 3.0 assay	Bayer HealthCare	Semiautomated bDNA	615 IU/ml	615–7,700,000 IU/ml
COBAS® TaqMan HCV Test	Roche Molecular Systems	Semiautomated real-time PCR	15 IU/ml	43–69,000,000 IU/ml
Abbott RealTime	Abbott Diagnostic	Semiautomated real-time PCR	30 IU/ml or 12 IU/ml[a]	12–100,000,000 IU/ml

Note: *RT* reverse transcriptase, *PCR* polymerase chain reaction, *TMA* transcription-mediated amplification, *bDNA* "branched DNA." *NA* not applicable
[a]For 0.2 ml or 0.5 ml of plasma analyzed, respectively

  - Use of centrifugation or Ultracolumn (QIAGEN) to process a large volume (1 ml), the LOD can be further improved
- Quantitative (Table 25.3)
  - On average 1–2 log10 units/ml less sensitive than qualitative tests
  - Used to establish baseline viral load (prior to therapy) and to monitor changes in viral load during therapy
  - PCR – Two real-time PCR platforms are currently available for the detection and quantification of HCV RNA: the COBAS TaqMan platform, which can be used together with automated sample preparation with the COBAS AmpliPrep system (CAP–CTM; Roche Molecular System, Pleasanton, CA), and the Abbott platform (Abbott Diagnostic, Chicago, IL), which uses the m2000RT amplification platform together with the m2000SP device for sample preparation (ART). The lower limits of detection were 12 IU/ml for ART and 15 IU/ml for CAP/CTM
  - VERSANT HCV RNA 3.0, quantiplex assay (bDNA) (Bayer)
    - Signal amplification directed to the 5′ NC region and core regions of the HCV genome
    - Microwell plate format
    - Equivalent detection of genotypes 1–6
    - LOD: 3200 HCV RNA copies/mL (5.2 HCV RNA copies/IU)
    - Broad dynamic range (615–7,690,000 IU/mL)
    - Comparative evaluations between Bayer bDNA and Roche PCR viral load assays demonstrated that PCR

reported significant lower viral load (as much as 1 log10) at the higher range
- HCV RNA detection and quantification are useful in clinical practice to diagnose chronic HCV infection, identify patients who need antiviral therapy, monitor the virological responses to antiviral therapy, and document treatment failure

– Genotyping
  - TRUGENE HCV 5′ NC Genotyping Kit (Bayer HealthCare LLC, Berkeley, California)
    – This technique utilizes PCR fragments previously generated by the diagnostic Roche Amplicor HCV test
    – Simultaneous PCR amplification and direct sequencing (CLIP sequencing) of the 5′ noncoding region (5′ NCR)
  - VERSANT HCV genotype assay (LiPA; Bayer HealthCare LLC)
    – The INNO-LiPA HCV II method uses 19 type-specific oligonucleotide probes attached to nitrocellulose strips to detect sequence variations found in the 5′ NC region of HCV
    – The biotin-labeled PCR product is hybridized to the probes on the strip under stringent conditions. After hybridization and washing, streptavidin-labeled alkaline phosphatase is added; followed by incubation with a chromogen, which results in the development of a purple-brown precipitate when there is a match between the probe and the biotinylated PCR product
    – Hybridization of the amplicon with one or more lines on the strip allows the classification of six major genotypes and their most common subtypes
  - Third Wave Technologies' Invader assay (Third Wave Technologies, Madison, Wisconsin) is a new DNA-scanning method application, which has been termed cleavase fragment length polymorphism (CFLP)
    – Relies on formation of unique secondary structure that results when DNA is allowed to cool following brief heat denaturation and serves as substrates for structure-specific cleavase I enzyme generating a set of cleavage products
    – Formation of secondary structures is sensitive to nucleotide sequences
    – The presence of sequence polymorphisms results in the generation of unique collections of cleavage products or structural fingerprints
    – It targets the well-conserved 5′ NCR of HCV
  - Determination of HCV genotype is needed before the initiation of therapy with pegylated IFNa and ribavirin because it determines both the dose of ribavirin and the treatment duration required

– HCV resistance testing
  - Several amino acid substitutions that confer resistance to directly acting antiviral molecules, such as protease inhibitors, have been identified. In case of a failure of the triple combination of pegylated IFNa, ribavirin, and either telaprevir or boceprevir, HCV variants that are resistant to these compounds are selected
  - Direct sequence analysis or reverse hybridization methods can be used to identify amino acid substitutions that confer resistance to antiviral drugs
  - Ultradeep sequencing methods, such as pyrosequencing, can detect minor resistant populations down to $<1\%$
  - HCV GenoSure NS3/4A represents the first in a series of HCV drug resistance assays that have been developed at Monogram Biosciences to support the clinical evaluation of HCV direct-acting antiviral (DAA) agents and their use in the management of HCV infection. HCV GenoSure NS3/4A analyzes the genetic sequence for the nonstructural proteins NS3 and NS4A of HCV

genotypes 1a and 1b that encode for an enzyme essential to viral replication. The assay detects mutations in NS3 and NS4A and specifically identifies those associated with boceprevir and telaprevir resistance

- Pitfalls
  - It is important to note that a "genotype bias" is possible for all HCV molecular assays because of the extensive genetic heterogeneity of the virus
  - False-positive results due to contamination (detected by negative control)
  - False-negative results due to amplification inhibition (detected by internal control) or due to a loss of bacteria during specimen preparation
  - "Home brew" PCR assays are not standardized and variations in sample handling and laboratory methods can affect the sensitivity of the assay

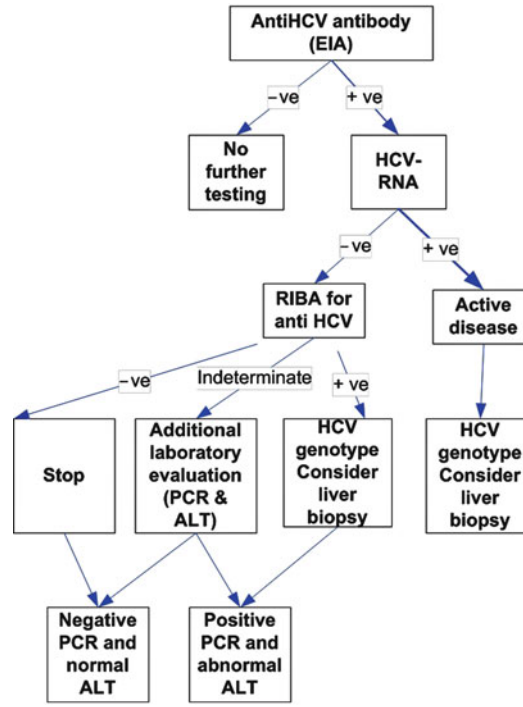


Fig. 25.12 Algorithm of HCV testing (Adapted from www.cdc.gov/hepatitis)

25.3.4 Clinical Utility

- HCV tests should be used in high-risk patients, such as intravenous drug users, children born to HCV-positive mothers, and HIV-positive patients. Figure 25.12 shows the algorithm of HCV testing
- Patients suspected of having chronic HCV infection should be tested for HCV antibodies. Patients suspected of having an acute infection should be tested for both HCV antibodies and also HCV RNA with a real-time PCR analysis. HCV RNA should be repeated as the patient is undergoing treatment, to better adjust the use of therapeutic agents
- HCV RNA testing should be performed in
  - Patients with a positive anti-HCV test
  - Patients for whom antiviral treatment is being considered, using a quantitative assay
  - Patients with unexplained liver disease whose anti-HCV test is negative and who are immune compromised or suspected of having acute HCV infection
- HCV genotype should be determined in all HCV-infected individuals prior to treatment in order to determine the duration of therapy and likelihood of response. Genotypic analysis has shown some amino acid substitutions to correlate with resistance to therapy. No commercial test is yet available, although home brew kits have been made
- The treatment of choice is peginterferon plus ribavirin

25.4 Hepatitis B Virus

25.4.1 General Characteristics

- Hepatitis B virus (HBV) is an enveloped dsDNA hepadnavirus. It is 47-nm spherical virus with three important polypeptides: HBV surface antigen (HBsAg) is an envelope protein, HBV core antigen (HBcAg) is a core protein, and HBV e antigen (HBeAg) is an early protein and a nonstructural protein coded by core gene. The envelope protein is involved in viral binding and released into

susceptible cells. The inner capsid relocates the DNA genome to the cell's nucleus where viral mRNAs are transcribed

- HBV is a circular, partially double-stranded DNA virus of approximately 3,200 nucleotides. This highly compact genome contains four open reading frames encoding the envelope (PreS1, PreS2, S), core (core, precore), polymerase, and X protein (Fig. 25.13)
- Although hepatocytes are most susceptible to infection, other cell types may be affected to a lesser extent. The life cycle of HBV begins when it attaches to the cell surface. In the cytoplasm, the DNA is still in the core but then capsid is removed and DNA passes into nucleus, where it forms a covalently closed circular DNA (cccDNA)
- HBV uses the host transcription machinery to replicate its genes and uses RNA polymerase II of the host. The (–) strand of the cccDNA will act as the template for this transcription. After transcription, the mRNAs are translated by the host's protein synthesis machinery to form viral proteins in the endoplasmic reticulum. The proteins are then assembled into virions that are secreted
- HBV is recognized as endemic in China and other parts of Asia. Over one-third of the world's population has been or is actively infected by HBV
- HBV strains are classified into at least 10 HBV genotypes (A to J) and several subtypes and based on the nucleotide homology of the surface gene. Except for the newly identified genotypes I and J, the geographic and ethnic distributions of HBV genotypes and subtypes are well characterized. Genotype A is mainly found in Northwestern Europe (subtype A2), North America, and Africa (subtype A1 or A3), whereas genotypes B and C have been described in Southeastern Asian populations. At present, genotype B is divided into B1–B6 subtypes. Among them, B1 is isolated in Japan, B2–5 are found in East Asia, and B6 is found in indigenous populations living in the Arctic, such as Alaska, Northern Canada, and Greenland. Genotype C, including subtypes C1–C5, mainly exists in East and Southeast Asia. Genotypes E and F are seen in East Africa and the New World, respectively. Genotype D is most often found in southern Europe, parts of Central Asia, India, Africa, and the Middle East. Genotype G is a recently determined genotype in France, America, and Germany while genotype H has been reported in patients from Central America. Recently, genotype I, a novel intergenotypic recombination among genotypes A, C, and G, was isolated in Vietnam and Laos. The newest HBV genotype, J, was identified in the Ryukyu islands in Japan, and this genotype has a close relationship with gibbon/orangutan genotypes and human genotype C
- Acute infection with genotypes A and D results in higher rates of chronicity than genotypes B and C. Compared to genotype A and B cases, patients with genotypes C and D have lower rates of spontaneous HBV e antigen (HBeAg) seroconversion; when this occurs, it tends to be delayed. Hepatitis genotypes C and D have lower response rates than genotypes A and B. Genotype C is also more associated with severe liver disease, including to hepatocellular carcinoma

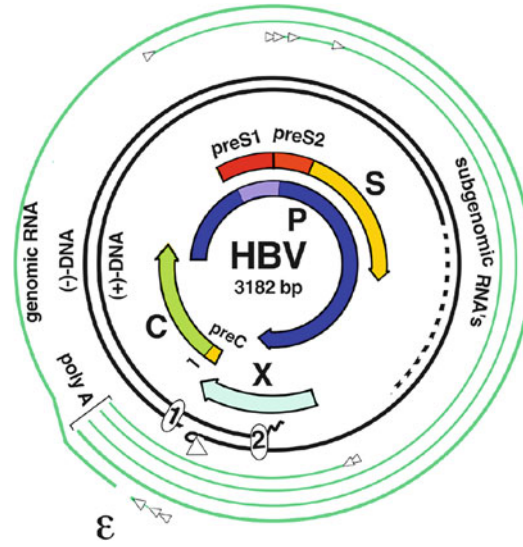


Fig. 25.13 HBV genome (Courtesy of Stephan Urban and Stefan Seitz, University of Heidelberg Dept. of Molecular Virology)

- The rate of new HBV infections has declined by approximately 82% since 1991, when a national strategy to eliminate HBV infection was implemented in the US. The decline has been greatest among children born since 1991, when routine vaccination of children was first recommended

25.4.2 Clinical Presentation

- Transmitted parenterally and sexually by contaminating open cuts or mucous membranes and has a long incubation period (45–120 days) (Fig. 25.14). Asian patients are more likely to be vertically infected (mother to child) than African or Western patients
- Majority of affected patients recover from the illness, characterized by
  - Anorexia, nausea, vomiting, headache, fever, abdominal pain, dark urine, and sometimes jaundice
  - Elevated transaminases, hyperbilirubinemia, and elevated alkaline phosphatase may also occur
  - Extrahepatic manifestations include arthralgias, arthritis, nephritis, and dermatitis
- 10% of patients continue to carry the virus or markers of the active viral infection greater than 6 months after initial infection
  - Small percentage may develop chronic persistent hepatitis with sequence fibrosis and cirrhosis
  - Incidence of HCC is increased with the viral genome found integrated in the cellular DNA in 75% of cases
  - May be associated with polyarteritis and cryoglobulinemia
- Recently, several clinical scoring systems, or nomograms, consisting of previously confirmed independent risk predictors such as sex, age, family history of HCC, alcohol consumption, serum alanine aminotransferase (ALT) level, HBeAg status, serum HBV DNA level, and/or HBV genotype have been introduced. These easy-to-use nomograms are based on noninvasive clinical characteristics and have been found to accurately predict HCC risk in either community- or hospital-based HBV-infected persons

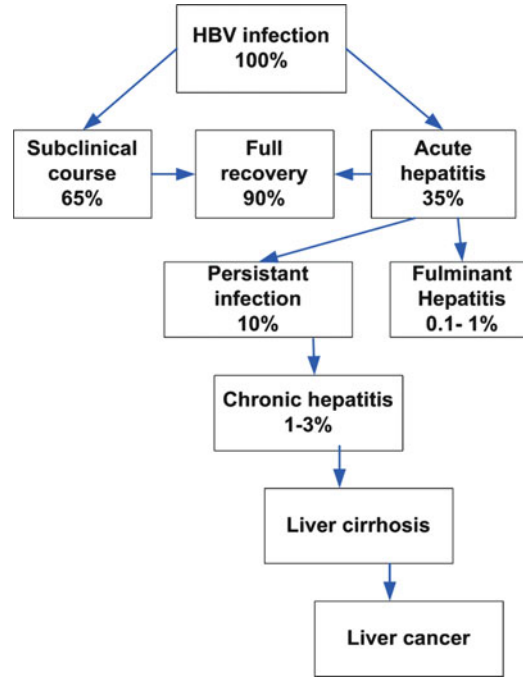


Fig. 25.14 Natural history of HBV

25.4.3 Diagnostic Methods

- Specimens: whole blood, serum, or plasma
- Conventional tests and problems
  - Serological studies
    - Viral antigens and particles (Fig. 25.15)
      - Dane particle
        - dsDNA bilayered sphere
        - 42 mm diameter; 22 nm core
        - Rarely identified in infectious serum
        - Thought to be infectious virus particle
      - HBsAg
        - Indicative of prior HBV exposure
        - Located on surface of Dane particle
        - Previously known as Australia antigen

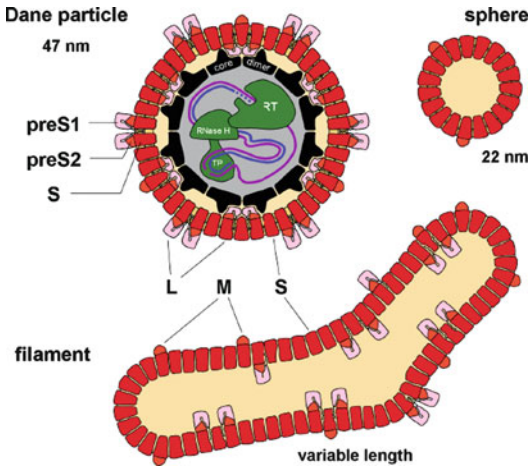


Fig. 25.15 HBV viral particle and antigens (Courtesy of Stephan Urban and Stefan Seitz University of Heidelberg Dept. of Molecular Virology)

  - HBcAg
    - Represents acute or chronic infection
    - 28 nm core of the Dane particle
  - HBeAg
    - Marker of HBV infection
    - Present in HBsAg-positive patients
    - Strong correlation with large serum concentrations of Dane particle and HbsAg
    - HBeAg is associated with high infectivity
- Antibodies (Fig. 25.16)
  - Anti-HBs
    - Antibody to surface antigen
    - Detected after disappearance of HbsAg
    - Protective properties
  - Anti-HBc
    - Antibody to core antigen
    - Detected after appearance of HbsAg
    - Used to confirm HBV infection when HBsAg and Anti-HBs are absent (window phase)
  - Anti-HBe
    - Antibody to HBeAg antigen protective properties
    - Associated with low risk of infectivity in presence of HBsAg
  - Interpretation of HBV serologic test results (Table 25.4)
- Molecular methods
  - Qualitative
    - COBAS AmpliScreen HIV1/HCV/HBV Tests (Roche Molecular Diagnostics)
      - It detects HBV DNA in human plasma
      - It is intended to be used to screen donors for HBV DNA
      - Detection limit is 100 copies/ml
      - It targets the S gene
  - Quantitative
    - Used to establish baseline viral load (prior to therapy) and to monitor changes in viral load during therapy
    - Digene HBV DNA hybrid capture II
      - Detection and quantitation of HBV DNA in serum
      - Limit of detection: 4,700 HBV DNA copies/ml
      - Quantitative range: 1.4×10^5 and 1.7×10^9 HBV copies/ml
    - PCR – Amplicor HBV Monitor and its semiautomated COBAS HBV Amplicor Monitor test (Roche)
      - Detection and quantitation of HBV DNA in serum or plasma
      - Use the primers HBV-104UB and HBV-104D to amplify a 104-bp sequence within the highly conserved precore/core region of the HBV genome
      - Amplify genotypes A to E equally and reduced amplification of genotypes F and G
      - Limit of detection: 200 copies/ml
      - Quantitative range: 10^3–4×10^7 copies/mL
    - Real-time PCR LightCycler/FRET hybridization probes
      - It targets 259-bp fragment of S gene,
      - Quantitative range: 250–5×10^8 copies/ml

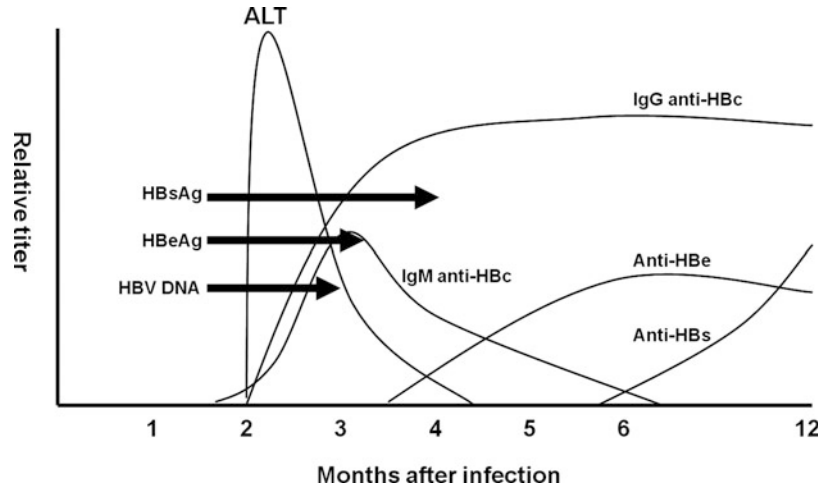


Fig. 25.16 Time course for appearance of viral antigens and antibodies in acute hepatitis B infection

Table 25.4 Hepatitis B antibody/antigen interpretation

Tests	Results	Interpretation
HBsAg	Negative	Susceptible
anti-HBc	Negative	
anti-HBs	Negative	
HBsAg	Negative	Immune due to natural infection
anti-HBc	Positive	
anti-HBs	Positive	
HBsAg	Negative	
anti-HBc	Negative	Immune due to vaccination
anti-HBs	Positive	
HBsAg	Positive	
anti-HBc	Positive	Acutely infected
IgM- Anti HBc	Positive	
anti-HBs	Negative	
HBsAg	Positive	
anti-HBc	Positive	Chronically Infected
IgM- Anti HBc	Negative	
anti-HBs	Negative	
HBsAg	Negative	*Results inconclusive:*
anti-HBc	Positive	Resolved infection (most common)
anti-HBs	Negative	False-positive anti-HBc "Low-level" chronic infection Resolving acute infection

- Real-time PCR – Roche TaqMan Assay
  - Utilizes FRET technology and probes based on the detection of amplicon during temperature cycling
  - It targets S gene
  - Limit of detection: 50 copies/ml
  - Quantitative range: 5 to 2 × 10^8 HBV IU/mL (30–10^7 copies/ml; 1 IU = 5.82 copies)
- bDNA assay – (VERSANT Hepatitis B Virus DNA 3.0 Assay) (Bayer Corporation)
  - Signal amplification directed to the 5′ NC region and core regions of the HCV genome
  - Microwell plate format
  - Limit of detection: 2,000 copies/ml
  - Quantitative range: 2.0 × 10^3 to 1.0 × 10^8 HBV DNA copies/ml
  - Equivalent detection of genotypes A through F

