Viruses with Single-Stranded, Positive-Sense RNA Genomes

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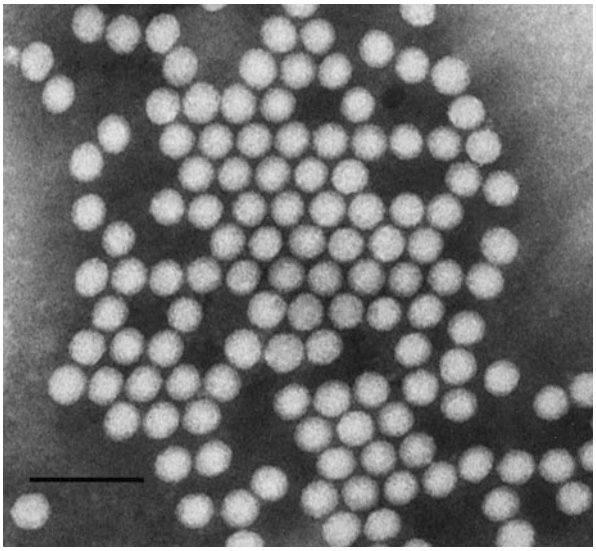
Eight virus families whose members infect vertebrates are currently known to possess single-stranded, positive-sense RNA genomes: the families *Picornaviridae*, *Astroviridae*, *Caliciviridae* and *Hepeviridae* have non-enveloped capsids, whereas the families *Flaviviridae*, *Togaviridae*, *Arteriviridae* and *Coronaviridae* are characterized by enveloped capsids. They all have in common the property of using their own genome as messenger RNA (mRNA), from which they synthesize one or several polyproteins that are subsequently cleaved into individual proteins by viral or cellular proteases. These viruses possess the genetic information for the synthesis of an RNA-dependent RNA polymerase. This enzyme transcribes the positive RNA strand as well as the complementary negative RNA strands, which arise as intermediate products of genome replication. In the course of this process, the new genomic RNA molecules are generated from the second transcription step. The classification into the different taxonomic families depends on the number, size, position and orientation of viral genes in the RNA molecule, the number of different polyproteins that are synthesized during viral infection and the existence of an envelope as a virion component.

14.1 Picornaviruses



The prototypic member of picornaviruses was discovered in 1898 by Friedrich Loeffler and Paul Frosch, who described the pathogen of foot-and-mouth disease (FMD; also referred to as hoof-and-mouth disease) as a filterable agent, thus demonstrating the existence of animal pathogenic viruses for the first time. Karl Landsteiner and Emil Popper published an article in 1909 which reported the identification of a virus as the pathogen of poliomyelitis, a disease that was described for the first time by Jacob von Heine in 1840 and later also by Oskar Medin. That these viruses are able to cause a cytopathic effect in cultures of human embryonic cells was demonstrated by John F. Enders, Thomas H. Weller and Frederick C. Robbins in 1949; however, the actual characterization as poliovirus was ascertained by Herdis von Magnus and co-workers only in 1955.

Coxsackievirus was discovered by Gilbert Dalldorf in 1947, when he infected newborn mice with virus-containing material and observed paralysis in the treated animals. The virus was named after the town Coxsackie in the federal state of New York, where it was isolated from a patient. Owing to different paralysis characteristics that they cause in newborn mice, coxsackieviruses have been subdivided into two groups, A and B. In addition to these viruses, there are also human pathogenic picornaviruses which in contrast to coxsackieviruses do not cause any signs of paralysis in newborn mice, but are associated with a lethal outcome within a few days: echoviruses and parechoviruses. The eponymous acronym "echo" stands for some viral properties: enteric, cytopathogenic, human, orphan; the last term indicates that no disease could be associated with the viral infection at that time. The multifarious symptoms that are caused by these viruses were discovered later and include diarrhoea, eczema and in rare cases also encephalitis and meningitis.

Another human pathogenic picornavirus was characterized much later: hepatitis A virus, the pathogen of an epidemic form of liver inflammation for which the genus *Hepatovirus* was created. Epidemiologically and diagnostically, hepatitis A was distinguished at an early date from hepatitis B. The first electron-microscopic detection of hepatitis A virus was performed by Stephen F. Feinstone and colleagues in 1973. However, the successful cultivation of this virus was only achieved in 1979; it was assigned to the picornavirus family in 1982. Besides the pathogens mentioned, there are also picornaviruses that can infect humans very frequently: namely rhinoviruses as the causative agent of the common cold.