– Genotyping and mutation analysis – currently used mainly for epidemiological purposes, rarely needed for clinical purposes
- Line probe assay-LiPA; INNO-LiPA HBV Genotyping assay, Innogenetics N.V., Ghent, Belgium
  - This method is based on the reverse hybridization principle, such that biotinylated amplicons hybridize to specific oligonucleotide probes that are immobilized as parallel lines on membrane-based strips. The amplified region analyzed overlaps the sequence encoding the major hydrophilic region of HbsAg

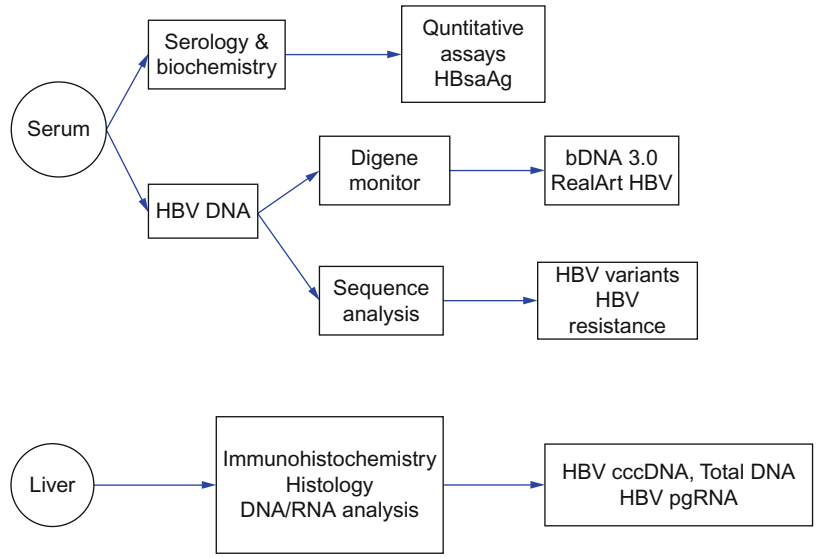


Fig. 25.17 An outline of the HBV assays available for testing of serum and liver biopsy samples

  - TRUGENE® HBV Genotyping Kit (Bayer Corporation)
    - Sequencing and phylogenetic analysis of the pre-S1/pre-S2 region of the HBV genome
    - Identifies HBV genotype, drug resistance mutations, and anti-HBs escape mutations based on comparison of DNA sequence
- Pitfalls
  - The analytical sensitivity and specificity of current real-time PCR assays allow for accurate quantification over a range of approximately 7–8 logs. They are not sufficient to quantify the very high HBV DNA levels that can be found in certain HBV-infected patients, which necessitates retesting these samples after dilution, a factor of quantification errors
  - Equal quantification of all HBV genotypes and robustness of quantification in case of nucleotide polymorphisms has not been validated for the current commercial real-time PCR assays
  - There is currently no uniform tendency to report HBV DNA levels in standardized units (such as copies/ml or genome equivalents/ml or IU/ml)
  - Not all assays are currently registered for use with plasma and serum
  - No precise thresholds of HBV DNA have been established that could guide medical decisions

25.4.4 Clinical Utility

- Viral load testing is used for the assessment and monitoring of responses to therapy in HBV infection (Fig. 25.17)
- In HBV carriers with active liver disease, HBV DNA loads are measured not only to assess patients regarding the need for either interferon alpha or lamivudine (a DNA polymerase inhibitor) antiviral therapy but also to monitor their effectiveness
- An increase in HBV viral load is also used as a marker of the emergence of lamivudine-resistant viral mutants
- Active chronic infections with HBV treated with lamivudine require surveillance for the emergence of lamivudine-resistant viral mutants. During lamivudine monotherapy, point mutations at the active site of the polymerase gene (YMDD variants) occur with a frequency of 14–32% after 1 year in phase III studies, and in 42% and 52% of Asian patients after 2 and 3 years of therapy, respectively. The emergence of lamivudine resistance is detected by a rise in HBV viral load and confirmed by sequencing of the active site of the DNA polymerase gene

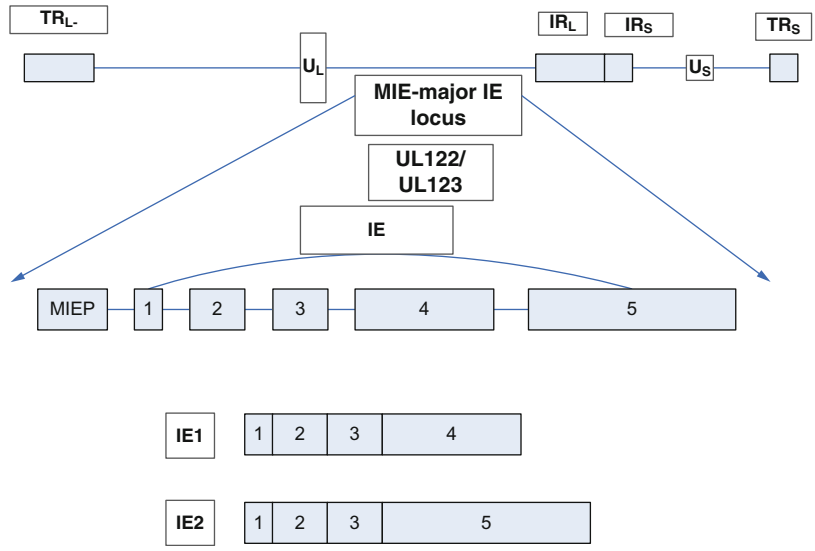


Fig. 25.18 CMV genome. *MIEP* major IE promoter, U_L unique long region, U_S unique short region, *TR* terminal repeat sequence, *IR* inverted repeat sequence

- The presence of HBV precore mutants may cause active liver disease despite the absence of HBeAg, the common marker for active hepatitis in HBV infection. This may be due to either a premature stop codon point mutation in the precore gene (G1896A) or a mutation in the basal core promoter region downregulating HBeAg production, both of which can only be reliably detected genotypically

25.5 Cytomegalovirus

25.5.1 General Characteristics

- Cytomegalovirus (CMV) is a viral genus of the viral group known as *Herpesviridae* or herpesviruses, sharing a number of characteristics with chicken pox, the various herpes viruses, and the Epstein–Barr virus (EBV). The species that infects humans is commonly known as human CMV (HCMV) or human herpesvirus (HHV) 5. It is the largest of the HHVs characterized by 240 kb double-stranded linear DNA virus (Fig. 25.18) with 162 hexagonal protein capsomeres surrounded by three distinct layers: a matrix or tegument, a capsid, and an outer envelope
- CMV can reside latent in the salivary gland cells, endothelium, macrophages, and lymphocytes. CMV infection is asymptomatic
- The virus acts by blocking cell apoptosis via the mitochondrial pathway and causing massive cell enlargement, which is the source of the virus name
- Clinically symptomatic patients are infants and immunocompromised adults. For infants, the mode of transmission is from the mother via the placenta, during delivery or during breast-feeding
- For adults, CMV transmission occurs from close contact with individuals excreting virus in saliva, urine, and other bodily fluids. Transmission of CMV has been reported from blood transfusion and organ transplant
- By the age of 30, approximately 40% of individuals are infected by CMV; by the age of 60, 80–100% of the population has been exposed to the virus
- Human CMV is difficult to culture as it slowly grows only in human fibroblasts since CMV is very specific in term of species and cell type to be infected

25.5.2 Clinical Presentation

- CMV elicits both humoral and cellular immune responses. CMV presents as primary, latent, reactivated, and reinfection

- CMV is transmitted by direct person-to-person contact with a person excreting the virus in their saliva, urine, or other body fluids; contact with infectious body fluids (e.g., urine on a diaper); or contact with fomites (e.g., a child's toy that has infectious virus on it). CMV can be sexually transmitted and can also be transmitted via transplanted organs and blood transfusions. Infectious CMV may be shed in the bodily fluids of any previously infected person, and thus may be found in urine, saliva, blood, tears, semen, and breast milk. The shedding of virus may take place intermittently, without any detectable signs
- The incidence of primary CMV infection in pregnant women in the US varies from 1% to 3%. Healthy pregnant women are not at special risk for disease from CMV infection. When infected with CMV, most people have no symptoms and very few have a disease resembling mononucleosis. But can be severe and life-threatening in immunocompromised patients including organ recipients and AIDS patients. It is their developing unborn babies that may be at risk for congenital CMV disease and occurs in about 1% of all newborns. 10–20% of these infants will develop complications before school age. CMV remains the most important cause of congenital viral infection in the US
- In infants and young children, typical features of the infection include hepatosplenomegaly, extramedullary cutaneous erythropoiesis and thrombocytopenia, and petechial hemorrhages. Encephalitis often leads to severe mental and motor retardation
- For immunocompromised patients, CMV disease is an aggressive condition. CMV hepatitis can cause fulminant liver failure. CMV infection can also cause CMV retinitis and CMV colitis

25.5.3 Diagnostic Methods

- Specimens
  - Whole blood, urine, CSF, amniotic fluid, bone marrow, biopsies
- Conventional tests (Table 25.5)
  - CMV cytology and histology tests
    - General
      - The CMV cytologic and histologic examination detects within cells evidence of CMV in urine or other body fluids or tissues. It also called CMV inclusion body detection. It is used to evaluate and manage CMV infection
      - The CMV cytologic and histological diagnosis is often considered the "gold standard" for diagnosing end-organ disease. Conventional H&E stains reveal enlarged (cytomegalic) cells that are often two- to fourfold larger than surrounding cells, usually with large eosinophilic intranuclear inclusions, sometimes surrounded by a clear halo, and smaller cytoplasmic inclusions. Its sensitivity shows ranging widely from 10% to 87%. It is important to note that 37.5% of patients with gastrointestinal CMV disease fail to demonstrate any inclusions
      - Immunohistochemistry with monoclonal antibodies directed against CMV immediate early antigen increases diagnostic yield of CMV compared to routine H&E staining. Sensitivity of immunohistochemistry for detecting CMV infection can approximate 93%
    - Advantages
      - Specific and definite diagnosis
      - Confirm end-organ disease along with virus infection diagnosis
    - Pitfalls
      - Invasive procedure required
      - Insensitive
  - Viral culture
    - Conventional culture
      - Viral culture is the traditional method of virus detection for demonstrating viremia and sometimes for establishing end-organ disease. Conventional CMV culture involves isolation of the virus in fibroblast tissue culture where it produces a distinctive

Table 25.5 CMV diagnostic tests

Assay	Specimen	Diagnostic utility and significance
CMV IgG antibody	Serum	Indication: to determine past exposure status. Diagnose primary infection Positive: indicates CMV infection in the past Negative: indicates absence of previous CMV infection Caveat: A negative result does not rule out recent CMV infection
CMV IgM antibody	Serum	Indication: to determine past exposure status. Diagnose primary infection Positive: indicates current primary CMV infection or possible reactivation of latent infection Negative: indicates absence of acute infection
CMV antigenemia	Whole blood	Indication: to diagnose and monitor CMV disease in immunocompromised patients Positive: indicates the presence of CMV viremia. May indicate impending CMV episode in absence of current disease Negative: CMV viremia not detected
Centrifuge-enhanced CMV Culture (shell vial)	BAL Bronch Gastric biopsy	Indication: to detect the presence of CMV infection Positive: indicates the presence of CMV infection Negative: does NOT rule out CMV disease, due to the low sensitivity of this test
Centrifuge-enhanced CMV Blood culture (shell vial)	Whole blood in heparin or EDTA	Indication: to diagnose and monitor CMV disease in immunocompromised patients Positive: indicates the presence of CMV viremia, but does not necessarily indicate clinical disease. May indicate impending CMV episode in the absence of current disease Negative: does NOT rule out CMV disease, due to the low sensitivity of this test
CMV DNA by hybrid capture	Whole blood in EDTA Refrigerate up to 48 h.	Indication: to diagnose and monitor CMV disease in immunocompromised patients Positive: indicates the presence of CMV DNA Negative: CMV DNA not detected
CMV DNA by PCR amplification	Serum/plasma Peripheral blood	Indication: to diagnose and monitor CMV disease in immunocompromised patients Positive: indicates the presence of CMV DNA Negative: CMV DNA not detected
CMV mRNA by Nucleic acid sequence-based Amplification (NASBA)	Whole blood in heparin or EDTA	Indication: to diagnose and monitor CMV disease in immunocompromised patients Positive: indicates the presence of CMV mRNA Negative: CMV mRNA not detected

cytopathogenic effect that is easily confirmed by fluorescent antibody stains

– Presence of the virus (positive cultures) can often be determined in as little as 1–2 days, but cultures that are negative for the virus must be held for 3 weeks to confirm the absence of CMV because the virus may be present in very low numbers in the original sample and/or the CMV strain may be slow-growing
– CMV culture can be performed on blood, tissue, urine, saliva, or respiratory swabs. Blood culture has a sensitivity of 45–78% but a very high specificity, approaching 89–100% for detecting disease. CMV detection in

blood is more strongly associated with disease than detection in urine or saliva, with a positive predictive value of 60%
  - Advantages
    - Gold standard test for CMV detection
    - Able to recover other viruses from the same specimen
  - Pitfalls
    - Low sensitivity compared to newer techniques, such as antigenemia and PCR and nucleic acid probe
    - Lack of virus quantitation
    - A long incubation period (1–3 weeks), lack of virus quantitation
    - False-negative results if cell culture inoculation is delayed
- Shell vial assay
  - Another CMV culture (shell vial culture) method is rapid viral culture. The shell vial culture with immunofluorescence staining is used for the early diagnosis of CMV infection
  - In immunocompromised patients, a sensitivity of 78% and a specificity of 100% have been claimed
  - Specimens are centrifuged into fibroblast monolayers, incubated briefly, and then stained with a fluorescent monoclonal antibody to early antigens, before the appearance of cytopathogenic effect. Results are available much earlier than with conventional CMV culture, usually within 24–48 h
  - The cells are read under a fluorescent microscope
  - Advantages
    - Higher sensitivity than conventional methods
    - Quantitative shell vial cultures are available, but not widely used
  - Pitfalls
    - May need large amount of biomass for virus recovery
- Serological studies
  - CMV antibody testing
    - Antibody testing can be used to determine if someone has had recent or past exposure. There are two types of CMV antibodies that are produced in response to a CMV infection, IgM and IgG, and one or both may be detected in the blood
    - IgM antibodies are the first to be produced by the body in response to a CMV infection. CMV IgM antibodies are detected within a week or two after primary infection and lasts 3–4 months. After several months, the level of CMV IgM antibody usually falls below detectable levels
    - It is not detectable in recurrent infection except in immunocompromised patients where it is detectable in about a third of the cases
    - CMV IgG antibody is produced early in primary infection and provides protection from primary infections. Levels of IgG rise during the active infection then stabilize as the CMV infection resolves and the virus becomes inactive. After a person has been exposed to CMV, he or she will have some measurable amount of CMV IgG antibody in their blood for the rest of their life
    - CMV IgG avidity test to distinguish primary CMV infection from past or recurrent infection (reactivation or reinfection). CMV IgG avidity is low (<30%) in primary infection
    - Prenatal diagnosis of congenital CMV infection is performed only in the case of primary maternal infection as transplacental transmission of CMV is higher in 40% of primary maternal CMV infection. Whereas it is low in the case of recurrent infection 1–4%
    - CMV IgG antibody testing can be used, along with IgM testing, to help confirm the presence of a recent or previous CMV infection
  - CMV antigenemia test
    - This test is based upon the detection of pp65, a structural protein expressed

on the surface of infected polymorph nuclear lymphocytes
  - The specimen must be processed within 6–8 h of collection, but results can be available within 8–24 h
  - The number of infected leukocytes present had been reported to correlate with the severity of infection
  - This test is usually applied to blood and cerebrospinal fluid, with a sensitivity of 60–100% and a specificity of 83–100%
  - Commercial kit
    - The CMV Brite™ Turbo Antigenemia Kit uses the well-defined C10/C11 antibody cocktail to detect the CMV lower matrix phosphoprotein (pp65), an early antigen in virus replication, which is abundantly present in antigen-positive polymorphonuclear cells
    - The CMV Brite Turbo Kit is a rapid new version of the first FDA-registered immunofluorescence antigenemia kit for in vitro CMV diagnosis
  - Advantages
    - Inexpensive kits are commercially available
    - May be able to detect CMV before development of symptoms
  - Pitfalls
    - Labor-intensive
    - Require skilled personnel
    - Subjective interpretation
    - Although an improvement over CMV viral culture, this technique is only semiquantitative, and the reading of results is somewhat subjective
    - Poor sensitivity in urine samples. The assay is adversely affected by low leukocyte counts
- Molecular methods (Table 25.5)
  - General
    - CMV DNA tests can be qualitative or quantitative. The two major techniques used are hybrid capture and PCR. PCR appears to be more sensitive than hybrid capture
    - Different tissues and body fluid compartments can be used for PCR including whole blood, plasma, leukocytes, buffy coat specimens, bronchoalveolar lavage (BAL) fluid, target organ tissue, or stool. Whole blood PCR testing is more sensitive than plasma PCR testing
    - Higher CMV viral loads seem to correlate with symptomatic disease
    - Stool CMV DNA testing is a technique that is noninvasive and would detect infection even when located in the right colon, out of the reach of a sigmoidoscopic examination. Stool CMV DNA also is possibly more sensitive than blood CMV DNA for detecting colonic disease, as it is more organ specific
    - CMV DNA testing in blood is a very promising technique for diagnosing CMV disease. Its advantages include quick results (6–48 h) and high sensitivity
  - Qualitative
    - CMV DNA by hybrid capture assay (Digene): FDA cleared
      - The Digene Hybrid Capture CMV test is a molecular assay that detects the presence of CMV DNA in white blood cells from blood collected in EDTA. It is more sensitive than CMV culture and has a sensitivity and specificity comparable to the CMV antigenemia assay for the detection of CMV viremia
      - Unlabeled CMV probes hybridized with viral DNA, then immobilized on a solid phase before being measured by conjugated antihybrid antibody
    - CMV mRNA by NASBA
      - Assay of CMV mRNA by nucleic acid sequence-based amplification (NASBA) has been investigated for the detection of active viral gene expression and replication
      - CMV-specific mRNA may be a more specific marker for CMV than culture or DNA PCR since it would

theoretically be indicative of CMV replication
  - Since late transcripts such as pp67 mRNA reflect a complete replication cycle of CMV, they could be indicative of disease
  - NucliSens CMV pp67 Assay (Organon Teknika Inc., Durham, NC) is an FDA-cleared test. NucliSens CMV pp67 measures replication of CMV in blood using NASBA RNA amplification technology
  - This assay detects messenger RNAs coding for the matrix tegument protein pp67 of CMV, a true late protein, which is only expressed during viral replication
  - The NASBA technology selectively amplifies RNA in a DNA background and allows direct testing in whole blood
  - It is a direct route for diagnosing an active CMV infection and monitoring treatment efficacy
- Quantitative
  - PCR
    - Amplicor CMV MONITOR test (Roche Molecular Systems) is a quantitative microtiter-based PCR assay
      - CMV viral DNA in the specimen was quantitated by coamplifying a region of the CMV DNA polymerase gene in the presence of a known quantity of quantitative standard
      - The primers used were specific for the CMV polymerase gene and amplified a 362-bp fragment of the gene
      - An internal quantitation standard (QS) that is added at a known concentration during specimen processing so that extraction and recovery of DNA, in addition to amplification and detection, can be monitored
      - The lower limit of sensitivity of the assay is 400 copies/ml of plasma. The linear range of the assay is 400–400,000 copies of CMV DNA per mL
      - The inherent sensitivity of molecular detection of CMV poses a problem since latent CMV genomes, present in most seropositive individuals, may be detected. Therefore, it is critical to adjust the sensitivity of the PCR so that latent genomes are not detected
    - Advantages
      - Sensitive enough to detect virus before symptom development
      - Specimen could be stored and transported
      - Rapid and less expensive
    - Pitfalls
      - False positive
      - Contamination must be prevented
  - Real-time PCR
    - The COBAS® AmpliPrep/COBAS® TaqMan® CMV Test is a real-time PCR-based quantitative assay for CMV DNA detection in human plasma using the COBAS® AmpliPrep Instrument for automated specimen processing and the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer for automated amplification and detection. The test can quantitate CMV DNA over the range of 150–10,000,000 copies/mL
    - Various home brew methods were developed either using real-time TaqMan PCR (ABI prism 7700) or real-time LightCycler PCR
    - Advantages
      - Quick result: turnaround time for the real-time PCR assay is 6–48 h
      - High sensitivity
      - CMV DNA testing also has greater than 80% concordance with CMV antigen test results, but also has the added benefits of increased specimen stability, smaller required

specimen volume, and with the ability to be performed in patients with depressed white blood cell counts results
  - Pitfalls
    - False-positive results due to contamination (detected by negative control)
    - False-negative results due to amplification inhibition (detected by internal control) or due to a loss of bacteria during specimen preparation
    - There is a lack of standardization of this process, with different techniques and assays, different quantitation methods, and different tissues and blood compartments being tested, thus making interpretation of results across studies challenging. Quantitative PCR is more sensitive than qualitative PCR, and CMV DNA values obtained with "in-house" quantitative assays are 3- to 10-fold higher than the commercial assay
    - Different quantitation methods include reporting results as genomic copies/mL, copies/mL, copies/microgram of total DNA, copies/106 leukocytes, and copies/2 $\times$ 10^5 leukocytes; such varied quantitation methods can make comparison of results almost impossible
    - No clearly defined cutoff values for determining CMV disease, this method has low specificity and low positive predictive value. Most of CMV cutoff on plasma ranging from 400 to 10,000 copies/mL of CMV DNA
- Antiviral susceptibility testing of CMV isolates
  - General
    - Resistance of CMV to antivirals was a major clinical problem in patients with AIDS. Currently marketed anti-CMV drugs, namely, ganciclovir (GCV), its oral prodrug valganciclovir (vGCV), foscarnet (FOS), and cidofovir (CDV), all target the viral DNA polymerase
    - CMV infection remains a major problem in transplantation, and resistance to antivirals is encountered in all forms of transplantation
    - In general, it takes weeks to months for CMV to develop resistance to antivirals. In patients with AIDS, some studies showed a 10% prevalence of resistance to ganciclovir by 3 months of therapy; similar time courses were found for foscarnet and cidofovir
  - Phenotypic methods
    - Plaque reduction assay
      - The gold standard for antiviral susceptibility testing of CMV is plaque reduction assay
      - In this assay, a standardized inoculum of a stock virus is inoculated into cultures and incubated in the presence of the antiviral agent
      - The cultures are then observed for the presence of viral plaques
      - The IC50 of the agent for the isolate is defined as the concentration of agent causing a 50% reduction in the number of plaques produced
      - Plaque reduction assays are labor-intensive
      - Plaque reduction assays are limited by the excessive time required completing the assay (4–6 weeks) and the lack of a standardized method validated across different laboratories
      - In addition, repeated passage of isolates to prepare viral stocks may influence the results of assays by selecting CMV strains that are not representative of the original population of the viruses
    - DNA hybridization assay
      - Whole genomic DNA is extracted and transferred by capillary action onto

negatively charged nylon membranes after incubation with a specific agent
  - The membranes are hybridized to a 125I-labeled human CMV probe (Diagnostic Hybrids, Athens, Ohio), rinsed, washed, and counted in a gamma counter
  - Mean hybridization values (in counts per minute [cpm]) for each concentration of antiviral agent are calculated and expressed as a percentage of the cpm in control cultures
  - The IC50 is defined as the concentration of antiviral agent resulting in a 50% reduction in viral nucleic acid hybridization values (i.e., DNA synthesis) compared with the hybridization values of controls
  - Disadvantage of DNA hybridization assays is that they require the use of radiolabeled probes
  - DNA hybridization assays have the advantage over plaque reduction assays of eliminating the variation due to subjective errors resulting from plaque counting by different individuals
- Viral load assays (e.g., antigenemia or quantitative DNA)
  - CMV viral load assay may rise as an indicator of antiviral resistance, but other factors (including compliance and declining immune function) may be responsible
  - The assay can measure viral DNA concentration exposed to a range of drug concentrations and is grown for 4 days in the presence or absence of drug then the IC50 is determined
  - Quantitative antigenemia assays are less exact than quantitative polymerase chain reaction
  - In many patients, certain CMV diseases (e.g., gastrointestinal disease or retinitis) are not always associated with measurable viral loads
- Other phenotypic methods: Viral production is measured by using immunofluorescence-, immunoperoxidase-, ELISA-, or flow cytometry-based methods for detection and quantitation of cells expressing CMV antigens (immediate–early, early, or late)

- Genotypic methods
  - General
    - Viral UL97 kinase and UL54 DNA polymerase gene mutations are well-documented mechanisms of resistance to current antivirals
    - The mutation of the viral phosphotransferase gene (UL97) coding sequence, which may confer resistance only to ganciclovir (GCV). One of seven canonical UL97 mutations (M460V/I, H520Q, C592G, A594V, L595S, and C603W) is found in over 80% of GCV-resistant clinical CMV strains
    - Although UL97 mutations do not affect susceptibility to FOS or CDV, UL54 mutations can confer resistance to all current drugs and may emerge after prolonged GCV therapy to increase the level of resistance conferred by a preexisting UL97 mutation. Mutations in UL54 are often accompanied by mutations in UL97, showing higher levels of resistance to ganciclovir with possible cross-resistance to foscarnet and/or cidofovir
    - The viral polymerase gene UL54 occurs in regions between codons 300 and 1,000 (Fig. 25.19)
  - Genotypes for CMV antiviral resistance
    - Detection of mutations is based on PCR amplification of the specific region of the genome followed by restriction enzyme analysis or direct sequencing of the amplification product
    - Standard dideoxy sequencing can detect an emerging resistance mutation when it exceeds approximately 20% of the sequence population

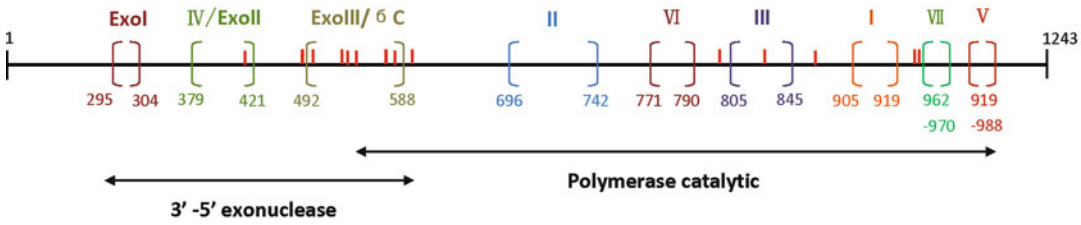


Fig. 25.19 Map of the CMV UL54 DNA polymerase gene showing conserved functional domains, ranges of codons containing drug resistance mutations, and the locations of drug resistance mutations and nonresistant sequence variants newly described (Adapted from Hakki et al. Curr Opin Infect Dis 2011)

- Pyrosequencing has been reported to detect mutant subpopulations at an approximately 6% level at codons 460, 520, and 592–607 of UL97, but the short sequence reads are unsuitable for analyzing the full range of codons needed for genotypic diagnosis (e.g., UL97 codons 335–670 and UL54 codons 300–1,000)
- An additional method "real-time PCR" with melting curve assay can also detect drug resistance mutations. The advantages are that low copy numbers without cell culture can be detected, mixed virus populations can be analyzed semi-quantitatively, and multiplex reaction to different mutations can be detected simultaneously. The disadvantages are that polymorphisms near known mutations may affect the melting curve and give false-positive result and different probes are needed to identify each mutated codon. Besides, the assay may not be able to differentiate different point mutations that occur in the same codon, such as M460I (ATT/ATA) and M460V (GTG)

25.5.4 Clinical Utility

- Quantitative PCR determination of CMV viral load in solid organ transplant recipients can predict CMV disease and relapse (Table 25.5) as well as for initiating antiviral therapy
- Viral load testing in patients with HIV infection is currently used to predict CMV disease and to monitor the efficacy of treatment
- Pitfalls
  - Using restriction enzyme analysis, not all of the presently confirmed resistance mutations are accompanied by alteration of known restriction enzyme recognition sites which lead to false-negative results, and base changes not associated with drug resistance can produce new restriction sites which lead to false-positive results
  - PCR assays are not standardized, and variations in sample handling and laboratory methods can affect the sensitivity of the assay
  - Well-defined CMV DNA standards are needed to avoid variation of viral load values obtained with commercial and home brew assays
- Clinical utility: The standardization of automated sequencing methods and the characterization of mutations associated with drug resistance will offer routinely genotypic resistance testing in a time frame that impacts clinical care

25.6 Epstein–Barr Virus

25.6.1 General Characteristics

- EBV is a dsDNA virus of the herpes family (HHV type 4) characterized by icosahedral capsid and a glycoprotein-containing envelope. It is one of most common viruses in human

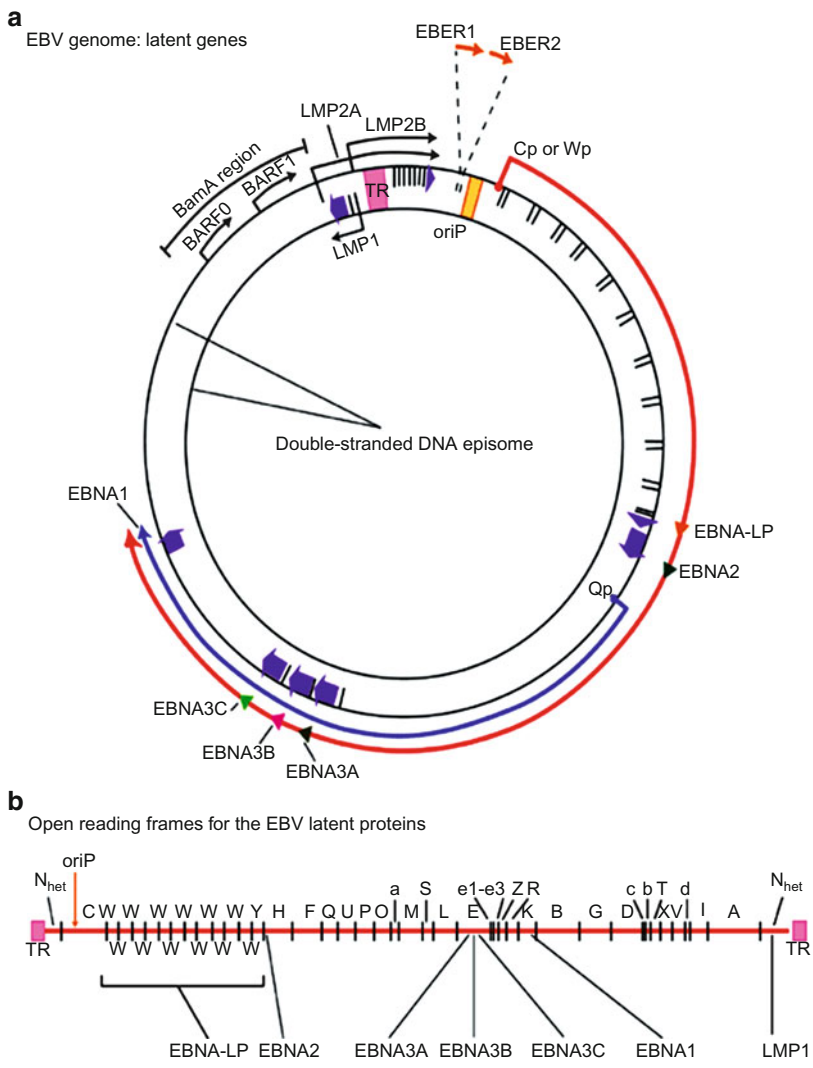


Fig. 25.20 Epstein–Barr virus genome structure (Adapted from Murray et al. 2001)

- The genome is a linear dsDNA molecule with 172 kbp. The viral genome does not normally integrate into the cellular DNA but forms circular episomes which reside in the nucleus
- Most common mode of transmission of EBV is through exposure to infected saliva from asymptomatic individuals. Virus is relatively fragile and does not survive long outside the human host fluids. Primary infection of the virus occurs by oral transmission from asymptomatic individuals. EBV preferentially infects B lymphocytes and remains latent, affecting more than 95% of population
- The genome is large enough to code for 100–200 proteins but only a few have been identified. The proteins characterized thus far fall into the following group: (1) Latent proteins, including EB viral nuclear antigen complex (EBNA), latent membrane protein (LMP), terminal protein, and lymphocyte-detected membrane antigen (LYDMA); and (2) lytic cycle proteins, including membrane antigen (MA), early antigen complex (EA), and viral capsid antigen complex (VCA). Critical viral target genes: EBNA1, LMP1, and LMP2 (Fig. 25.20)

25.6.2 Clinical Presentation

- EBV causes infectious mononucleosis, an acute but self-limiting disease affecting children and young adults. After primary infection, the virus persists indefinitely in B lymphocytes, only to reactivate when cellular immunity is impaired
- In infected young children remain asymptomatic or developed nonspecific viral illness
- EBV causes infectious mononucleosis, associated with PTLD, linked to several malignancies such as nasopharyngeal carcinoma (NPC) among Chinese males and Burkitt lymphoma (BL) in children of Central Africa/New Guinea. In HIV-infected individuals, EBV is associated with diseases such as oral hairy leukoplakia and AIDS-related non-Hodgkin lymphoma; EBV might imply a higher risk of some autoimmune diseases such as systemic lupus erythematosus (SLE) and multiple sclerosis
- EBV is a ubiquitous virus which causes persistent, latent infection that can be reactivated. More than 90% of the adult population is estimated to demonstrate serologic evidence of prior exposure with EBV
- Primary infection in young children is often asymptomatic or causes nonspecific minor illness
- For adolescents and young adults, primary infection is typically manifested as infectious mononucleosis (IM), usually a self-limiting condition characterized by fever, sore throat, myalgias, lymphadenopathy, and hepatosplenomegaly
- A strong association between EBV and Burkitt lymphoma in children of Central Africa/New Guinea and nasopharyngeal carcinoma among Chinese males
- In HIV-infected individuals, EBV is associated with diseases such as oral hairy leukoplakia and AIDS-related non-Hodgkin lymphoma, i.e., oral hairy leukoplakia and CNS lymphoma
- Patients undergoing transplantation are prone to develop posttransplant lymphoproliferative disease (PTLD)
- Although the correlation between EBV burden and disease status is incompletely understood, several studies have shown an association between symptomatic infection and elevated DNA loads in clinical samples. Increasing virus burden is also believed to be a rapid indicator of immunopathological changes preceding and/or underlying the B lymphocyte-driven changes caused by EBV. Therefore, determining EBV DNA loads in EBV-related disorders in immunocompromised populations is an important step toward disease diagnosis, management, and treatment

25.6.3 Diagnostic Methods

- Specimens
  - Whole blood, plasma, CSF, biopsy
- Conventional tests and problems (Table 25.6)
  - EBV antibodies: EBV antibodies are used to help diagnose Mono if people are symptomatic but have a negative Mono test. EBV antibodies include (1) viral capsid antigen (VCA)-IgM, VCA-IgG, and D early antigen (EA-D) – to detect a current or recent infection; and (2) VCA-IgG and Epstein–Barr nuclear antigen (EBNA) – to detect a previous infection
    - Heterophile IgM antibody
      - Present in 90% of adults during the course of illness
      - Nonspecific serologic response to EBV infection
    - Classic Paul–Bunnell test
      - Measures agglutination of sheep RBCs by patient serum; limited by false-positive agglutinins in sera of normal individuals (Forssman agglutinins), patients with serum sickness, etc
    - Monospot test: detects agglutinins to formalized horse RBCs not removed by prior absorption with guinea pig kidney
    - Viral capsid antigen antibody (Fig. 25.21)
      - IgM – indicates recent infection, lasts only 4–8 weeks

Table 25.6 EBV diagnostic tests

EBV test	Diagnostic utility and significance	Comments
Infectious mononucleosis slide test	Initial testing to confirm infectious mononucleosis or recent EBV infection (Monospot test) Negative Monospot test is common in children and immunocompromised adults	If test results are negative but a strong clinical suspicion exists, repeat testing in 7–14 days
Epstein–Barr virus (EBV) antibody to viral capsid antigen, IgM and IgG	Clarify or confirm equivocal or negative Monospot test Discriminate EBV from other IM-like diseases (e.g., CMV, toxoplasmosis)	Repeat testing in 10–14 days may be helpful if results are equivocal
EBV antibody to nuclear antigen, IgG	Confirm previous infection with EBV	Repeat testing in 10–14 days may be helpful if results are equivocal
EBV by PCR	Detect EBV in cerebrospinal fluid and serum specimens Diagnose EBV-related diseases in immunocompromised patients or patients with lymphoproliferative tumors Do not use to confirm acute mononucleosis	Negative result does not rule out the presence of PCR inhibitors in patient specimen or EBV DNA in concentrations below assay detection
EBV, quantitative PCR	Monitor disease (whole blood, plasma, serum, or CSF specimens) Do not use to confirm acute mononucleosis	
EBV antibody to early D antigen (EA-D), IgG	Confirm chronic active mononucleosis, posttransplant lymphoproliferative disease, and nasopharyngeal carcinoma This antibody test is more useful and appropriate than early antigen R for mononucleosis assessment	Repeat testing in 10–14 days may be helpful if results are equivocal
EBV by in situ hybridization	Virus identification of EBV	

  - IgG – peaks during week 3–4 of infection, can persist for more than 1 year or entire lifetime
- Early antigen antibody, Anti-D
  - Diffusely nuclear and cytoplasmic staining of infected cells
  - Present in 40% of infectious mononucleosis patients
  - Persists for 3–6 months
  - Detected in patients with nasopharyngeal carcinoma
- Early antigen antibody, Anti-R
  - Stains cytoplasmic aggregates
  - Found in atypical protracted cases of infectious mononucleosis
  - Found in patients with African Burkitt lymphoma
- Epstein–Barr nuclear antigen antibody
  - Appears 3–4 weeks after infection
  - Persistent for life
  - Found in patient with Burkitt lymphoma
- Molecular methods (Table 25.6)
  - In situ hybridization (Biogenex, San Ramon, CA)
    - Used for tissue biopsy
    - The EBV EBER Probe is specific for EBER RNA transcripts and is intended for the detection of latent EBV infection
    - The EBV Not I/Pst I DNA Probe is specific for the Not I/Pst I repeat sequence of EBV and is intended for the detection of active EBV infection
  - Quantitative competitive PCR
    - Specific primers are specifically designed to the EBV viral latent membrane protein 2a (LMP2a) and

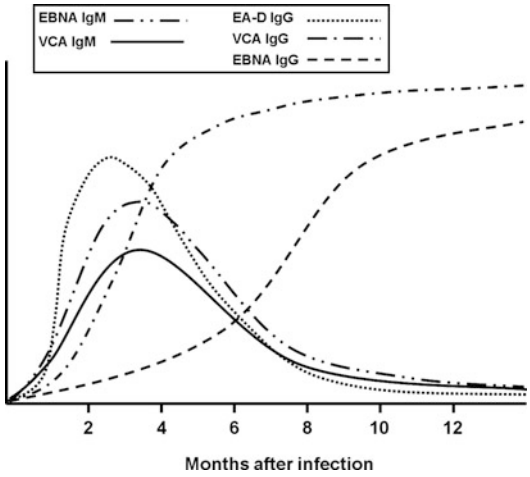


Fig. 25.21 Time course for appearance of antibodies in EBV infection

internal competitor DNA (ssDNA) that is confirmed against a known number of Namalwa cells (B cell lymphoma cell line containing two integrated copies of the EBV genome per cell)
- Four separate PCR reaction tubes each containing internal competitor DNA (8 copies/μl, 40 copies/μl, 200 copies/μl, or 1,000 copies/μl) are placed in competition with EBV-specific primers for amplification of patient DNA
- PCR amplicons are examined by electrophoresis through a 2% agarose gel and visualized using a gel imaging documentation system. The band densities are quantitatively measured using Bio-Rad Quantity One software and used to calculate EBV copies
- Although highly accurate and reproducible, such assays are rather laborious and require intensive post-PCR handling. Each sample has to be spiked with different amounts of internal standard to achieve precise quantification

– Real-time PCR
  - LightCycler® EBV quantitative kit (Roche)
    – Detection of LMP gene in EBV viral genome
    – EBV is amplified with specific primers in a PCR reaction. The amplicon is detected by fluorescence using a specific pair of hybridization probes
    – A melting curve analysis is performed after the PCR run to differentiate positive samples from non-EBV species; i.e., other herpes virus family
    – The internal control is a synthetic double-stranded DNA molecule with primer binding sites identical to the EBV target sequence, comprising a unique hybridization probe binding region that differentiates the internal control from the target-specific amplicon. It is added already to the lysed sample before the purification step and copurified/amplified with the EBV DNA from the specimen in the same PCR reaction (dual color detection)
    – The kit allows quantification in a range of 10^2–106 copies per reaction. The lower detection limit of the kit is $\leq$10 copies per reaction (95% confidence interval; probit analysis)
- Other commercial assays and reagents
  – Other commercial assays and reagents included Nanogen EBV Q-PCR Alert, Argene EBV R-gene™, QIAGEN artus® EBV (LC and RG) PCR Kits, Cepheid affigene® EBV trender and SmartEBV™, ELITech/Epoch EBV ASR, and Quantification of HHV4 PrimerDesign™ Ltd
  – The range of EBV PCR targets included EBNA1, EBNA2, BNRF1 p143, BXLF1, EBER1, BALF5, and BamHI-W
  – Amplification platforms included Roche LightCycler® 1.5, 2.0, and 480 systems, Applied Biosystems™ 7300, 7500, 7500 Fast, and 7900 HT Fast Real-Time PCR Systems, Agilent Mx3000P® quantitative PCR System, QIAGEN Rotor-Gene™ Q and Rotor-Gene™ 3000, and Cepheid SmartCycler™ II

- Real-time PCR using SYBR Green I dye
  - To maximize detection rates and reduce false-negative results, two primer sets targeting the highly conserved EBV regions, (1) Epstein–Barr nuclear antigen 1 (EBNA1) and (2) BamHI fragment H rightward open reading frame 1 (BHRF1), used to detect and measure absolute EBV DNA load in clinical settings with different EBV-associated diseases. Two separate real-time quantitative PCR assays using SYBR Green I dye and a single quantification standard containing the two EBV genes
  - PCR products analyzed by an amplification curve, melt analyses, and amplification efficiency
  - The lower limit of detection for both EBV regions was 2.0×10^3 copies/ml
  - Sensitive and cost-effective
- Pitfalls
  - Quantitative PCR requires analysis of absolute lymphocyte count, which inversely affects viremia; real-time PCR does not
  - Important to note that real-time PCR assay requires sequential analysis of run data prior to result reporting to prevent false positives (i.e., pseudoamplification and amplification of non-EBV species) and false negatives (i.e., shifted melting curve for EBV variants)
  - PCR assays are not standardized and variations in sample handling and laboratory methods can affect the sensitivity of the assay