14.1.1 Classification and Characteristic Prototypes

The family *Picornaviridae* is classified into the order *Picornavirales* together with the families *Dicistroviridae*, *Iflaviridae*, *Marnavidirae* and *Secoviridae*. Whereas members of the families *Dicistroviridae* and *Iflavividae* infect various species of insects and other invertebrates, those belonging to the families *Marnaviridae* and *Secoviridae* are pathogenic in algae and plants. The family *Picornaviridae* comprises a large number of virus groups and virus types (Table 14.1) that infect vertebrates. The members of this family can cause completely different disorders in humans, and partially also severe diseases. The name "picorna" is an abbreviation that alludes to two molecular properties of the virus family, i.e. small (*pico*) viruses with an RNA genome. The classification of picornaviruses into 17 genera is based on their respective molecular-biological properties, sequence homologies as well as on the diseases they induce: *Enterovirus*, *Parechovirus*, *Hepatovirus*, *Cardiovirus*, *Sapelovirus*, *Teschovirus*, *Kobuvirus*, *Aphthovirus*, *Erbovirus*, *Avihepato*virus, *Tremovirus*, *Senecavirus*, *Aquamavirus*, *Cosavirus*, *Dicipivirus*, *Megrivirus*, and *Salivirus*. More than 150 known human pathogenic viruses that belong to the genus *Enterovirus* have been newly classified on the basis of new sequence data of the viral genomes. Until recently, human rhinoviruses were also assigned to the genus *Enterovirus*, which comprises a large number of different species and types of viruses which can infect both animals and humans. Today, this genus is subdivided into 12 species, namely enteroviruses A-I and rhinoviruses A, B and C. All species differ by certain molecular properties (e.g. human enteroviruses A and B differ from human enteroviruses C and D by distinct sequence elements within the 5′ untranslated region, UTR) and/or in regard to their infection courses. Owing to its similarity to human enterovirus C, poliovirus has been assigned to this species; however, poliovirus differs from human enteroviruses by its property of causing poliomyelitis. Genome sequencing revealed that many of the different types of coxsackieviruses, enteroviruses and echoviruses have arisen by genetic recombination between various enteroviruses. For example, the animal pathogenic swine vesicular disease virus is a recombinant between the genomes of human coxsackievirus B5 – from which the coding region for the structural proteins is derived – and echovirus 9, from which the sequences encoding the

Table 14.1 Characteristic members of picornaviruses

Genus	Human virus	Animal virus
Enterovirus	**Enterovirus A**	**Enterovirus A**
	Coxsackievirus A2-A8, A10, A12, A14, A16 Enterovirus A71, A76, A89-91, A114, A119	Enterovirus A19, A43, A46, A92 (simian) Enterovirus A13 (baboon)
	Enterovirus B	**Enterovirus B**
	Coxsackievirus A9, B1-B6 Echovirus 1–7, 9, 11–21, 24–27, 29–33 Enterovirus B69, B73–75, B77–88, B93, B97, B98, B100, B101, B106, B107	Enterovirus B110 (chimpanzee) Simian enterovirus SA5
	Enterovirus C	
	Poliovirus 1–3	
	Coxsackievirus A1, A11, A13, A17, A19-22, A24	
	Enterovirus C95, C96, C102, C104, C105, C109, C113, C116-118	
	Enterovirus D	**Enterovirus D**
	Enterovirus D68, D70, D94, D111	Enterovirus D120 (gorilla)
		Enterovirus D111 (chimpanzee)
		Enterovirus E
		Bovine enterovirus/BEV A1–A4
		Enterovirus F
		Bovine Enterovirus/BEV B1–B6
		Enterovirus G
		Porcine enterovirus/PEV 9, 10, 14, 15, 16
		Ovine enterovirus/OEV 1
		Enterovirus H
		Simian enterovirus/SEV A1
		Enterovirus J
		Simian virus 6
		Enterovirus J103, J108, J112, J115, J121 (simian)
	Rhinovirus A	
	Rinovirus A1, A2, A7–13, A15, A16, A18–25, A28–34, A36, A38–41, A43–47, A49–51, A53–68, A71, A73–78, A80–82, A85, A88–90, A94–96, A98, A100–103	
	Rhinovirus B	
	Rhinovirus B3-6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69, B70, B72, B79, B83, B84, B86, B91, B93, B97, B99	
	Rhinovirus C	
	Rhinovirus C1–51	

(*continued*)