25.6.4 Clinical Utility

- Serial viral load testing can be used to monitor disease burden and assess efficacy of immunosuppressive therapy in posttransplant patients
- Detection of EBV in tissue biopsy assists the diagnosis of EBV-related malignancies, including lymphoma and nasopharyngeal carcinoma

25.7 Herpes Simplex Virus

25.7.1 General Characteristics

- Family of enveloped icosahedral nucleocapsid viruses with total nine members
- Herpes simplex virus (HSP), type 1 and type 2, demonstrates an 83% DNA homology in protein-coding regions
- The genetic map of the two viruses is colinear, and the genomes are of approximately the same size, HSV1 of 152 kbp and HSV2 of 155 kbp
- Humans are the only known reservoir
- Direct contact with lesion or secretions necessary for transmission. After direct exposure to infectious material (i.e., saliva, genital secretions), initial viral replication occurs at either the skin or mucous membrane entry site, typically of epithelial cells
- HSV1 and HSV2 most common. HSV1 acquired early in life, usually associated with oral lesions. HSV2 acquired after onset of sexual activity, associated with genital lesions. Both viral types can cause oral–facial and genital infections and maybe clinically indistinguishable
- Risk of transmission of HSV from HSV-infected mother during vaginal delivery to infant is 50%, estimated to be between 1 in 2,000 and 1 in 5,000 births
- Beyond the neonatal period, most childhood HSV infections are caused by HSV1. The seroprevalence of HSV1 antibodies increases with age and is 20% by age 5 years. No increase occurs until age 20–40 years, when 40–60% of individuals are HSV1 seropositive
- Latent infections reside in neurons (trigeminal, sacral, and vagal ganglia) and can be activated by a stimulus (e.g., physical or emotional stress, fever, ultraviolet light) causes reactivation of the virus in the form of skin vesicles or mucosal ulcers, with symptoms less severe than primary infection

25.7.2 Clinical Presentation

- Primary infection usually with a 2–20 days incubation period
- Cutaneous vesicles characterized by ulcers that eventually pustulate, dry, and crust; mucosal vesicles appear as shallow punctuate ulcers that often coalesce
- Primary herpetic gingivostomatitis/pharyngotonsillitis (HSV1): Most cases are asymptomatic. Most cases are between 6 months and 5 years. Characterized by generalized malaise, fever, linear gingivitis, and lymphadenopathy
- Primary herpes genitalis (HSV2): Genital HSV2 infection is twice as likely to reactivate and recurs 8–10 times more frequently than genital HSV1 infection. A classic vesicular rash may be noted or progressive lesions (pustules or painful ulcerative lesions). Lesions may persist for as many as 3 weeks. Painful inguinal lymphadenopathy, dysuria, and vaginal discharge are frequent complaints. Most primary genital HSV infections are asymptomatic, and 70–80% of seropositive individuals have no history of symptomatic genital herpes. HSV can be transmitted in the presence or absence of symptoms
- Primary cutaneous herpetic infections can occur in wrestlers and rugby players with contaminated abrasions (herpetic gladiatorum or scrumpox)
- HSV keratitis presents with an acute onset of pain, blurring of vision, chemosis, conjunctivitis, and characteristic dendritic lesions of the cornea
- HSV meningitis
  - 1–7% of all cases of aseptic meningitis
  - HSV2 > HSV1
  - 20–45% with meningitis have recurrent episodes
  - HSV accounts for 10–20% of all cases of sporadic viral encephalitis in the US. The clinical hallmark of HSV encephalitis has been the acute onset of fever and focal neurologic (especially temporal lobe) symptoms. Clinical differentiation of HSV encephalitis from other viral encephalitides, focal infections, or noninfectious processes is difficult
  - Neonates (<6 weeks) have the highest frequency of visceral and/or CNS infection of any HSV-infected patient population
- HSV infection of visceral organs usually results from viremia, and multiple organ involvement is common
- Recurrent infection at sites of primary infection
  - Activation of latent virus form neurons of cervical ganglia (herpes labialis, HSV1) or sacral ganglia (HSV2)
  - Self-inoculation of fingers and thumbs (herpetic whitlow) can occur in children with orofacial herpes, although less common
  - Antiviral prophylaxis recommended for persistent recurrent cases
  - Some cases of erythema multiforme (EM) are believed to represent an allergic response to recurrent HSV infection

25.7.3 Diagnostic Methods

- Specimens
  - Vesicular fluid, ulcerated lesions, pharyngeal and throat swabs, urine, CSF, autopsy and biopsy material, ocular exudates, and vaginal swabs
  - Specimen is best collected within the first 3 days after appearance of lesion but no more than 7 days
- Conventional tests and problems
  - Viral culture
    - Conventional
      - Cell culture requires the collection of live virus samples that require special care in transport to the laboratory to retain viability. When viable samples are used, culture can be highly specific (if typing is performed) and positive results are generally reliable
      - The sensitivity of culture declines rapidly as lesions begin to heal,

and for this reason, frequently nonpositive result can be falsely negative. Type-specific serology tests should be used in these cases to confirm a clinical diagnosis of genital herpes
    - Many commercial cell lines are used (A549, RK, ML, HNK, MRC5, etc.)
    - Diagnosed by observation of cytopathic effect (CPE) induced by virus which usually occurs in 1 week after initial inoculation
  - Shell vial assay
    - A centrifugation-enhanced culture technique used to obtain rapid culture results. Generally less sensitive than conventional culture
    - The test can detect HSV in shell vial cultures (MRC5 cells) before the development of cytopathic effect (pre-CPE)
    - Immunofluorescent assay (IFA) staining of shell vial for viral detection and typing
- Cytology
  - Intranuclear inclusion bodies
  - Multinucleated, molded giant cells
  - Margination of nuclear chromatin
  - Indicates the presence of herpesvirus (HSV1/2 or varicella zoster virus [VZV]) and excludes coxsackievirus and nonviral entities
- Serological studies
  - Limited by cross-reacting antigens between HSV1 and HSV2
  - ELISA
    - Performed on fluids or other samples using HSV-specific antibody that is bound to a solid surface
    - Antibody captures antigen to which anti-HSV antibodies labeled with enzymes are added. These attach to the bound antigen and cause a color change
  - IFA and immunoperoxidase (IPA) assay
    - Detect HSV antigen in smears or tissues. HSV-specific antibodies are labeled with fluorescent dyes or enzymes (peroxidase)
    - Labeled antibodies are incubated with the specimen and bind to HSV antigens in the specimen, if present
    - Attached fluorescent dye or enzyme can be visualized in appropriate regions of infected cells under a microscope
    - Used in conjunction with shell vial culture
  - ELVIS® (enzyme-linked virus inducible system)
    - Technique combines cell culture amplification with HSV-activated reporter genes
    - The test produces results that are equal to conventional culture
- Molecular methods
  - Real-time PCR (LightCycler® – HSV1/2 detection kit, Roche)
    - Detection and differentiation of HSV1/2
    - HSV1/2 is amplified with specific primers in a PCR reaction. The amplicon is detected by fluorescence using a specific pair of hybridization probes
    - A melting curve analysis is performed after the PCR run to differentiate positive samples in HSV1 or HSV2. Melting points for HSV1 and HSV2 are significantly different (HSV1 at 53.9 °C, whereas HSV2 at 67.1 °C) and allow clear determination of the HSV type
    - The internal control is added already to the lysed sample before the purification step and copurified/amplified with the HSV DNA from the specimen in the same PCR reaction (dual color detection)
  - Real-time PCR (HSV1/2 PCR kit, Abbott Molecular)
    - The HSV1/2 PCR kit is for the detection and differentiation of HSV 1 and 2 DNA using PCR in the ABI PRISM® 7000 and 7900HT sequence detection system (Applied Biosystems)

    - The HSV TM Master contains reagents and enzymes for the specific amplification of a 148-bp region of the HSV genome
    - The amplicon is detected by measuring the FAM fluorescence (HSV1) and NED fluorescence (HSV2) in the ABI PRISM® SDS
    - In addition, the HSV1/2 PCR kit contains a second heterologous amplification system to identify possible PCR inhibition. This is detected as an internal control (IC) by measuring the VIC fluorescence
    - External positive controls (HSV1 LC/RG/TM QS 1–4 and HSV2 LC/RG/TM QS 1–4) are used to allow the determination of the pathogen load
  - Advantages
    - HSV real-time PCR-based assay is highly sensitive and specific, and it can detect the virus even during the low viral shedding
    - PCR-based technique was shown to increase the overall rate of HSV detection by 61–71%. Even in patients with visible genital ulcerations, PCR detected 88% more infections than virus culture
- Sensitivity and specificity
  - The lower limit of detection (analytical sensitivity) for HSV qualitative PCR is 25 copies/reaction (~1,250 copies/ml)
  - The sensitivity of PCR
    - HSV in skin lesions: sensitivity 83–100% and specificity 100%
    - CSF: sensitivity 70–100%
  - HSV was detected more frequently by PCR than by viral culture regardless of whether samples were obtained from HSV lesions, or from genital or oral secretions during a period of subclinical shedding. Yield of virus positivity is four times greater by PCR than by culture, and the results are more reliable, especially in settings in which transport or climate may interfere with the yield from viral culture
  - Due to the sensitivity of PCR, many labs now only offer PCR tests and culture is used only when sensitivity test is needed
  - CSF culture
    - ~80% positive with first attack
    - 0% with recurrent episodes
    - Multiplex PCR could be performed for HSV1, HSV2 and VZV, CMV, and HSV6 from the same sample
- Pitfalls
  - Important to note that PCR assays are not standardized and variations in sample handling and laboratory methods can affect the sensitivity of the assay
  - PCR cannot always diagnose HSV encephalitis in the first few days of illness, and serial evaluations of CSF by PCR during the first week of illness are necessary

25.7.4 Clinical Utility

- Diagnosis of herpes encephalitis in neonates and immunocompromised patients by detection of HSV in CSF
- CSF PCR for HSV DNA should be performed in patients with febrile encephalopathy even in the absence of focal features, initial CSF pleocytosis, or abnormal CT. Mild or atypical HSV encephalitis may be associated with infection from HSV1 or HSV2
- In addition to CSF, other specimens can be used for PCR, including mucosal secretion, skin lesion, etc
- Current treatment guidelines for herpes include three antiviral therapies, acyclovir, famciclovir, and valacyclovir, and should begin as soon as possible after symptoms begin. Antiviral therapy may be effective when taken during onset of prodromal symptoms; i.e., tingling
- Antiviral therapy will reduce the duration of outbreak by approximately 2 days
- Suppressive therapy is highly effective and can dramatically reduce the frequency of recurrences. Suppression can be continued for years with very low risk of toxicity or development of drug-resistant HSV. Suppressive therapy will also reduce the frequency of asymptomatic HSV shedding

Table 25.7 Varicella zoster virus (VZV) diagnostic tests

Test name	Diagnostic utility and significance	Comments
VZV – viral culture	Culture considered gold standard	Not recommended for CSF samples
VZV – DFA (direct fluorescent antibody)	Rapid confirmation of VZV High sensitivity and specificity	
VZV – PCR	VZV test need be performed by PCR when vesicle fluid specimens negative for VZV by culture and/or DFA	PCR is the most sensitive and rapid test
VZV – antibodies, IgG and IgM	Diagnose clinical infections with varicella or herpes zoster Identify hospitalized children with varicella Assess immune status of individuals exposed to varicella, especially pregnant women	

25.8 Varicella Zoster Virus

25.8.1 General Characteristics

- VZV is a human alpha herpes virus, a member of herpes family (HHV3) with a linear, double-stranded DNA genome
- The VZV genome is 125kb
- Isolated in patients with chicken pox (primary), subsequent latency followed by reactivation of virus, known as shingles (recurrent). The virus is spread via contact with vesicular fluid or inhalation of respiratory droplets
- Multiple recurrences are common and can be triggered by immunosuppression, exposure to cytotoxic drugs, radiation, and malignancy

25.8.2 Clinical Presentation

- Varicella (chicken pox)
  - Chicken pox, which is caused by the VZV, is one of the most contagious childhood diseases. Nearly every unvaccinated child becomes infected with it
  - Mild self-limited illness common in school-aged children with fever followed by vesicular eruption on skin and mucous membranes
  - Spreads by respiratory secretions with a 10–14 days incubation period
  - More severe in adults, pneumonia common
- Herpes zoster (shingles)
  - Recurrent infection, usually in adults that may be activated by trauma, neoplasm, or immunosuppression
  - Virus remains latent in sensory ganglia of spinal or cranial nerves causing dermatomal pain and vesicular eruptions, fever, and malaise. Commonly occurs in trunk, but may affect any dermatome
  - Associated with encephalitis and delayed cerebral vasculitis
- Zoster sine herpete occurs in the event of recurrence in the absence of vesicle formation
- Post herpetic neuralgia: pain lasting longer than 1 month after an episode, occurs in as many as 14% of affected individuals, particularly those over 60 years of age. Most neuralgias resolve within one year with 50% experiencing resolution within 2 months
- Ramsay Hunt syndrome: combination of cutaneous involvement of herpes zoster infection of external auditory canal and ipsilateral facial and auditory nerve. Syndrome can cause facial paralysis, hearing deficits, and vertigo

25.8.3 Diagnostic Methods

- Specimens
  - Skin vesicle fluid, cerebrospinal fluid, nasopharyngeal secretion, bronchial washings, blood, amniocentesis fluid, and urine
- Conventional tests and problems (Table 25.7)
  - Viral culture
    - Conventional

  - Virus is difficult to grow in cell culture. Unlike other HSVs (HSV1 and HSV2), VZV manifests a very narrow range of hosts
  - Viral isolation should be attempted in cases of severe disease, especially in immunocompromised persons
  - The best results are obtained from vesicular fluid with lower yield from other sites (nasopharyngeal secretion, blood, urine, bronchial washings, and cerebrospinal fluid)
  - Diagnosed by observation of cytopathic effect (CPE) induced by virus which usually occurs in 1 week after initial inoculation
- Shell vial assay
  - A centrifugation-enhanced culture technique used to obtain rapid and more sensitive culture results
  - It provides results within 2–3 days
- Cytology
  - Intranuclear inclusion bodies
  - Multinucleated, molded giant cells
  - Margination of nuclear chromatin
- Serological studies
  - Enzyme-linked immunosorbent assays (ELISA) range in sensitivity from 86% to 97% and range in specificity from 82% to 99%
  - Latex agglutination (LA) is a rapid, simple to perform assay to detect antibodies to VZV glycoprotein antigen
    - 96% is positive in convalescent-phase serum specimens
    - 61% is positive in persons after vaccination
  - Fluorescent antibody to membrane antigen (FAMA) test
    - It is highly sensitive and is the gold standard for screening for immune status for VZV
    - 100% positive in convalescent-phase serum specimens
    - 77% positive in persons after vaccination
  - Direct fluorescent antibody (DFA)
    - Using fluorescein-labeled monoclonal antibodies specific for either HSV or VZV antigens
    - Results are obtained within several hours
    - Specimen is best collected from the base of a skin lesion, preferably a fresh fluid-filled vesicle
    - The use of DFA may be positive when viral cultures are negative because infected cell viral proteins persist after cessation of viral replication
- Molecular methods (Table 25.7)
  - Conventional PCR targets VZV orf 29 gene, and detection limit is 500 copies/ml
  - Real-time PCR (artus® VZV LC PCR kit, QIAGEN)
    - artus® PCR Kits are used for identification of pathogens including the herpes viruses CMV, EBV, HSV1/2, and VZV
    - Harmonized amplification profiles allow the parallel testing of these four different herpes viruses in a single run on LightCycler® Instruments
    - Analytical sensitivity: five copies/reaction
    - Specificity: 100%
  - LightCycler® VZV Qualitative Kit
    - It targets orf gene 28, DNA polymerase, orf gene 29, orf gene 38, or DNA binding protein
    - It is 91% more sensitive than the shell vial cell culture assay from dermal specimens
  - Real-time quantitative PCR (TaqMan®) technique
    - It targets orf gene 28, orf gene 38, or glycoprotein B
    - Assay results range from 10 copies/ml to 1×10^{10} copies/ml
    - It is 53.8% more sensitive than cell culture from dermal specimens
- Pitfalls
  - Important to note that PCR assays are not standardized and variations in sample handling and laboratory methods can affect the sensitivity of the assay

25.8.4 Clinical Utility

- Although the varicella vaccine is routinely given as a childhood vaccine, certain high-risk groups and scenarios require careful monitoring (i.e., immunocompromised patients)
- Intrauterine infection of the fetus with VZV can be detected by PCR testing of amniocentesis fluid
- It can be applied on different specimens including mucosa secretion, skin lesion, etc
- Diagnosis of encephalitis in immunocompromised patients by detection of VZV in CSF
- Early initiation of VZV-specific antiviral therapy may prevent serious morbidity among HIV-infected patients

25.9 Human Papillomavirus

25.9.1 General Characteristics

- Human papillomavirus (HPV) is a member of the *Papillomaviridae* family that can completely integrate with the DNA of the host cell. Humans are the only known reservoir for HPV
- Papillomaviruses are nonenveloped viruses of icosahedral symmetry with 72 capsomeres that surround a genome containing double-stranded circular DNA with approximately 8,000 base pairs
- The expression of viral genes is closely associated with an epithelial localization and linked to the state of cellular differentiation. Most viral genes are not activated until the infected keratinocyte leaves the basal layer. Production of virus particles can occur only in highly differentiated keratinocytes. Therefore, virus production only occurs at the epithelial surface where the cells are ultimately sloughed into the environment
- Over 100 genotypes of HPV have been identified based on DNA sequence heterology. A specific group, termed high-risk genital HPV types (especially 16, 18, 31, 45, and 58, but also 33, 35, 39, 51, 52, 56, 59, 68, 73, 82), is recognized as a necessary factor for the development of cervical cancer
- The genome HPV virus is circular (Fig. 25.22). The genome has eight open reading frames that encode ten proteins. The genes for these are divided into an early region that are expressed in the skin's infected basal cells that have yet to differentiate, and a late region with two genes whose protein products exist only in cells after cell differentiation
- The E5 (changes the cellular responses to programmed cell death or apoptosis), E6 (binds to tumor suppressor protein, p53), and E7 (binds and inactivates retinoblastoma protein, Rb) proteins are early viral proteins expressed upon infection and cause destabilization of the infected cell and induces replication
- As the cell differentiates, it migrates upward and induces expression of the E1, E2, and E4 genes; E1 and E2 cause viral replication and E4 destabilizes the cytoskeleton and prevents cellular differentiation
- In the upper epithelial cell layers, the late viral proteins L1 (major capsid protein) and L2 (minor capsid protein) are expressed. They bind the viral DNA and autoassemble, giving rise to the complete virions, ready for a new infection that is released as the keratinocytes desquamate
- The most common mode of transmission is via contact; i.e., sexual or autoinoculation

25.9.2 Clinical Presentation

- HPV is by far the most common sexually transmitted disease. An estimated 80% of sexually active adults have been infected with one or more genital HPV strains. The vast majority of infected adults experience transient infectivity and are unaware of the condition; however, they may be able to infect others
- However, most women infected with high-risk HPV, especially women under 30 years of age, do not develop cervical cancer. Their immune system effectively clears the infection over the course of several months
- Specific factors that determine which HPV infections persist and develop into squamous intraepithelial lesions currently are unknown.

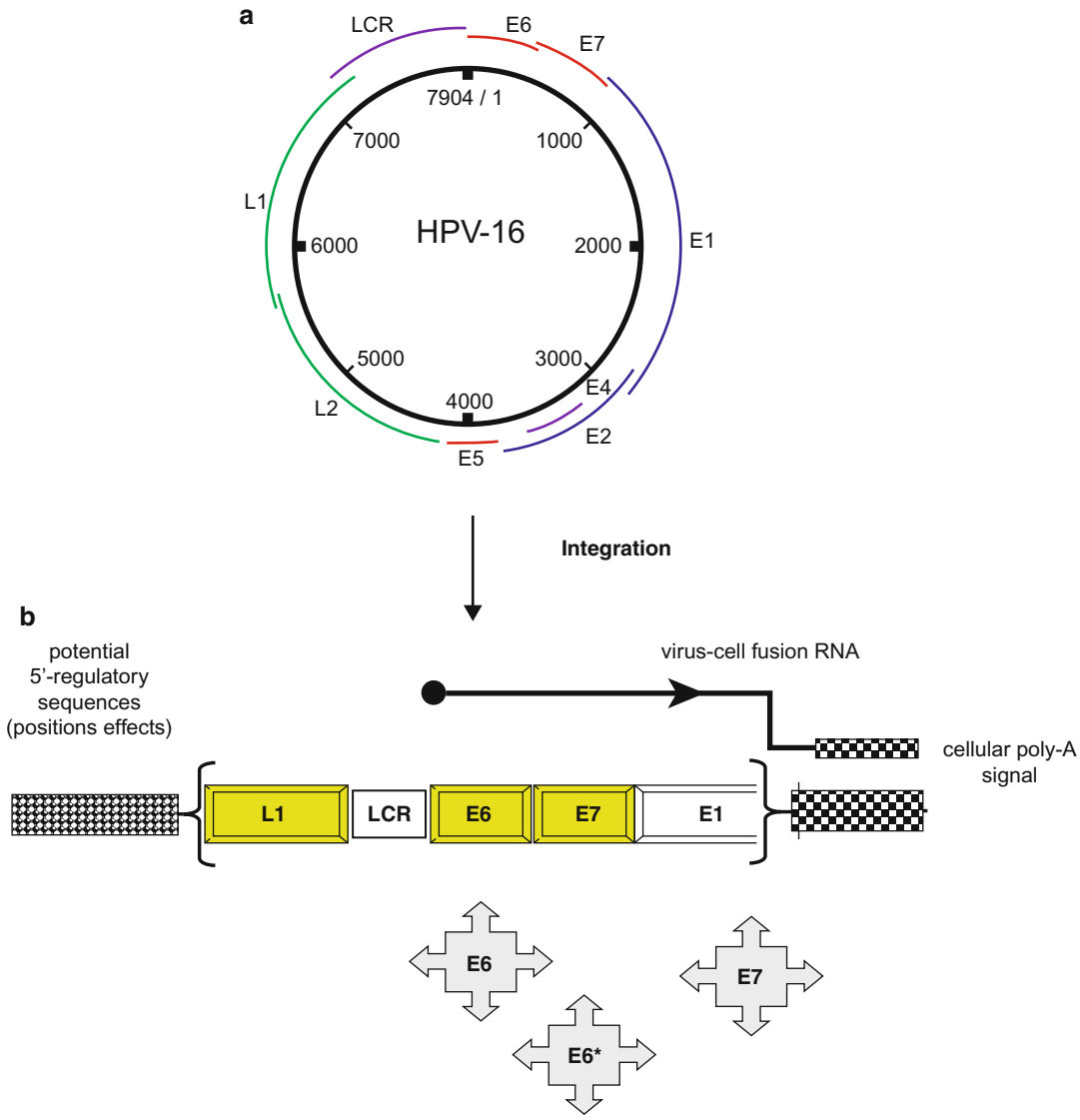


Fig. 25.22 The genome HPV16 virus (Adapted from Finzer et al. Cancer Lett 2002)

Cigarette smoking, ultraviolet radiation, pregnancy, folate deficiency, and immune suppression have been implicated as possible cofactors

- Low-risk HPV types (6, 11, 42, 43, and 44) produce benign epithelial tumors of the skin and mucous membranes. Infection with certain types of HPV (high risk) can also increase the risk of developing cervical and other cancer types. Conditions associated with HPV
  - Verruca vulgaris (common wart) – associated with HPV2, HPV4, and HPV40. Highly contagious and can spread to other sites of skin or mucous membranes via autoinoculation
  - Condyloma acuminatum (venereal wart) – associated with HPV6, HPV11, HPV16, and HPV18 and is considered a sexually transmitted disease with lesions occurring in sites of sexual contact or trauma

(i.e., mucous membranes of genitalia, perianal region, oral cavity, and larynx)
- Flat warts are most commonly found on the face or forehead and are most common in children and teens
- Plantar warts are found on the soles of the feet
- Subungual and periungual warts form under the fingernail (subungual) and around the fingernail or on the cuticle (periungual) and are a subtype of the common skin wart. They may be more difficult to cure than warts in other locations
- Butcher warts are caused by HPV7 and occur in people handling meat, poultry, and fish
- Focal epithelial hyperplasia (Heck disease) is caused by HPV13 (and possibly HPV32) and commonly occurs in Native American and Inuit populations. A childhood condition characterized by multiple soft, nontender flat papules and plaques of the oral mucous membrane
- Laryngeal papillomatosis – frequently recurs and may require repetitive surgery when interferes with breathing. Rare cases can progress to laryngeal cancer (HPV30 and HPV40)
- HIV-associated papillomatosis – HPV7 and immunocompromised states
- Cervical cancer – History of HPV (high-risk types) infection is strongly associated with development of cervical cancer. However, most HPV infections do not progress to cervical cancer. Because the progression of transforming normal cervical into cancerous cells is a slow process, cancer occurs in people who have been infected with HPV for a long time, usually over a decade. High-risk HPV types 16 and 18 are together responsible for over 70% of cervical cancer cases; type 16 alone causes 41–54% of cervical cancers
- Anal/rectal cancer – Although rare, anal/rectal cancer is becoming more prevalent in the US. Similar to cervical cancer, the main cause of anal cancer is HPV and is most commonly acquired through anal intercourse. However, anal cancer can also be acquired from other genital areas that are infected with HPV, particularly from the vulva or penis. High-risk HPV types (16, 18, 31, 33 and 35) are associated with anal squamous intraepithelial lesions and account for approximately 80% of cervical and anal cancers
- Head and neck squamous cell cancer – HPV16 is the most common type detected in head and neck squamous cell cancers (HNSCCs). HPV16 accounts for 78.6–100% of HPV-positive oropharyngeal cases. HPV type 18 accounts for 1% of oropharyngeal, 8% of oral cavity, and 4% of laryngeal HPV-positive SCCs. HPV16 and HPV18 genotypes are associated with a better prognosis in squamous cell carcinomas of the head and neck. Furthermore, the HPV-positive tumors are also more sensitive to radiation and chemotherapy. Coinfections are possible and most frequently include HPV16. HPV33 is often reported and has been identified in up to 10% of HPV-positive HNSCCs. Numerous other HPV types have been rarely detected in HNSCCs and include types 6, 11, 35, 45, 51, 52, 56, 58, 59, and 68
- Other cancers – HPV (including 16, 18, and 31) may also cause vulvar, nonmelanoma skin cancers, and (rarely) penile cancer. High-risk types of HPV can cause intraepithelial neoplasias, or abnormal and precancerous cell growth, in the vulva and cervix, which can progress to cancer

25.9.3 Diagnostic Methods

- Specimens – cervical washings/brushings collected in liquid media (i.e., PreservCyt), Digene specimen collection tube (Hybrid Capture only), or biopsies
- Conventional tests and problems
  - Viral culture
    - HPV cannot be reliably cultured and is not identified using this technique

- Cytology
  - Koilocytosis describes the combination of perinuclear clearing (halo) with a pyknotic or shrunken nucleus
- Serological studies are not useful for diagnosis

- Molecular methods and genotyping
  - Nucleic acid hybridization
    - In situ hybridization (INFORM HPV DNA test, Ventana Medical Systems Inc., Tucson, AZ)
      - Use tissue sections, liquid-based cytology specimens, and cervical smears
      - On slide detection of high- and low-risk HPV genotypes
      - 16 probe cocktail for high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 51, 52, 56, 58, and 66
      - 6 probe cocktail for low-risk HPV genotypes 6 and 11
  - Nucleic acid hybridization
    - Digene Hybrid Capture II (Digene Corporation, Gaithersburg, MD)
      - Method utilizes an RNA probe mix for the detection of the L1 gene of HPV. Assay can identify HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. In addition, a kit detecting low-risk virus (6, 11, 42, 43, 44) is also available
      - Manual, semiautomated (rapid capture), and automated (utilizing QIAGEN's QiaSymphony extraction system) platforms available
      - Signal amplification is based on immunocapture of DNA/RNA hybrids that are immobilized on a 96-well microplate, reacted with alkaline phosphatase-conjugated antibodies specific for the RNA: DNA hybrids and detected with a chemiluminescent substrate
      - Can detect 5,000 viral copies per sample, or one picogram of HPV DNA per sample
  - Signal amplification
    - Invader Assay Cervista HPV assays (Hologic, Madison, WI)
      - The Invader assay is an isothermal linear signal amplification using structure-specific oligonucleotide cleavage and has been applied to DNA-based genotyping. This method uses two types of isothermal reactions: a primary reaction that occurs on the targeted DNA sequence and a secondary reaction that produces a fluorescent signal
      - Invader utilizes an internal control for human HIST2HBE to assure DNA quality and quantity in each reaction
      - Two diagnostic qualitative assay formats that utilize the Invader chemistry are available: Cervista HPV HR (detects pool of 13 HPV genotypes) and Cervista HPV16/18 (detects HPV16 and/or HPV18)
      - Cervista HPV HR uses isothermal signal amplification to detect 13 h HPV types utilizing three probe pools based on phylogenic relatedness. 3 probe pools include A5/A6 [51, 56], A7 [18, 39, 45, 59, 68], and A9 pool [16, 31, 33, 35, 52, 58]. Assay cannot determine the specific HPV genotype present
      - The Cervista HPV16/18 test is a diagnostic test to genotype HPV16 and HPV18 in cervical specimens
      - Can detect 1,250–5,000 viral copies per specimen
  - Target amplification
    - TMA – APTIMA HPV assay (Gen-Probe; San Diego, CA)
      - The APTIMA HPV assay is an in vitro nucleic acid amplification test for the qualitative detection of E6/E7 viral messenger RNA (mRNA) from 14 high-risk types of HPV in cervical specimens
      - Detects HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, but does not distinguish between the 14 high-risk types
      - The assay is used with the TIGRIS DTS system, one of the first

diagnostic instruments to truly automate nucleic acid testing (NAT) from start to finish by from sample preparation, amplification, and detection to reporting results
  - The TIGRIS system can process approximately 450 samples in an 8-h shift, and up to 1,000 samples in approximately 13.5 h
  - The APTIMA HPV assay involves three main steps, mainly: target capture, target amplification by transcription-mediated amplification (TMA), and detection of the amplification products (amplicon) by the hybridization protection assay (HPA) that measures the emitted relative light units (RLU) in a luminometer
  - Can detect 100–300 viral copies per sample
- Real-time PCR – COBAS® HPV test (Roche Diagnostics, Indianapolis, IN)
  - The COBAS HPV test is a clinical diagnostic qualitative assay to detect HPV in patient samples using the COBAS 4800 system that automates specimen extraction, amplification, and detection. The assay utilizes amplification of HPV DNA by real-time PCR and nucleic acid hybridization to detect 14 high-risk HPV genotypes in a single reaction tube that target the polymorphic L1 region of the HPV genome
  - The assay specifically genotypes HPV16 and HPV18 while concurrently detecting the other high-risk genotypes in a pooled fashion (31, 33, 35, 39, 45, 51, 51, 56, 58, 59, 66, 68)
  - The COBAS HPV test is based on two major processes: (1) automated specimen preparation to simultaneously extract HPV and cellular DNA and (2) PCR amplification of target DNA sequences
  - Using both HPV- and β-globin-specific complementary primer pairs and real-time detection of cleaved fluorescent-labeled HPV- and β-globin-specific oligonucleotide detection probes
  - The master mix reagent for the COBAS HPV test contains primer pairs and probes specific for the 14 high-risk HPV types and β-globin DNA
  - The detection of amplified DNA (amplicon) is performed during thermal cycling using oligonucleotide probes labeled with four different fluorescent dyes. The amplified signal from 12 high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) is detected using the same fluorescent dye, while HPV16, HPV18, and β-globin signals are each detected with their own dedicated fluorescent dye
  - An additional primer pair and probe target the human β-globin gene (330-bp amplicon) to provide a process control
  - Can detect 300–1,200 viral copies/ml
- Genotyping
  - Roche linear array (Fig. 25.23)
    - Qualitative test that utilizes amplification of HPV target DNA by PCR and nucleic acid hybridization bases on four major steps: (1) sample preparation, (2) PCR amplification of target DNA using HPV-specific complementary primers, (3) hybridization of the amplified products to oligonucleotide probes, and (4) colorimetric detection of the probe-bound amplified products
    - Uses a pool of biotinylated primers to define a sequence of nucleotides for the L1 region of the HPV genome designed to amplify HPV DNA from 37 HPV genotypes, including 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68)
    - β-globulin gene is concurrently isolated and ensures adequacy of cellularity, extraction, and amplification for each processed sample

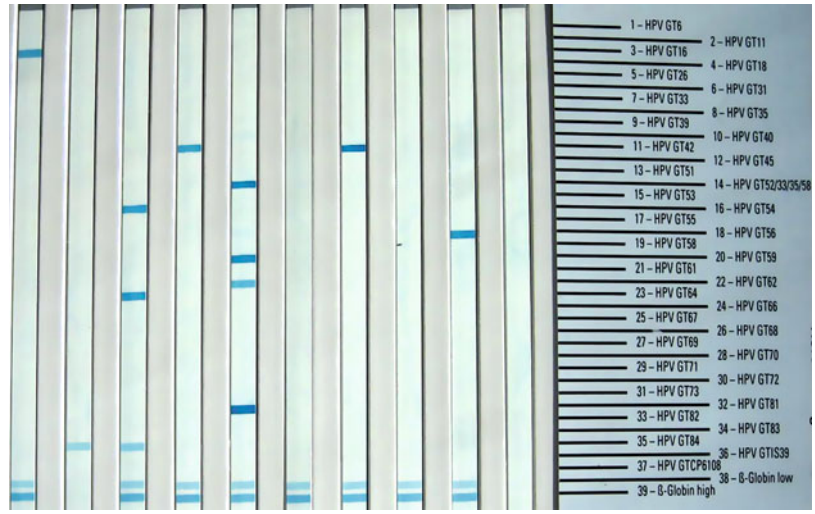


Fig. 25.23 Roche linear array HPV genotyping assay with reference guide utilized for interpretation. *P* positive control, *N* negative control, *ßgL* ß globulin low, *ßgH* ß globulin high

Table 25.8 hc2 high-risk HPV DNA test performance versus consensus histology results (CIN 2–3). Age-specific characteristics. Kaiser study data

	Age <30	Age 30–39	Age >39
Number of cases	287	233	365
Prevalence of disease (%)	12.2	11.2	2.7
Sensitivity (%)	100.00 (35/35)	88.46 (23/26)	80.00 (8/10)
95% confidence interval	90.0–100	69.9–97.6	44.4–97.5
Specificity (%)	31.4 (79/252)	66.2% (137/207)	79.15 (281/355)
95% confidence interval	25.7–37.5	59.3–72.6	74.6–83.3
Negative predictive value (%)	100 (79/79)	97.86 (137/140)	99.29 (281/283)
Positive predictive value (%)	16.83 (35/208)	24.73 (23/93)	9.76 (8/82)

Adapted from Digene package insert

- Sensitivity and specificity
  - Overall, the sensitivity for cytology for detecting HGSIL ranges from 50% to 70% and specificity of 86–98%
  - Overall, the sensitivity of HPV DNA test for detecting HGSIL is about 80–98% and specificity of 64–95%
  - Overall, the sensitivity of COBAS HPV assay for detecting HGSIL is about 69–71% and specificity of 90–94%
  - Overall, the sensitivity of Cervista HPV assay for detecting HGSIL is about 93–100% and specificity of 43–44%
  - Overall, the sensitivity of APTIMA HPV test for detecting HGSIL is about 87–93% and specificity of 60–63%
  - However, the sensitivity and specificity are influenced by the age and prevalence (Table 25.8)
- Pitfalls
  - Presently available assays provide only qualitative results and do not correlate the magnitude of the positive assay signal to meaningful quantitative results
  - The effects of other potential variables such as vaginal discharge, use of tampons, douching, personal lubricants, topical medicaments, and specimen collection variables may affect the performance of the assay
  - A negative HPV result does not exclude the possibility of present or future cytologic abnormalities
  - COBAS HPV assay

Table 25.9 Summary of recommendations for cervical cancer screening

Variable	ACS-ASCCP-ASCP Draft 2011	ACOG 2009	USPSTF Draft 2011
Age to start HPV testing	21 years	21 years	21 years
Testing frequency			
Age 21–29 year (PAP alone)	Every 3 years	Every 2 years	Every 3 years
Age 30 year and older			
PAP alone	Every 3 years	Every 3 years	Every 3 years
PAP and HPV cotesting	Recommended – no more than every 3 years	Allowed – no more than every 3 years	No recommendation – insufficient data available
Age to stop HPV testing	65 years after 3 negative Pap tests or two negative HPV tests in past 3 years	65–70 after 3 negative tests in a period	65 years after adequate screening
After hysterectomy	Discontinue, if not dysplasia or neoplasia	Discontinue, if not dysplasia or neoplasia	Discontinue, if not dysplasia or neoplasia
Screening after HPV vaccination	Same as if unvaccinated	Same as if unvaccinated	Unaddressed

Note: *ACOG* American College of Obstetricians and Gynecologists, *ACS* American Cancer Society, *ASCCP* American Society for Colposcopy and Cervical Pathology, *ASCP* American Society for Clinical Pathology, *HPV* human papillomavirus, *PAP* Papanicolaou, and *USPSTF* US Preventive Services Task Force

    - Strong laboratory information system needed to support automation of bar coding
    - Invalid results frequent
- APTIMA HPV assay
    - Assay does not detect E6/E67 mRNA of HPV low-risk types
    - Performance characteristics based only on ThinPrep 2000 processor
- Cervista HPV assay
    - Indeterminate test results caused by insufficient mixing, pipetting error, or inadequate genomic DNA
    - Exhibits cross-reactivity to HPV67 and HPV70 genotypes
- Digene hybrid capture assay
    - Limited sensitivity (1 pg/ml)
    - Mixed high-risk and low-risk probes, cannot distinguish specific HPV types
    - Manual platform is labor-intensive

25.9.4 Clinical Utility

- To screen patients with ASCUS (atypical squamous cells of undetermined significance), Pap smear results to determine the need for referral to colposcopy. The results of this test are not intended to prevent women from proceeding to colposcopy (Table 25.9)
- In women 30 years and older, the hc2 high-risk HPV DNA test can be used with Pap smear to adjunctively screen to assess the presence or absence of high-risk HPV types. This information, together with the physician's assessment of cytology history, other risk factors, and professional guidelines, may be used to guide patient management (Table 25.9)
- Recently, a new test scheme was proposed (Fig. 25.24)

25.10 Influenza A, B, and C Viruses

25.10.1 General Characteristics

- Influenza is part of the Orthomyxoviridae family and can be classified into three basic types, influenza A, B, or C (Table 25.10). Each influenza virus type is an enveloped single-stranded RNA virus that shares

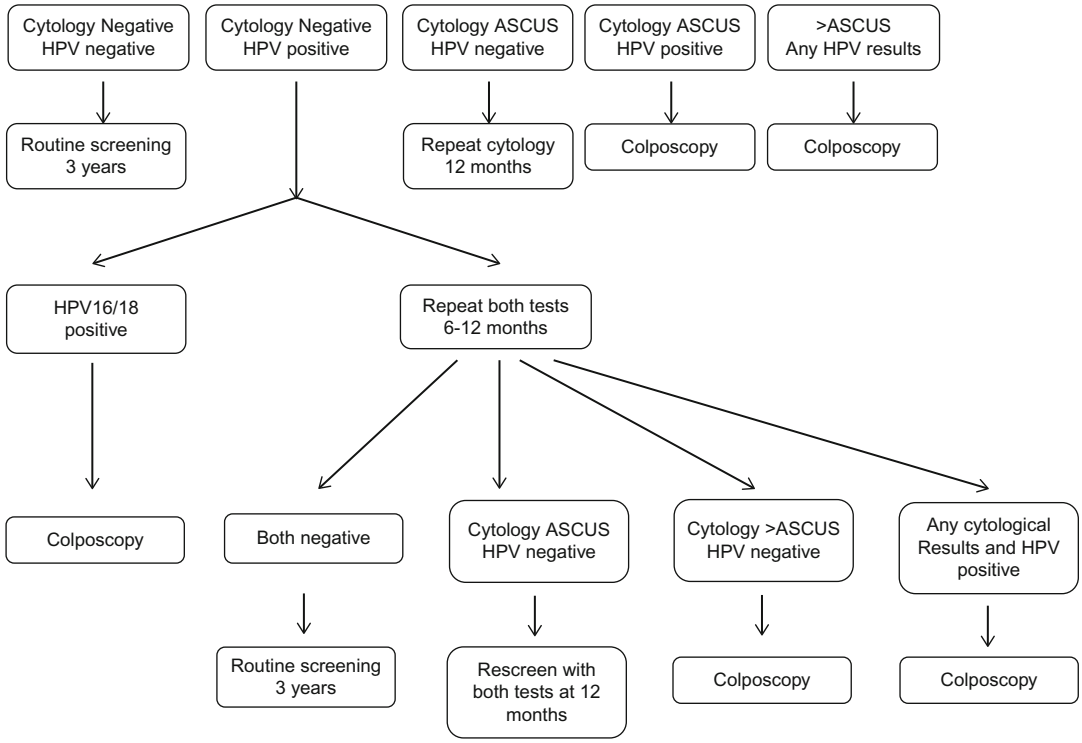


Fig. 25.24 Proposed management scheme of ASCUS based on cytology and/or high-risk HPV DNA test (Adapted from Wright et al. Obstet Gynecol 2004)

Table 25.10 Comparison of influenza A, B, and C

	Type A	Type B	Type C
Severity of illness	++++	++	+
Animal reservoir	Yes	No	No
Human pandemics	Yes	No	No
Human epidemics	Yes	Yes	No (sporadic)
Antigenic changes	Shift, drift	Drift	Drift
Segmented genome	Yes	Yes	Yes
Amantadine, rimantadine	Sensitive	No effect	No effect
Zanamivir (Relenza)	Sensitive	Sensitive	
Surface glycoproteins	2	2	(1)

structural and biological similarities but differs antigenically. Type A influenza virus which causes pandemic is found in a variety of warm-blooded animals. Types A and B are predominantly human pathogens. Type C is found in humans and pigs