Table 14.1 (continued)

Genus	Human virus	Animal virus
Sapelovirus		**Porcine sapleovirus** (PEV 8)
		Simian sapelovirus
		Avian sapelovirus (duck)
Parechovirus	**Parechovirus**	**Ljungan virus** (rodents)
	Human Parechovirus 1–16	
Hepatovirus	**Hepatitis A virus**	
Avihepatovirus		**Duck hepatitis virus A**
Senecavirus		Seneca Valley virus (natural host unknown)
Cardiovirus	**Theilovirus**	**Theilovirus** (rodents)
	Saffold virus 1–8; Vilyuisk human encephalomyelitis virus	Theiler's murine encephalomyelitis virus; Theravirus **Encephalomyocarditis virus** Mengovirus (mice)
Teschovirus		**Porcine teschovirus** 1–13
Tremovirus		**Avian encephalomyelitis virus**
Kobuvirus	**Aichivirus A**	**Aichivirus B**
		Bovine kobuvirus
		Aichivirus C
		Aka porcine kobuvirus, canine kobuvirus, murine kobuvirus
Aphthovirus		**Bovine rhinitis Avirus**
		Bovine rhinitis B virus
		Equine rhinitis A virus
		Foot-and-mouth disease virus types O, A, C, SAT1-3, Asia 1
Erbovirus		**Equine rhinitis B virus**
Cadicivirus		**Cadicivirus A** (canine)
Cosavirus	**Cosavirus A–D**	
	Dekavirus	
Salivirus	**Salivirus A**	
	Human klassevirus 1	
Megrivirus		**Melegrivirus A**
		Turkey hepatitis virus 1
Aquamavirus		**Aquamavirus A**
		Seal picornavirus 1

The newly defined virus species are indicated in bold, benath are given the traditional names of the virus types/isolates, which are partially still in use

non-structural proteins originated. *Aichi virus*, a member of the genus *Kobuvirus*, is widely spread as a pathogen of gastrointestinal infections especially in Asia. Similar manifestations have been observed with Cosa- and Saliviruses. Human rhinoviruses are grouped into three distinct species: rhinoviruses A, B, and C.

This subdivision is based on differences in the amino acid sequences of capsid proteins. Nonetheless, many of the currently known virus types have still not been assigned to any of these species. The genera *Cardiovirus*, *Sapelovirus*, *Teschovirus*, *Erbovirus*, *Cadicivirus*, *Aquamavirus*, and *Aphthovirus* comprise primarily animal pathogenic picornaviruses that partly cause animal epidemic diseases and have considerable importance for animal breeding and food technology; e.g. FMD virus, which has been classified into the genus *Aphthovirus*. Seven different serotypes can be distinguished worldwide today. The name of this genus alludes to the blisters or cysts (aphthae) that arise in the mucosa of infected animals. The genera *Avihepatovirus*, *Megrivirus* and *Tremovirus* have been created for species that have been isolated from birds. Only one viral species has been assigned to the genus *Senecavirus*: Seneca valley virus was identified in the cultured cell line PER C6; its natural host has not yet been identified. The 5′ UTR of this new virus displays some similarities to that of pestiviruses and hepaciviruses (Sect. 14.5).

In addition to this taxonomic classification, picornaviruses can be subdivided into two principal groups according to their molecular properties that are closely associated with pathogenesis: (1) acid-stable viruses that can survive in the acid environment of the stomach without a loss of infectivity, thus infecting the host organism preferentially through the digestive tract – to this group belong enteroviruses, parechoviruses, hepatoviruses, avihepatoviruses, sapeloviruses and teschoviruses; (2) acid-labile viruses that infect the host organism preferentially via the upper respiratory tract, such as rhinoviruses, aphthoviruses and erboviruses.

14.1.2 Structure

14.1.2.1 Virus Particle

All picornaviruses have a very similar structure, which consists of a non-enveloped icosahedral nucleocapsid with a diameter of approximately 30 nm. The capsids are composed of four viral proteins: VP1, VP2, VP3 and VP4. Occasionally, a fifth polypeptide is found in inconstant quantities; it is denominated VP0 and is the precursor of components VP2 and VP4, which arise by proteolytic cleavage during viral maturation. Especially in parechoviruses, VP0 is barely cleaved; therefore, their particles contain large quantities of the precursor protein. Inasmuch as the particle structure of several picornaviruses has been elucidated by X-ray diffraction analysis, the arrangement of the different viral structural proteins in the particles is well known. The virions are composed of 60 units of each of the four single proteins VP1, VP2, VP3 and VP4, in which VP4 is localized inside the particle and in association with the RNA genome. The hepatovirus VP4 is very small (21–23 amino acid residues), and its presence in the virion has not been definitively resolved. VP1, VP2 and VP3 form the surface of the icosahedron (Fig. 14.1).