- Influenza viruses have a segmented RNA genome (Fig. 25.25). Influenza A and B contain eight distinct segments and are covered with surface glycoproteins, hemagglutinin (HA), neuraminidase (NA), and matrix 2. Influenza C has seven segments and one surface glycoprotein. The viruses are typed based on these proteins. For example, influenza A (H3N2) expresses hemagglutinin 3 and neuraminidase 2

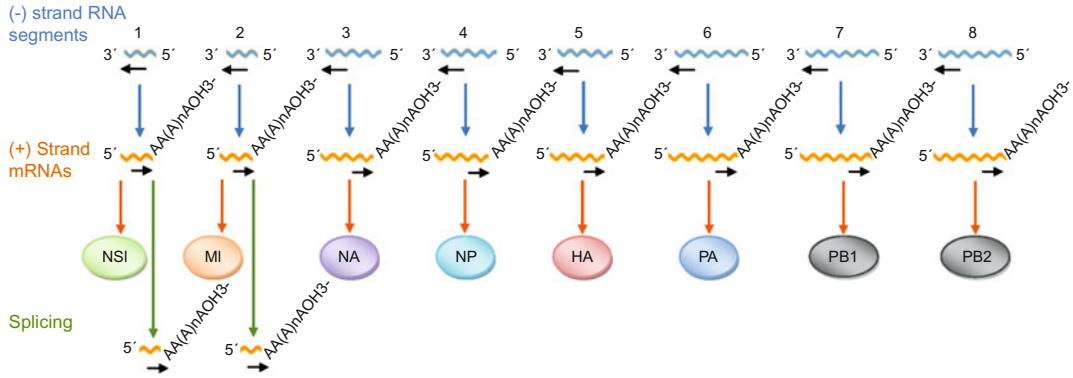


Fig. 25.25 Influenza virus genome: The virus contains 7–8 single-stranded RNAs (influenza A and B contains 8 RNAs, and influenza C contains 7 RNAs). The RNAs code for 9–11 viral proteins: hemagglutinin *HA*, neuraminidase *NA*, polymerase complex *PA, PB1*, and *PB2*, nucleoprotein *NP*, matrix protein *M*, and nonstructural protein *NS*. PCR primers usually target HA and NA consensus region

- Influenza is a dynamic virus that may evolve in two different ways via antigenic drift and antigenic shift resulting in genetic diversity. Antigenic shift occurs when two different strains of influenza viruses combine with antigenically different HA and NA by reassortment of viral RNA segments; this process occurs every 10–40 years. Antigenic drift occurs by random point mutation in viral RNA leading to amino acid substitutions in HA glycoproteins. Influenza type A viruses undergo both antigenic shift and drift; influenza type B viruses undergo antigenic drift
- Each influenza RNA segment is further encapsulated by nucleoproteins to form ribonucleotide–nucleoprotein complexes surrounded by matrix proteins
- Influenza virus infections rank as one of the most common infectious diseases in humankind. However, influenza may potentially cause severe epidemics and kills an average of 20,000 individuals in the US
- Influenza virus infection occurs after transmission of respiratory secretions from an infected individual to a person who is immunologically susceptible
- A number of different subtypes are classified within the influenza A type based on two viral surface proteins: hemagglutinin and neuraminidase. The most common prevailing human influenza A subtypes are H1N1 and H3N2. Each year, the distributed vaccine contains A strains from H1N1 and H3N2, along with an influenza B strain. A novel influenza A H1N1 strain emerged in March 2009 and is the causative agent of the current public health emergency
- In March 2009, an outbreak of influenza-like illness occurred in Mexico and the US; the CDC reported seven cases of novel A/H1N1 influenza. Preliminary genetic characterization found that the hemagglutinin gene was similar to that of swine flu viruses present in US pigs since 1999, but the neuraminidase and matrix protein genes resembled versions present in European swine flu isolates. The six genes from American swine flu are themselves mixtures of swine flu, bird flu, and human flu viruses
- Recently, several studies reported the novel A/H1N1 strains carried a prominent amino acid change at position 222 (D222N) within the primary hemagglutinin receptor binding site. Previously, enhanced virulence associated with the change, D222G, has been clinically linked to severe morbidity and mortality

25.10.2 Clinical Presentation

- Although the presentation of influenza virus infection is variable, typical symptoms may

Table 25.11 Comparison of available influenza diagnostic tests

Influenza diagnostic tests[a]	Types detected	Method	Typicalprocessing time[b]	Sensitivity[d] for 2009 H1N1 influenza	Distinguishes 2009 H1N1 influenza from other influenza A viruses?
Viral cell culture (conventional)	A and B[c]	Virus isolation	2–10 days	N/A	Yes[f]
Rapid cell culture (shell vials; cell mixtures)	A and B[c]	Virus isolation	1–3 days	N/A	Yes[f]
Immunofluorescence, direct (DFA) or indirect (IFA) antibody staining	A and B[c]	Antigen detection	2–4 h	47–93 %	No
RT-PCR[d] (single plex and multiplex; real-time and other RNA-based)	A and B[c]	RNA detection	Varied (generally 6–8 h)	86–100%	Yes
Rapid influenza diagnostic tests[e]	A and B	Antigen detection	<30 min.	10–70%	No

[a]Serologic (antibody detection) testing is not recommended for routine patient diagnosis
[b]The amount of time needed from specimen collection until results are available
[c]May be adapted to identification of specific subtypes
[d]Compared with rRT-PCR tests; rRT-PCR tests are compared to other testing modalities including other rRT-PCR assays
[e]Rapid influenza diagnostic tests include tests that are CLIA waived (can be performed in an outpatient setting) and tests that are moderately complex (can be performed only in a laboratory). Clinical specimens approved for RIDTs vary by test and may not include all respiratory specimens
[f]Requires additional testing on the viral isolate

include the following: fever, sore throat, myalgia, headache, rhinitis, fatigue, and coughing. Onset of illness may be abrupt
- Patients with a preexisting immunity or received vaccination may have mild and less severe symptoms
- Acute encephalopathy has recently been described to be associated with influenza A virus. Clinical features included altered mental status, coma, seizures, and ataxia
- In young infants, influenza may produce a sepsis-like picture with shock; occasionally, influenza viruses can cause croup or pneumonia
- In April 2009, a novel influenza A (H1N1) virus was identified in Mexico and has since spread rapidly worldwide. The unique genetic and antigenic features of this virus have resulted in a high incidence of infection, with an epidemiologic profile that is different from that of previous seasonal influenza infections. As a consequence, a surge of pediatric patients has been presenting to emergency departments and physician's offices across the country during this 2009–2010 flu season
- Vomiting and diarrhea have been reported more often with 2009 H1N1 influenza virus infection than with seasonal influenza
- The incubation period typically ranges from 18 to 72 h

25.10.3 Diagnostic Methods

- Specimens (Table 25.11)
  - Nasopharyngeal aspirate/swab/washing, tracheal aspirate, or bronchoalveolar lavage
  - Transport
    - Culture/DFA – 3 mL (minimum 1 ml) of respiratory sample in viral transport media (Microtest M4) or in sterile leakproof container at 2–8 °C
    - Serologic – 1 mL (minimum 0.5 ml) serum in an SST tube at 2–8 °C
  - Unacceptable specimens: dry swabs or wood and calcium alginate swabs that may inactivate the virus for culture. Plasma or hemolyzed, lipemic, icteric, turbid, bacterially contaminated, or heat-inactivated serum is inadequate for serological test

  - All respiratory specimens should be kept at 4°C for no longer than 72 h before testing and ideally should be tested within 24 h of collection. If storage longer than 72 h is necessary, clinical specimens should be stored at –70 °C. Freezing at higher temperatures (e.g., –20 °C) can reduce the likelihood of virus detection
- Conventional tests and problems (Table 25.11)
  - Viral culture
    - The criterion standard for diagnosing influenza A and B is via viral propagation in embryonated hen eggs or Madin–Darby canine kidney (MDCK) cells
    - Laboratory diagnosis of influenza is established once specific cytopathic effect is observed or hemadsorption testing findings are positive
    - After culture isolation, final identification via immunoassays or immunofluorescence
    - Staining the infected cultured cell lines with fluorescent antibody confirms the diagnosis
    - The viral culture process requires 2–10 days to complete
    - Primary method for vaccine production
  - DFA testing
    - DFAs are widely available, have variable sensitivity (range 47–93%) for 2009 H1N1 influenza virus, and a high specificity (≥96%). DFAs detect and distinguish between influenza A and B viruses but do not distinguish among different influenza A subtypes
    - The technique is more rapid (24 h) to result; it is less sensitive than culture methods
  - Serologic studies
    - Two samples should be collected per person. One sample within the first week (acute) of symptoms and a second sample (convalescent) 2–4 weeks later. If antibody levels increase from the first to the second sample, influenza infection likely occurred
    - Because of the length of time needed for a diagnosis of influenza by serologic testing, other diagnostic testing should be used if a more rapid diagnosis is needed
    - Inability to differentiate between current and previous infection. Cannot be used for rapid diagnosis
  - Rapid influenza diagnostic test (RIDT) (Table 25.12)
    - RIDTs are widely available but have variable sensitivity (range 10–70%) for detecting 2009 H1N1 influenza when compared with real-time reverse transcriptase polymerase chain reaction (rRT-PCR), and a negative RIDT result does not rule out influenza virus infection
    - RIDTs have a high specificity (>95%). Depending on which commercially available RIDT is used, the test can either (1) detect and distinguish between influenza A and B viruses or (2) detect both influenza A and B but not distinguish between influenza A and B viruses
    - Fastest method of currently available diagnostic tools. Result may be obtained in <30 min
    - However, the technique has a sensitivity of 70–80%
- Molecular methods (Table 25.11)
  - General information
    - Nucleic acid amplification tests, including rRT-PCR, are the most sensitive and specific influenza diagnostic tests
    - But they may not be readily available, obtaining test results may take one to several days, and test performance depends on the individual rRT-PCR assay. As with any assay, false negatives can occur
    - Not all nucleic acid amplification assays can specifically differentiate 2009 H1N1 influenza virus from other influenza A viruses
    - Several rRT-PCR assays have been evaluated and authorized by the FDA under an emergency use authorization (EUA) to diagnose 2009 H1N1 influenza virus infection, such as Roche

Table 25.12 Commercially available rapid point of care influenza detection kits (Adapted from http://www.cdc.gov/flu/professionals/diagnosis/testing_algorithm.htm)

Procedure (manufacturer/distributor)	Influenza virus types detected	Approved specimens[a]	Test time
3M™ Rapid Detection Flu A + B Test[d, f] (3M)	A and B	NP[b] swab/aspirate Nasal wash/aspirate	15 min
BinaxNOW® Influenza A and B[e, f] (Alere)	A and B	NP[b] swab Nasal wash/aspirate/swab	15 min
BioSign® Flu A + B[d, f] (Princeton BioMedtech)	A and B	NP[b] swab/aspirate/wash, nasal swab	15 min
Clearview® Exact Influenza A and B[d, f] (Alere)	A and B	Nasal swab	15 min
Directigen™ EZ Flu A and B[d, f] (Becton–Dickinson)	A and B	NP[b] wash/aspirate/swab Throat swab	15 min
OSOM® Influenza A and B[d, f] (Genzyme)	A and B	Nasal swab	10 min
QuickVue® Influenza Test[c, e] (Quidel)	A or B	Nasal wash/aspirate/swab	10 min
QuickVue® Influenza A and B Test[e, f] (Quidel)	A and B	NP[b] swab Nasal wash/aspirate/swab	10 min
SAS™ FluAlert A and B[d, f] (SA Scientific)	A and B	Nasal wash/aspirate	15 min
SAS™ FluAlert A[c, e] (SA Scientific)	A only	Nasal wash/aspirate	15 min
SAS™ FluAlert B[c, e] (SA Scientific)	B only	Nasal wash/aspirate	15 min
TRU FLU®[d, f] (Meridian Bioscience)	A and B	NP[b] aspirate/swab Nasal wash	15 min
XPECT™ Flu A and B[d, f] (Remel/Thermofisher)	A and B	Nasal wash/swab Throat swab	15 min

[a]List may not include all test kits approved by the US Food and Drug Administration. Discontinued tests not included. Approved respiratory specimens according to manufacturer's package insert. Note that test performance may vary if other respiratory specimens are used
[b]*NP* nasopharyngeal
[c]Does not distinguish between influenza A and B virus infections when used alone
[d]Moderately complex test – requires specific laboratory certification
[e]CLIA-waived test. Can be used in any office setting. Requires a certificate of waiver or higher laboratory certification
[f]Distinguishes between influenza A and B virus infections

real-time-ready influenza A/H1N1 test, Cepheid Xpert® flu A panel for the diagnosis of 2009 H1N1 Influenza virus infection, ELITech Molecular Diagnostics 2009 H1N1 influenza A virus real-time RT-PCR test, Focus Diagnostics influenza A H1N1 (2009) real-time RT-PCR, IMDx 2009 influenza A H1N1 real-time RT-PCR assay from IntelligentMDx, and QIAGEN *artus*® influenza A H1N1 2009 LC RT-PCR kit

– RT-PCR: artus® influenza A H1N1 2009 LC RT-PCR kit (QIAGEN Diagnostics)
  - The assay is for the detection of influenza A viral RNA and the detection and differentiation of 2009 H1N1 influenza virus RNA in nasopharyngeal swabs of symptomatic patients
  - The assay utilizes the EZ1 Advanced instrument with the EZ1 DSP Virus Card v. 2.0 (QIAGEN) and the EZ1 DSP virus kit (QIAGEN) for viral nucleic acid extraction. The LightCycler 2.0 instrument with software v. 4.1 (Roche) is used for amplification and detection
  - The limit of detection for influenza A (seasonal H1N1) is 57.0 TCID 50 (tissue culture infectious dose 50) /ml, and the limit of detection for 2009 H1N1 influenza is 4.217 PFU (plaque-forming unit)/ml
– RT-PCR: Cepheid Xpert® flu panel (ASR)
  - Accurate detection and differentiation of Influenza A from Influenza B infection and simultaneous identification of 2009 H1N1 flu strain

- Less than 2 minutes hands-on time improves lab workflow efficiencies
- Accepts nasal aspirate/washes (NA/W) or nasopharyngeal (NP) swab to accommodate wide range of specimen types

– RT-PCR: Roche real-time-ready influenza A/H1N1 detection set
  - In mid-November 2009, the test received emergency use authorization (EUA) from the US FDA
  - The test for the 2009 H1N1 influenza virus detects RNA from the 2009 H1N1 influenza A virus and provides a rapid means of identification of patients infected with this virus duration of the declaration of emergency
– Influenza antiviral resistance test
  - In the US, four antiviral drugs are FDA approved for use against influenza: amantadine, rimantadine, zanamivir (Relenza®), and oseltamivir (Tamiflu®). The adamantane drugs (amantadine and rimantadine) are approved for influenza A, while the neuraminidase inhibitor drugs (zanamivir and oseltamivir) are approved for both influenza A and influenza B
  - To date, 2009 H1N1 influenza, influenza A (H3N2), and influenza B viruses have been detected by surveillance. All three virus strains typically are sensitive to oseltamivir and zanamivir, but the influenza A strains have been resistant to the adamantanes. While, sporadic oseltamivir-resistant 2009 influenza A (H1N1) virus infections were identified
  - The oseltamivir-resistant 2009 influenza A (H1N1) is commonly described point mutation in the virus neuraminidase gene (histidine to tyrosine at position 275 of the N1 neuraminidase, commonly referred to as H274Y in N2 numbering), which is known to confer high-level resistance to oseltamivir
  - Influenza viruses A (H1N1) carrying the H274Y mutation have reduced ability to replicate and transmit efficiently when compared with parental, susceptible virus, but the clinical implications of infection with these viruses have been largely unknown
  - RT-PCR along with sequencing (Sanger sequence or next gen sequence such as pyrosequence) is usually used for influenza antiviral resistance test

- Sensitivity and specificity
  – Sensitivity of DFA methodology is dependent upon adequacy of the specimen, i.e., >20 cells. Otherwise, specimen may be inadequate for accurate interpretation resulting in false negatives
  – Rapid diagnostic testing is approximately >70% sensitive for detecting influenza and approximately >90% specific. Thus, as many as 30% of samples that would be positive for influenza by viral culture may give a negative rapid test result; some rapid test results may indicate influenza when a person is not infected with influenza
- Pitfalls
  – Due to the length of time required to perform viral culture, the assay has poor efficacy as results are obtained much after the patient has left the office or well past the time when drug therapy could be effective
  – Development of PCR-based assays must always consider antigenic drift and random mutations due to viral evolution that may result in false negatives

25.10.4 Clinical Utility

- Because of cost, availability, and sensitivity issues, diagnosis of influenza is often based on clinical criteria and presentation
- RT-PCR assays provide a rapid and specific diagnosis of influenza to allow for early therapeutic intervention and prophylactic treatment in high-risk patients, i.e., geriatric care facility
- Molecular diagnosis will play a large role in epidemiologic surveillance, vaccine strain selection, and surveillance of emergent novel influenza viruses, i.e., the Hong Kong H5N1 outbreak with sequence analysis

Fig. 25.26 Influenza A epidemic information

25.11 Avian Influenza (Bird) and Influenza (Flu) A Viruses

25.11.1 General Characteristics

- Influenza viruses that infect birds are called avian influenza viruses, and commonly known as "bird flu." Only influenza A viruses and subtypes infect birds
- There are substantial genetic differences between the subtypes that typically infect both people and birds. Within subtypes of avian influenza A viruses, there also are different strains
- The incubation period of avian influenza A virus is typically 2–5 days but can be as long as 8–17 days
- These influenza viruses occur naturally among birds
  - Wild birds worldwide carry the viruses in their intestines, but usually do not get sick from them
  - However, avian influenza is very contagious among birds and can make some domesticated birds, including chickens, ducks, and turkeys, very sick and kill them
- There are many different subtypes of type A influenza viruses (Fig. 25.26)
  - These subtypes differ because of changes in certain proteins on the surface of the influenza A virus (hemagglutinin and neuraminidase proteins)
  - There are 16 known hemagglutinin subtypes and 9 known neuraminidase subtypes of influenza A viruses
  - Many different combinations of hemagglutinin and neuraminidase proteins are possible. Each combination represents a different subtype
  - Avian influenza A H5 and H7 viruses can be distinguished as "low pathogenic" and "high pathogenic" forms on the basis of genetic features of the virus and the severity of the illness they cause in poultry; influenza H9 virus has been identified only in a "low pathogenicity" form
  - Each of these three avian influenza A viruses (H5, H7, and H9) theoretically can be partnered with any one of nine neuraminidase surface proteins; thus, there are potentially nine different forms of each subtype (e.g., H5N1, H5N2, H5N3, H5N9)

25.11.2 Clinical Presentation

- The reported signs and symptoms of avian influenza in humans have ranged from eye infections (conjunctivitis) to influenza-like illness symptoms (e.g., fever, cough, sore throat, muscle aches) to severe respiratory illness (e.g., pneumonia, acute respiratory distress, viral pneumonia) sometimes accompanied by nausea, diarrhea, vomiting, and neurologic changes
- CDC and WHO recommend oseltamivir, a prescription antiviral medication, for treatment and prevention of human infection with avian influenza A viruses. Analyses of available H5N1 viruses circulating worldwide suggest that most viruses are susceptible to oseltamivir. However, some evidence of resistance to oseltamivir has been reported in H5N1 viruses isolated from some human H5N1 cases. Monitoring for antiviral

resistance among avian influenza A viruses is important and ongoing
- The first avian influenza virus to infect humans occurred in Hong Kong in 1997. The epidemic was linked to chickens and classified as avian influenza A (H5N1). Human cases of avian influenza A (H5N1) have since been reported in Asia, Africa, Europe, Indonesia, Vietnam, the Pacific, and the Near East. Hundreds of people have become sick with this virus. Slightly more than 60% of those who became ill have died

25.11.3 Diagnostic Methods

- Specimen: see Influenza A, B, and C section
- Viral culture: see Influenza A, B, and C section
- Serological test: see Influenza A, B, and C section
- Molecular test
  - Real-time RT-PCR
    - On February 3, 2006, the FDA announced clearance of the influenza A/H5 (Asian lineage) virus real-time RT-PCR
    - Primer and probe set and inactivated virus as a source of positive RNA control for the in vitro qualitative detection of highly pathogenic influenza A/H5 virus (Asian lineage)
    - Two genetic lineages of influenza A/H5 viruses exist: Eurasian (Asian) and North American. The primer and probe set, developed at CDC, is designed to detect highly pathogenic influenza A/H5 viruses from the Asian lineage associated with recent laboratory-confirmed infections of avian influenza in humans in east Asia and, most recently, in Turkey and Iraq
    - The test is limited to laboratories designated by the Laboratory Response Network (LRN), which consists of approximately 140 US laboratories in 50 states
- Limitation
  - Due to the limitation of the assay, negative results do not preclude influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions

25.12 Adenovirus

25.12.1 General Characteristics

- Adenovirus is ubiquitous in humans and is endemic
- Adenoviruses are medium-sized (90–100 nm), nonenveloped icosahedral viruses containing double-stranded DNA (Fig. 25.27)
- There are at least 52 known immunologically distinct types (seven subgenera: A–G) that can cause human infections. Types 4 and 7 are common in military recruit outbreaks; type 14 is commonly associated with severe and sometimes fatal respiratory illnesses
- Adenovirus transcription can be defined as a two-phase event, early and late, occurring before and after DNA replication
- Early transcription is accompanied by a complex series of splicing events, with four early "cassettes" of gene termed E1, E2, E3, and E4. Early genes facilitate DNA replication and result in the transcription and translation of the late genes (Fig. 25.28)
- Adenovirus produces cytolysis in different tissues and induces host inflammatory responses and cytokine production
- The route of entry is usually by droplet nuclei or by oral infection. Transmission of adenovirus is via direct contact, the fecal–oral route, and occasionally waterborne

25.12.2 Clinical Presentation

- Most adults have measurable titers of antiadenovirus antibodies, implying prior infection. Most adenovirus infections occur early in life, and by age 10, most children

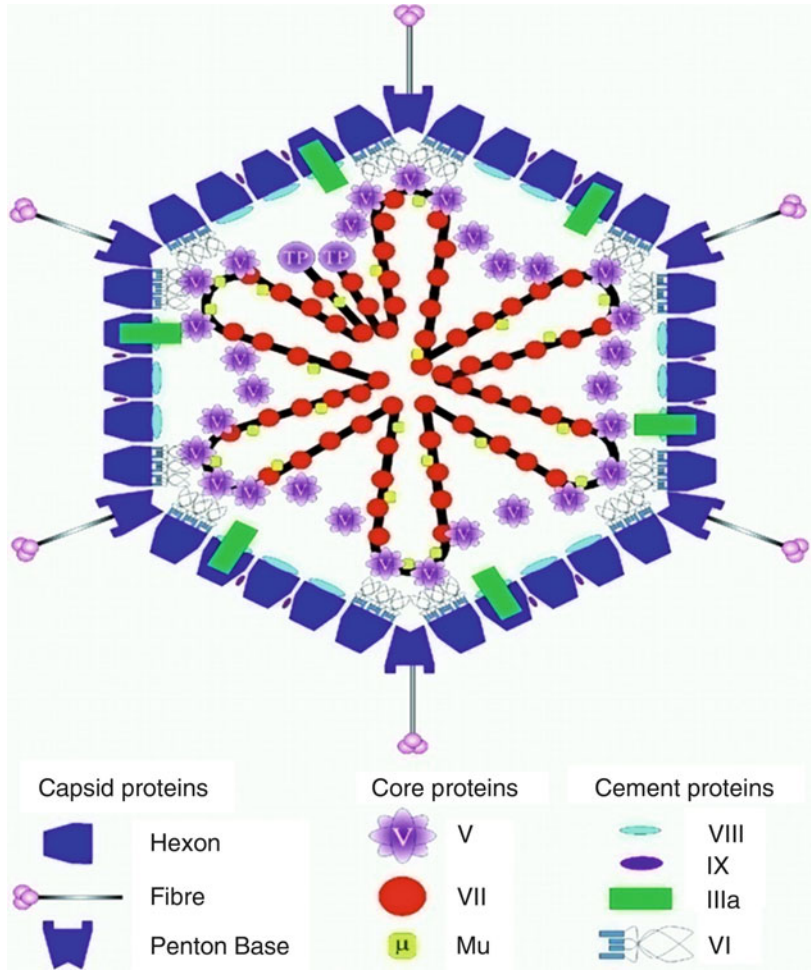


Fig. 25.27 Structure of adenovirus (Adapted from Russell WC. J Gen Virol 2008)

have been infected by at least one serotype. However, most infections are asymptomatic

- Some adenovirus types can establish persistent subclinical infections in tonsils, adenoids, and intestines of infected hosts with viral shedding occurring for as long as several months to years
- Adenovirus may infect multiple organ systems and is recognized as the etiologic agent of a variety of diverse syndromes: acute respiratory disease (ARD), pharyngoconjunctival fever, epidemic keratoconjunctivitis, acute hemorrhagic cystitis, gastroenteritis, and adenoviral infections in immunocompromised hosts
- Infection with serotypes 1, 2, and 5 is most frequent in the first years of life, and all serotypes can occur during any season, but infections are most frequent in late winter and early spring. Historically, 1–5% of all respiratory infections have been caused by adenovirus. The prevalence of adenovirus respiratory infection in children has ranged from 2% to 14%. Adenovirus also causes 5–15% of acute diarrheal infections in children

25.12.3 Diagnostic Methods

- Specimens
  - Respiratory, stool, and blood
- Conventional tests and problems
  - Viral culture
    - The "gold" standard for adenovirus testing is viral culture

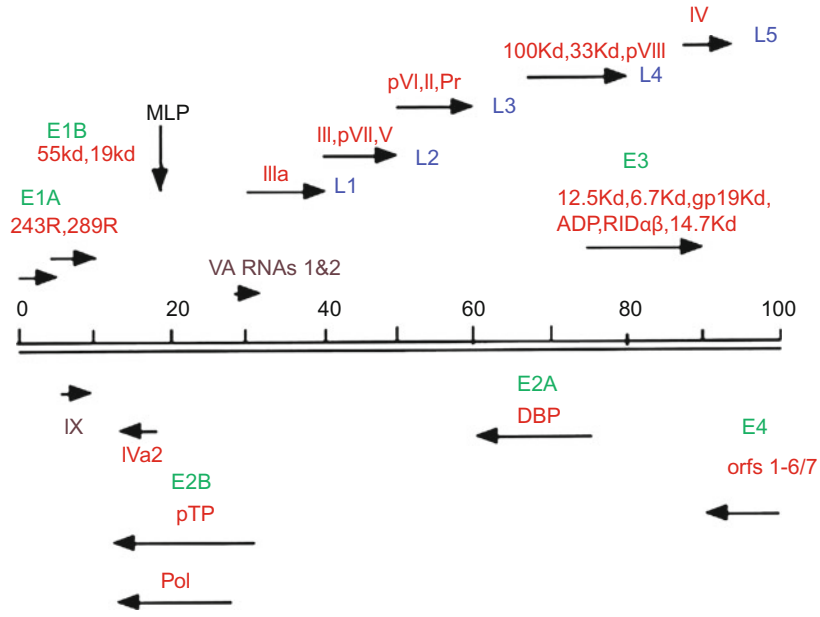


Fig. 25.28 Transcription of the adenovirus genome. The early transcripts are outlined in *green*, the late in *blue*. *Arrows* indicate the direction of transcription. The gene locations of the VA RNAs (nontranslated RNAs) are denoted in *brown*. MLP, major late promoter (Adapted from Russell WC. J Gen Virol 2008)

- Collected samples are layered on a monolayer of cells in test tubes. If present, adenovirus will present as cytopathic effect of rounded cells
- The best cell line for testing adenovirus is A549. This is a human carcinoma continuous cell line
- When samples are collected within 1–3 days of clinical onset, cell culture generally is positive within 4–7 days. Samples collected after 3 days may take 1–3 weeks to produce cytopathic effect
- Slow growth – not ideal for acute identification: The viral culture can confirm an adenovirus diagnosis, but it may not provide timely results for immediate patient care

– Shell vial culture
  - Shell vial is another cell culture test but the results are ready in 3 days
  - Vials of A549 cells are inoculated with collected samples and centrifuged. The vials are then incubated and stained at day three with immunofluorescent antibodies specific to adenovirus
  - The cells infected with adenovirus will light up with examination under a fluorescent microscope
  - It is recommended that the shell vial testing is for diagnosing ocular adenovirus infection

– Serologic studies
  - Seroreactivity to adenovirus is common; measuring adenovirus-specific IgM or rising level of IgG antibody can be helpful. Positive adenovirus titers occur in 50% of individuals >4 years old
  - Serology is less useful in the acute clinical setting
  - For a serologic diagnosis, serum should be obtained as early as possible in the clinical course, followed by a second titer 2–4 weeks later. A 4-fold rise in acute titers to convalescent titers is diagnostic
  - Serological assays have been used over the years to measure adenovirus-specific IgM or rising levels of IgG antibody; however, serology is no longer for diagnosis because it is slow and less specific than antigen or DNA detection

– Immunofluorescence
  - DFA or indirect IFA may be used for direct examination of tissue. It uses a mouse antibody against an adenovirus group-specific hexon antigen

  - This method has shown to have poor sensitivity compared to other tests, and shell viral cultures are run concurrently to improve sensitivity
  - Several commercial kits are (1) Light Diagnostics™ IFA and DFA respiratory viral screening kits for respiratory syncytial virus (RSV), Flu A, B, parainfluenza 1, 2, 3, and adenoviruses (Millipore Corp.); and (2) MonoFluo® RSV, Flu A, B, parainfluenza 1,2, 3, and adenoviruses (Bio-Rad Laboratory)
- Molecular methods
  - PCR: Marked improvement in sensitivity when compared to viral culture
  - Most of these assays amplify conserved regions of the hexon gene
  - Most of real-time quantitative PCRs are designed to detect adenovirus DNA from all major subgroups of the virus
  - Four common multiplex assays are the MultiCode-Plx assay (EraGen), the ResPlex III assay (Millipore), ADENOVIRUS R-gene™ CE Marked PCR Assay, which detects and quantifies all the 52 adenovirus serotypes, and the xTAG RVP assay (Luminex Molecular Diagnostics). The xTAG RVP is the only FDA-approved test and has a reported sensitivity of 78.3% and a specificity of 100%
- Pitfalls
  - Development of PCR-based assays must always consider antigenic drift and random mutations due to viral evolution that may result in false negatives
  - Important to note that PCR assays are not standardized and variations in sample handling and laboratory methods can affect the sensitivity of the assay

25.12.4 Clinical Utility

- PCR assays provide a rapid and specific diagnosis of adenovirus to allow for early therapeutic intervention and prophylactic treatment in high-risk patients, i.e., geriatric care facility and immunocompromised patients
- Detection of high viral load in blood and monitoring of viral load during treatment can correlate with disseminated adenovirus disease in immunosuppressed patients. Adenovirus is increasingly important in solid organ and bone marrow transplant patients; infections in pediatric transplant cases can exceed 22% with mortality rates as high as 60%

25.13 Respiratory Syncytial Virus

25.13.1 General Characteristics

- RSV is a negative sense, enveloped RNA virus. The virion is variable in shape and size (120–300 nm), is unstable in the environment (surviving only a few hours on environmental surfaces), and is readily inactivated with soap and water and disinfectants
- RSV is a member of the paramyxovirus family that produces a characteristic fusion of human cells (syncytial effect) in tissue culture
- RSV is a medium-sized, enveloped virus with an antisense single-stranded RNA genome
- RSV has two heterotypic strains of viruses that are antigenically distinct, and are classified as subgroups A and B
- The major difference between these subgroups is the antigenic properties of the G surface glycoprotein
- Transmission is from aerosolized respiratory droplets via close contact with infected persons or contact with contaminated surfaces
- Most prevalent in infants aged 2–6 months, but children of any age with underlying cardiac or pulmonary disease or who are immunocompromised are at risk for serious complications from RSV infection

25.13.2 Clinical Presentation

- RSV infections typically occur in temperate climates during late fall through early spring and account for 5–15% of community-acquired pneumonias. 50%

of children ≤1year are infected and 100% are infected by 3 years. Immunity wanes with age. Disease may reoccur in patients >65 years
- Two subtypes have been identified. Subtype A involves a severe clinical presentation and predominates in most outbreaks. Subtype B predominates in most asymptomatic strains of the virus that the majority of the population experiences
- RSV bronchiolitis presents with a 2–3-day "prodromal" phase, which resembles a common viral upper respiratory tract infection. Additional symptoms include rhinorrhea, wheezing, coughing, low-grade fever, and pneumonia. Circumoral and nail bed cyanosis may occur in severely affected infants
- In the majority of patients with RSV bronchiolitis, symptoms resolve within 5–7 days

25.13.3 Diagnostic Methods

- Specimens – respiratory swabs and bronchoalveolar lavage
- Conventional tests and problems
  - Viral culture
    - RSV has a high liability and any specimens should be transported to the laboratory promptly and inoculated into cell cultures
    - Nasopharyngeal aspirates, nasal washes, or tracheal secretions are generally the best specimens for isolation
    - Specimens should not be subjected to major temperature changes such as freezing and thawing
    - Human heteroploid cells, such as HEP2 and HeLa, generally provide the best tissue culture for the isolation of RSV
    - RSV produces a characteristic CPE consisting of syncytia formation and appears in 4– 5 days
    - Decreased sensitivity in adults from reduced viral shedding during acute infections as compared to adolescents
  - Rapid antigen detection
    - Direct and indirect immunofluorescence (IF) methods
      - Ability to perform direct screening with low cost
      - Both direct and indirect IF utilizing either polyclonal or monoclonal antibodies are available which possess a high degree of sensitivity and specificity
      - The general sensitivity of IF is 80–90% and for monoclonal antibody 95–100%. The specificity is at least 94%
      - IF techniques are fast and easy to perform, but the interpretation of results is subjective and the specimen must contain adequate nasopharyngeal cells
      - Incorrect and indeterminate results may occur for specimens with few epithelial cells or when nonspecific antibody reagents are used
    - ELISA (BD Directigen™ RSV)
      - ELISA assays do not require expensive laboratory equipment, take only 15–20 min, and are inexpensive compared to cell culture
      - ELISA techniques offer the advantages of objective interpretation, speed, and the possibility of screening a large number of specimens. Disadvantages include a generally poorer sensitivity and a "gray zone" of equivocal results, which requires confirmation by a time-consuming blocking ELISA procedure
    - Serology
      - Acute and convalescent phase sera are required for the serologic diagnosis of RSV
      - A fourfold increase in antibody titer or the appearance of specific IgM antibody is required for serologic confirmation of infection
      - It includes complement fixation (CF) antibody titers, ELISA, neutralization to specific A and B subtypes, and indirect IF

  - It is unlikely to be of help in the management of the patient because of the length of time required. Furthermore, the serological response in young infants may be poor and not detectable by some antibody assays
  - Seroconversion does not occur for at least 2 weeks and may require 4–6 weeks. CFTs are less sensitive than neutralization and ELISA assays
- Molecular methods
  - NASBA-beacon
    - NucliSens EasyQ RSV A + B assay (bioMérieux)
    - Real-time PCR-based assay utilizing NASBA technology containing internal control and specific molecular beacon mix targeting fusion protein of RSV
    - Limit of detection is 22 input copies of RSV
    - Improved time to result, <4 h
  - Cepheid SmartCycler® System – RSV (ASR)
    - The RSV Type A or B Primer ASR contains primers designed to detect RSV type A or B
    - The RSV Type A or B Probe ASR contains FAM-labeled probes designed to detect RSV type A or B
  - xTAG® Respiratory Viral Panel (RVP) (Luminex)
    - The RVP is a qualitative nucleic acid multiplex test intended for the simultaneous detection and identification of multiple respiratory virus nucleic acids in nasopharyngeal swabs from individuals suspected of respiratory tract infections
    - The xTAG RVP has been designed to simultaneously probe for 12 viral targets in a single patient specimen: influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, RSV subtype A, RSV subtype B, parainfluenza 1, parainfluenza 2, and parainfluenza 3, human metapneumovirus, rhinovirus, and adenovirus. All viruses are probed for in a single multiplex reaction
    - xTAG RVP incorporates multiplex Reverse RT-PCR and multiplex target-specific primer extension (TSPE) with Luminex Molecular Diagnostic proprietary Universal Tag sorting system on the Luminex xMAP platform. xTAG RVP is compatible with both the Luminex 100/200 IS systems
- Sensitivity and specificity
  - Improved sensitivity and specificity when compared to conventional tests, particularly in the adult population
  - No cross-reactivity was shown for PIV13; influenza A and B; measles; adenovirus types 1 and 5; hMPV A1, A2, B1, and B2 indicating that the assay is specific for RSV
- Pitfalls
  - Although the RSV virus can be cultured from an infected individual, the delay in definitive diagnosis of 3–5 days decreases the clinical utility

25.13.4 Clinical Utility

- Ease of assay, rapid turnaround time, and improved sensitivity have enhanced clinical utility in early detection of respiratory illness

25.14 Severe Acute Respiratory Syndrome

25.14.1 General Characteristics

- Severe acute respiratory syndrome (SARS) is a recently identified respiratory illness that first infected individuals in parts of Asia, North America, and Europe in late 2002 and early 2003
- The SARS-associated coronavirus belongs to the *Coronaviridae* family, a family of large, enveloped positive-stranded RNA viruses. It is the first example of a coronavirus causing serious disease in humans
- The SARS-coronavirus (SARS-CoV) genome is 29,272 nucleotides in length with 41% being G/C residues

- SARS is spread mainly through contact with infected saliva or droplets from coughing. Vertical transmission from mother to infant does not appear to occur

25.14.2 Clinical Presentation

- The SARS virus produces an atypical pneumonia that often leads to respiratory failure, with pulmonary edema and hyaline membrane formation similar to that seen with adult respiratory distress syndrome (ARDS)
- During the early phase of the disease, fever greater than 38 °C (100.4 °F) is the hallmark symptom. This finding is often associated by myalgia, rigors, and other flu-like symptoms
- During the second week, patients develop a dry, nonproductive cough, shortness of breath, and lung infiltrates with rapid progression to respiratory distress
- The cause of death is respiratory failure, with the best predictor of mortality being old age. The fatality of SARS is less than 1% for people aged 24 or younger, 6% for those 25–44, 15% for those 45–64, and more than 50% for those over 65
- Except for ventilation, no effective treatment is currently available

25.14.3 Diagnostic Methods

- Specimens
  - Respiratory sample: nasal wash, nasopharyngeal swab, BAL, bronchial wash, or sputum
  - Transport: 1 ml (minimum volume 0.5ml for adults and pediatrics) respiratory sample in viral transport media (Microtest M4) or in sterile leakproof container at 2–8 °C
  - Unacceptable conditions: Dry swabs are not acceptable. Respiratory aspirates collected in containers with tubing as samples tend to leak, compromising the specimen
- Conventional tests and problems
  - Viral culture
    - Requires BSL3 facility
    - Difficulty in culturing the virus from infected individual late in the outbreak during late stages of illness due to possible genetic drift of virus
  - Serologic studies
    - Utility of serologic testing is poor due to late seroconversion of infected patients, i.e., 2–4 weeks
    - Antigen detection with monoclonal antibodies or monospecific polyclonal antibody against the N protein was found to be a sensitive and specific test for the diagnosis of SARS
    - For antibody testing, the indirect immunofluorescent antibody test is more commonly performed than the neutralizing antibody test since the former involves minimal manipulation of infectious virus and therefore carries less risk of a biohazard. The test is generally not useful during the first week of illness
- Molecular methods
  - RT-PCR
    - Multiple RT-PCR assays have been developed to detect SARS RNA in clinical specimens utilizing nested, nonnested, one-step or two-step conventional, or real-time RT-PCR assays
    - Most nucleic acid amplification tests are designed with the Orf1b or nucleoprotein gene. The latter gene has the theoretical advantage of being more abundant in infected cells and therefore of higher sensitivity, but this has not been clearly proven in clinical studies
    - LightCycler SARS-CoV quantitation kit [Roche Diagnostics Corporation] for use with the LightCycler instrument
      - Ready to use which amplifies a 180-bp target sequence of the replicase 1AB/polymerase gene of SARS CoV
      - The analytical sensitivity for the SARS-CoV test is about 20 copies/PCR reaction
    - RealArt HPA-coronavirus RT-PCR kits [artus] for use with the LightCycler

instrument, the ABI PRISM 7000, 7700, and 7900H instruments
  - Amplifies an 80-bp region of the SARS-CoV genome
- EraGen Biosciences MultiCode-RTx (research only)
  - EraGen's platform increases size of the genetic "alphabet" from the two DNA base pairs to six pairs with the development of eight new synthetic bases
  - It is a new multiplexed real-time PCR platform
  - Only standard PCR primers need to be designed. Since reporters are placed directly onto the primers and not on probes
  - It targets nucleocapsid *(nuc)* or polymerase *(pol)* gene
- Sensitivity and specificity
  - Sensitivity of commercial assays ranged from 36% to 80% and specificity ranged from 80% to 100%
  - The absolute sensitivity of the RT-PCR assays ranged from 10 to 100 genome equivalents per reaction
- Pitfalls
  - When present, SARS antibodies can be detected in serum at any point during the course of the disease. However, most patients do not seroconvert until after the second week, highlighting the importance of an RT-PCR assay for early diagnosis of the virus
  - Positive results must be confirmed by repeat testing using an aliquot of the original specimen and/or another laboratory before reporting. Alternatively, testing of a second gene region may be helpful. Furthermore, testing of one sample from a single source does not rule out the presence of SARS-associated coronavirus
  - A negative result does not rule out SARS as the presence of PCR inhibitors in the patient specimen, poor RNA quality, or nucleic acid concentrations below the level of detection of the assay may occur

25.14.4 Clinical Utility

- During the first week, serum and plasma are preferred for RT-PCR. Between 1 and 3 weeks, these sample types are less effective; stool and respiratory samples are the preferred types. After 3 weeks, stool is the preferred sample type for RT-PCR. Viral load in the upper respiratory tract and feces is low during the first 4 days of infections and peaks at approximately the 10th day of illness
- During the 10th–15th day of illness, high viral loads are independent predictors of poor clinical outcomes

25.15 Enterovirus

25.15.1 General Characteristics

- Enteroviruses represent one of the most common human viruses, affecting an estimate 50 million individuals in the US and potentially one billion worldwide. Enterovirus infections most commonly occur in temperate zones during the summer and early fall
- Enteroviruses are a diverse group of small, nonenveloped ssRNA viruses of 6–7 kb that are transmitted by the fecal–oral route. Enteroviruses comprise a group of human viruses that includes polioviruses, echoviruses, coxsackie A viruses, coxsackie B viruses, and various enterovirus subtypes. The original classification of human enteroviruses has been substituted by a taxonomic scheme based on molecular and biological properties of the viruses. This revised classification recognizes at least 90 subtypes and separates them into four species
- Several modes of transmission exist for these viruses, including fecal–oral, respiratory, transplacental, perinatal, and self-inoculation modes, but the majority are fecal–oral
- Although enteroviruses undergo rapid replication in the GI tract, they rarely cause significant GI disease. Instead, they travel via the bloodstream to target organs where they further replicate and induce pathologic alteration

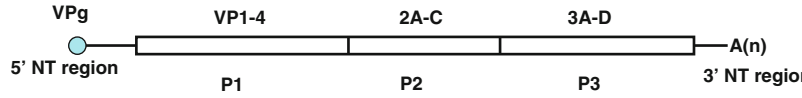


Fig. 25.29 Enterovirus genome: 7,450 nucleotide long single-stranded RNA virus with a 5′ NT region of 743 nt, a 6625 coding region, and 3′ polyA region (*VP* viral protein, *P* polypeptide, *NT* nontranslational region). PCR primers usually design to target to 5′ NT region

25.15.2 Clinical Presentation

- Most infections are subclinical, although may cause a variety of acute and chronic diseases
  - Acute: mild upper respiratory illness (common cold), febrile rash (hand, foot, and mouth disease and herpangina), aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis, and neonatal sepsis-like disease
  - Chronic: myocarditis, cardiomyopathy, type 1 diabetes mellitus, and neuromuscular disease
- The highest incidence of enterovirus infection is in infants and young children

25.15.3 Diagnostic Methods

- Specimens
  - Non sterile sites: nasal/throat swabs, and feces where the presence of the virus might merely indicate coincidental carriage
  - Sterile sites: vesicular fluid, CSF, serum, urine, or gathered at autopsy, are more reliable
  - Samples transported in viral transport media, were either transported directly to the laboratory or were stored at 4 °C for a maximum of 24
- Conventional tests and problems
  - Viral culture and shell vial culture
    - Gold standard to detect enterovirus
    - Time-consuming methods and insensitive methods, relying on the presence of viable virus
    - Inability to fully characterize some enterovirus strains associated with late inadequate collection, handling and processing of samples, or because of intrinsic insensitivity to cell lines used
    - It can take up to 8 days for CPE to appear when virus is present in low titers (e.g., in CSF specimens), and some type A coxsackievirus do not grow in cell culture
    - Although shell vial culture using monoclonal antibodies has decreased the culture time compared with that for tube culture, it is less sensitive than conventional culture
  - Serology
    - Serotype is usually irrelevant to individual management
    - The absence of a widely shared antigen has hampered the development of immunoassays for the enterovirus
    - Reports of monoclonal antibodies that cross-react with multiple enterovirus serotypes are promising, but further testing is required to determine the clinical relevance of those observations
- Molecular methods
  - Real time RT-PCR – ABI Prism (Applied Biosystems, Foster City, Calif.)
    - Improved speed and accuracy using TaqMan assay platform
    - Targets conserve sequences of the 5′ NTR and VP 1 and 2 (capsid protein). The 5′ NTR is the most highly conserved region and is involved in viral protein translation (Fig. 25.29)
    - An enterovirus real-time TaqMan PCR analysis of serum or plasma may be a good alternative for the enterovirus culture of feces, particularly in neonates with sepsis

- AnDiaTec® Enterovirus real time RT-PCR Kit
  - The kit is a screening assay for the detection of enteroviruses (coxsackie A, coxsackie B, and echovirus) in the capillary system of the LightCycler (Roche)
  - Targets conserve sequences of the 5′ NTR
- Cepheid SmartCycler® System
  - It detects a 115-bp region of the 5′ NTR
- NASBA
  - NASBA-electrochemiluminescently (ECL) and NASBA-beacon are not significantly different in sensitivity and specificity
    - Targets conserve sequences of the 5′NTR
  - NASBA-ECL
    - NucliSens basic kit has proved of equal or greater sensitivity for detection of enteroviruses
    - In the NucliSens basic kit, amplified RNA products are detected by hybridization using ECL-labeled probes, a highly sensitive methodology
  - NASBA-beacon
    - NucliSens EasyQ Enterovirus Test (bioMerieux, Durham, NC), which utilizes real-time molecular beacons as probes (NASBA-beacon)
    - Real-time RT-PCR using TaqMan to shorten both technical hands-on time and time to result
- Enterovirus consensus, Argene Biosoft (for research use only in the US)
  - One-step RT-PCR of all enterovirus serotypes in one single reaction tube
  - Amplified region is in the 5′ NCR of the genome
  - Detection is performed with a biotinylated enterovirus generic probe

- Sensitivity and specificity
  - NASBA-ECL and NASBA-beacon were similar in sensitivity, 100% and 94.5%, respectively
  - RT-PCR sensitivity is 97%, while culture sensitivity is 54.5%
  - Real-time RT-PCR sensitivity is 100% and the specificity is 96.2%
- Pitfalls
  - Parechoviruses may cause similar clinical illnesses but are not detected by enterovirus testing
  - Poor handling of CSF or CSF collected during late infection can yield false-negative results
  - Enteroviruses can be shed in high titers in stool for prolonged periods. Therefore, a positive result in stool alone may not correlate with current disease

25.15.4 Clinical Utility

- Enteroviral meningitis is common in the US and leads to a large number of hospitalizations per year due to an inability to distinguish from bacterial meningitis. Therefore, enterovirus differentiation from bacterial illness can significantly reduce hospitalizations, antimicrobial use, and diagnostic testing
- Rarely, dual infections (enteroviral and bacterial) can occur. Therefore, a positive enterovirus result with clinical features incompatible with benign viral meningitis should not lead to discontinuation of antibiotics

25.16 JC/BK Virus

25.16.1 General Characteristics

- The BK virus (BKV) and JC virus (JCV) are small, nonenveloped, closed circular, double-stranded DNA virus and belong to human polyomaviruses, members of the Papovaviridae family
- They were first isolated in 1971 and named JC and BK after the initials of the patients in which they were first discovered. JCV was isolated from the brain tissue of a patient with progressive multifocal leukoencephalopathy (PML); BKV was isolated from the urine of a renal transplant

patient who developed ureteral stenosis postoperatively
- BKV and JCV share 75% homology at the level of nucleotide sequence. Each is 70% homologous to simian virus 40 (SV40), which belongs to polyomaviruses and was introduced in the human population, between 1955 and 1963, by contaminated polio vaccines produced in SV40-infected monkey cells
- The two are not cross-reactive serologically, and serologic tests for antibodies are able to distinguish between BKV and JCV
- More than 70% of the adult population has antibodies to BKV and JCV, with primary infections typically occurring in childhood
- After an initial infection, polyomaviruses establish latency in various tissues. The primary sites of latency are uroepithelial cells for BK virus and B lymphocytes and renal tissue for JCV. Additional sites of latency for both viruses include the ureters, brain, and spleen

25.16.2 Clinical Presentation

- Both BKV and JCV have been associated with human tumors. The recent evidence that SV40 may be a cofactor in the etiology of specific human tumor types has raised again the interest on the two human polyomaviruses as possible agents involved in human oncogenesis
- In immunocompetent individuals, primary BKV infections usually cause a mild respiratory illness and, rarely, cystitis, whereas primary JCV infections are typically asymptomatic. BKV seroprevalence peaks at ~90% at age 5–9 years old
- Reactivation of latent as well as primary BKV and JCV infections may occur in immunocompromised individuals, i.e., organ transplantation, AIDS, and leukemia. BKV infections can lead to interstitial nephritis, tubulitis, hemorrhagic cystitis, and kidney allograft rejection
- JCV is responsible for progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system seen in up to 70% of AIDS patients

25.16.3 Diagnostic Methods

- Specimens: urine, plasma, CSF, and tissue biopsy
- Conventional tests and problems
  - Cytology and immunohistocytochemistry for urine sample
    - It can be used to confirm BKV reactivation diagnosis
    - The urine sample can be stained with JC/BK and SV40 monoclonal antibodies
  - Viral culture
    - JC virus is difficult to culture
    - The most sensitive cell type for JCV is primary human fetal glial cells, which is not an easy reagent to acquire
    - BK virus will grow in common cell lines, such as human diploid fibroblasts, but several days and weeks are required before CPE is evident
  - Serologic studies
    - Hemagglutination inhibition or enzyme-linked immunosorbent assay methods can measure titers of antibodies to JCV and BKV
    - Serological tests of blood and CSF for anti-JCV and BKV antibodies are not useful in the diagnosis of PML and immunosuppressed individuals because antibodies to JCV and BKV are common and many patients with PML or immunosuppressed patients fail to develop a significant rise in antiviral antibody titers in serum or CSF
- Molecular methods (Fig. 25.30)
  - PCR, quantitative
    - Seminested PCR to measure serum BKV DNA has been shown to have a higher specificity of 88% (50% positive predictive value) to detect BKV nephropathy. It was also shown to have a sensitivity of 100% for detecting BKV nephropathy
    - Serial quantification of BKV DNA levels through PCR can aid in managing BKV nephritis by measuring increasing or decreasing activity levels noninvasively

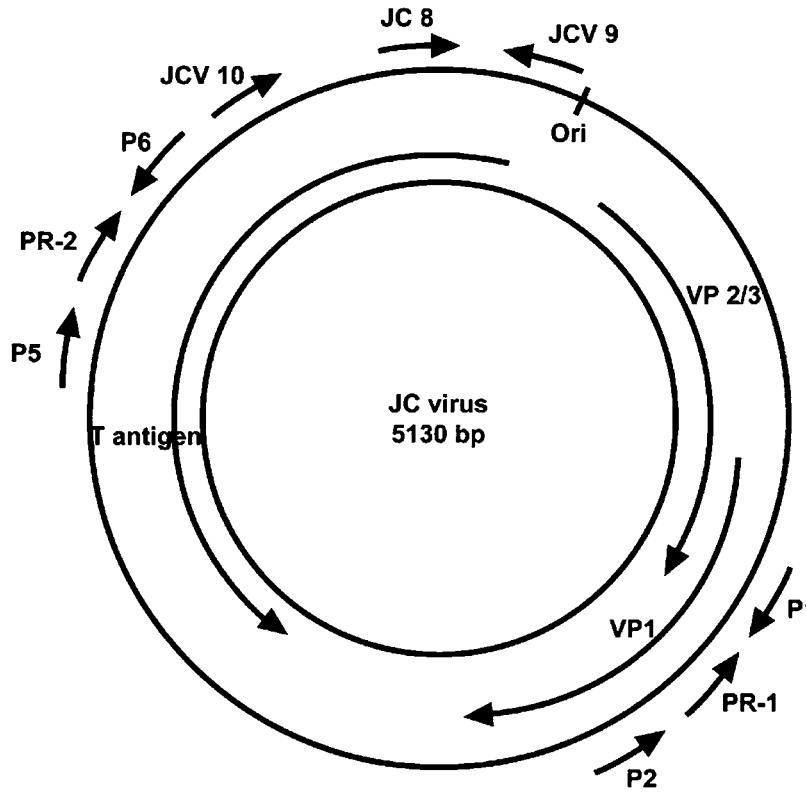


Fig. 25.30 JC virus genome structure

- PCR analysis of JCV DNA in spinal fluid is a noninvasive method of detecting active JCV infection which is 95% specific and 80% sensitive
- JC/BK Consensus Complete kit, Argene Biosoft (US: For research use only). The kit is for the detection and typing of JC and BK viruses by PCR and hybridization on microwell plates, and offers high sensitivity, up to 1 copy per PCR for BKV and 10 copies per PCR for JCV primers/probe product is designed to amplify JCV/BKV using 5′ nuclease real-time assay. The targeted sequence corresponds to a fragment of 197/198 bp located in the gene of large T antigen
- Real-time TaqMan PCR and LightCycler Probes (Homebrew) targets highly conserved sequence of JCV/BKV genomes (VP2 gene)

- Sensitivity and specificity
  - Analytical specificity: no cross-reactivity with HSV family viruses, simian virus, adenovirus, and HIV. Absolute sensitivity: 10 JC/BK virus detection
  - PCR has been able to detect JCV in CSF in 80–90% of PML patients
  - The specificity of diagnosis is influenced by the choice of primers and extraction methods but can approach 100%
- Pitfalls
  - Sequence variation of polyomavirus genome and within various JC/BK subtypes that may cause difficulty in primer and probe design
  - Competition between JC and BK viruses due to sensitivity may lead to false-negative PCR result

25.16.4 Clinical Utility

- Detection of the virus by PCR may be indicative of an active infection. Therefore, the identification of viral DNA may warrant the institution of antiviral therapies

and/or a decrease of immunosuppressive therapies
- Determination of viral DNA presence or concentration in transplant patients is useful in establishing the cause of allograft rejection. Viral load may also be useful in immunocompromised patients
- BKV nephropathy is associated with BK viremia of >5,000 copies/mL (plasma) and BK viremia >107 copies/mL and is seen in approximately 8% of kidney transplant recipients
- Though latency is typically associated with the absence of viremia, low levels (<2,000 copies/mL plasma) are seen in some asymptomatic individuals

25.17 HHV6

25.17.1 General Characteristics

- HHV6: two variants (A and B)
  - Type B is most common as primary infections in US, Japan, and Europe
  - Type A is most common among asymptomatic children in Africa
- Nearly 100% of population infected by 3 years of age
- Virus becomes latent or persistent until reactivated (immunocompromised hosts). Can be present in mononuclear cells, saliva, and the central nervous system
- 1% of population shows vertical transmission
- Congenital infection also reported

25.17.2 Clinical Presentation

- Most infants develop an undifferentiated febrile illness
- 20% present as roseola infantum
  - High fever, followed by maculopapular rash on face, neck, and trunk
  - Febrile seizures are most common neurologic complication
  - Encephalitis and encephalopathy is also reported
- Reactivated HHV6 presents as fever, rash, and encephalitis
- Differentiating reactivated from latent infection is very difficult, especially following stem cell or organ transplant

25.17.3 Diagnostic Methods

- Specimens: HHV6 can be isolated from peripheral blood mononuclear cells
- Conventional tests and problems
  - Serologic testing
    - Primary infection causes an IgM spike in the first week of illness followed by IgG rise approximately 2 weeks later. IgG antibody persists indefinitely
    - Tests include indirect immunofluorescence, immunoblot, and enzyme immunoassays. Viral culture can also be used but is time-consuming
    - However, gap between onset of infection and rise of titer does not allow a timely diagnosis
- Molecular methods
  - PCR
    - Qualitative nested polymerase chain reaction
    - Can be performed on plasma, mononuclear cells, whole blood, saliva, urine, cervical swabs, and placenta
    - Studies have shown results in plasma showed a sensitivity to diagnose reactivated or primary HHV6 infection with 75–95% sensitivity
    - Additionally, DNA can be detected in absence of viral replication
    - Specificity was reported to be 84% compared with viral culture
  - RT-PCR
    - Increased sensitivity and specificity (90% and 98%, respectively)
  - Quantitative PCR
    - Viral load may be helpful
    - Primary infection usually ranges from 4.4 to 4.9 log (10) genome equivalent copies (gec)/106 PBMCs

    - Asymptomatic individuals are lower (1.5–3.7)
    - Viral load with individuals with latent infection does overlap with immunocompromised host. However, plasma can be used to distinguish between the two (as latent individuals do not have plasma positive viral DNA)
    - Viral loads can be separated into very high (integrated virus), intermediate (primary, reactivated, or integrated virus) and low (latent infection)
- Pitfalls
  - Some overlap does occur with latent and immunocompromised hosts (as listed above)
  - Contamination is an issue, given that the techniques use signal amplification

25.18 Clinical Utility

- HHV6 can be especially dangerous in immunocompromised hosts
- HHV6 is present in a very large percentage of the population (100% of individuals greater than 3 years of age)
- Testing is required to determine if the host has a primary or latent infection
- Standard testing (viral capture or serology) is too time-consuming
- Quantitative PCR and RT-PCR can especially be useful

Further Reading

Anzola M, Burgos JJ. Hepatocellular carcinoma: molecular interactions between hepatitis C virus and p53 in hepatocarcinogenesis. Exp Rev Mol Med. 2003;5:1–16.

Bautista E, Chotpitayasunondh T, Gao Z, Harper SA, Shaw M, Uyeki TM, et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. N Engl J Med. 2010;362:1708–19.

Boeckh M, Boivin G. Quantitation of cytomegalovirus: methodologic aspects and clinical applications. Clin Microbiol Rev. 1998;11:533–54.

Cockerill 3rd FR, Smith TF. Response of the clinical microbiology laboratory to emerging (new) and reemerging infectious diseases. J Clin Microbiol. 2004;42:2359–65.

Comanor L, Hendricks D. Hepatitis C virus RNA tests: performance attributes and their impact on clinical utility. Expert Rev Mol Diagn. 2003;3:689–702.

Ellermann–Eriksen S. Macrophages and cytokines in the early defence against herpes simplex virus. Virol J. 2005;2:59.

Erice A. Resistance of human cytomegalovirus to antiviral drugs. Clin Microbiol Rev. 1999;12:286–97.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, et al. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev. 2006;19:165–256.

Finzer P, Aquilar-Lemarroy A, Rosl F. The role of human papillomavirus oncoproteins E6 and E7 in apoptosis. Cancer Lett. 2002;188:15–24.

Hakki M, Chou S. The biology of cytomegalovirus drug resistance. Curr Opin Infect Dis. 2011;24:605–11.

Kesli R, Polat H, Terzi Y, Kurtoglu MG, Uyar Y. Comparison of a newly developed automated and quantitative hepatitis C virus (HCV) core antigen test with the HCV RNA assay for clinical usefulness in confirming anti-HCV results. J Clin Microbiol. 2011;49:4089–93.

Landry ML, Garner R, Ferguson D. Comparison of the nuclisens basic kit (nucleic acid sequence-based amplification) and the argene biosoft enterovirus consensus reverse transcription-PCR assays for rapid detection of enterovirus RNA in clinical specimens. J Clin Microbiol. 2003;41:5006–10.

Landry ML, Garner R, Ferguson D. Real-time nucleic acid sequence-based amplification using molecular beacons for detection of enterovirus RNA in clinical specimens. J Clin Microbiol. 2005;43:3136–9.

Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. Clin Microbiol Rev. 2010;23:689–712.

Mahony JB, Richardson S. Molecular diagnosis of severe acute respiratory syndrome: the state of the art. J Mol Diagn. 2005;7:551–9.

Moore C, Valappil M, Corden S, Westmoreland D. Enhanced clinical utility of the NucliSens EasyQ RSV A + B Assay for rapid detection of respiratory syncytial virus in clinical samples. Eur J Clin Microbiol Infect Dis. 2006;25:167–74.

Moret H, Brodard V, Barranger C, Jovenin N, Joannes M, Andreoletti L. New commercially available PCR and microplate hybridization assay for detection and differentiation of human polyomaviruses JC and BK in cerebrospinal fluid, serum, and urine samples. J Clin Microbiol. 2006;44:1305–9.

Murray PG, Young LS. Epstein–Barr virus infection: basis of malignancy and potential for therapy. Expert Rev Mol Med. 2001;3:1–20.

National Institutes of Health. National institutes of health consensus development conference statement: Management of hepatitis C. Hepatology. 2002;36:S3–20.

Neville BW, Waldron CA, Herschraft EE. Oral and maxillofacial pathology. 1st ed. Philadelphia: Saunders; 1995.
Oberste MS, Pallansch L. Enterovirus molecular detection and typing. Rev Med Microbiol. 2005;16:163–71.
Ooi MH, Wong SC, Lewthwaite P, Cardosa MJ, Solomon T. Clinical features, diagnosis, and management of enterovirus 71. Lancet Neurol. 2010;9:1097–105.
Posthuma CC, van der Beek MT, van der Blij-de Brouwer CS, van der Heiden PL, Marijt EW, Spaan WJ, et al. Mass spectrometry-based comparative sequencing to detect ganciclovir resistance in the UL97 gene of human cytomegalovirus. J Clin Virol. 2011;51:25–30.
Russell WC. Update on adenovirus and its vectors. J Gen Virol. 2000;81:2573–604.
Shafer RW. Genotypic testing for human immunodeficiency virus type 1 drug resistance. Clin Microbiol Rev. 2002;15:247–77.
Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis. 2010;10:778–90.
Taubenberger JK, Layne SP. Diagnosis of influenza virus: coming to grips with the molecular era. Mol Diagn. 2001;6:291–305.
Whiley DM, Mackay IM, Sloots TP. Detection and differentiation of human polyomaviruses JC and BK by LightCycler PCR. J Clin Microbiol. 2001;39:4357–61.
Wright TC Jr, Schiffman M, Solomon D, Cox JT, Garcia F, Goldie S, et al. Interim guidance for the use of human papillomavirus DNA testing as an adjunct to cervical cytology for screening. Obstet Gynecol. 2004;103:304–9.
Zoulim F. New nucleic acid diagnostic tests in viral hepatitis. Semin Liver Dis. 2006;26:309–17.