The virions of enteroviruses, parechoviruses, kobuviruses, cardioviruses, teschoviruses and hepatoviruses are very stable and cannot be compromised even by an acid environment with pH values of 3 and below. This suggests that a very

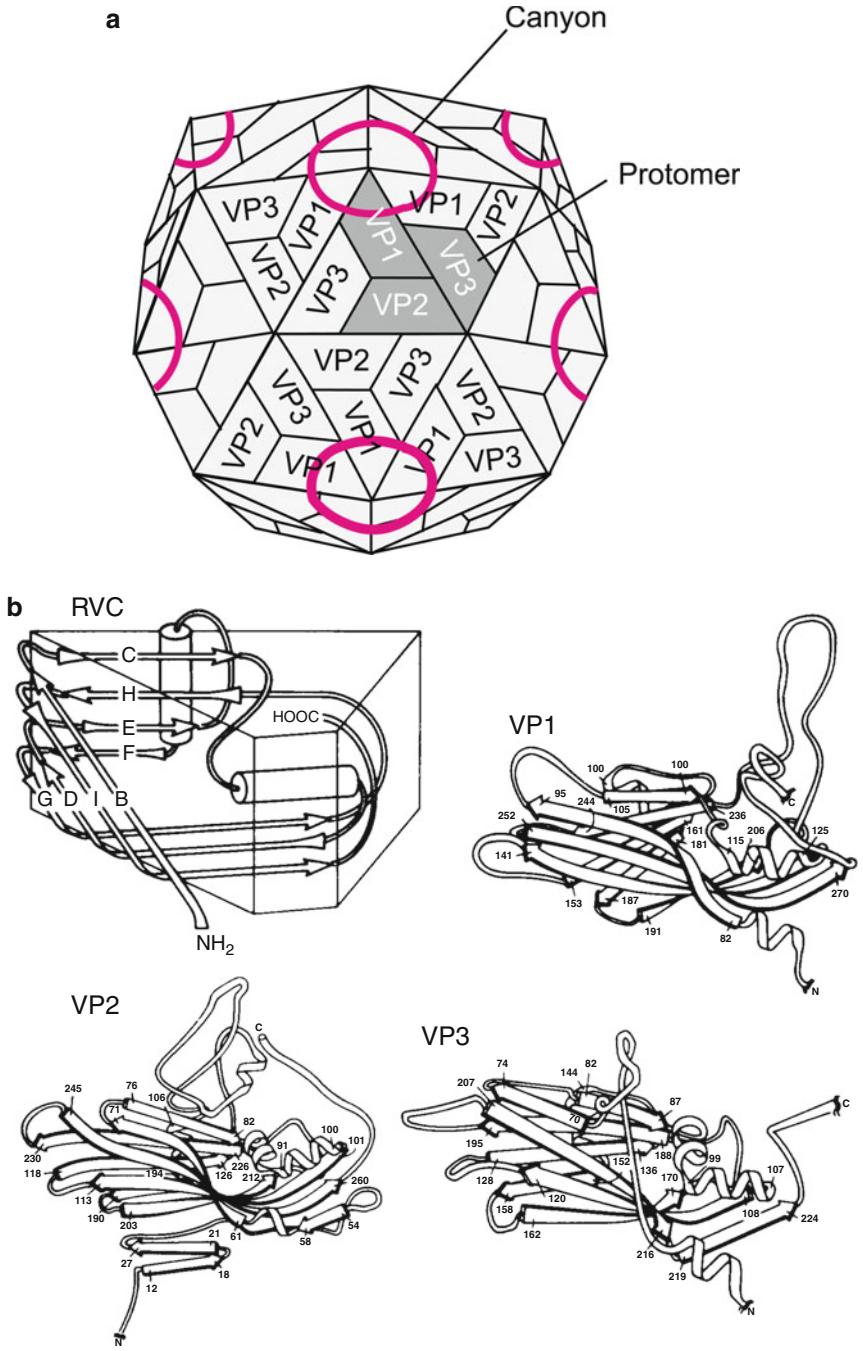


Fig. 14.1 (continued)

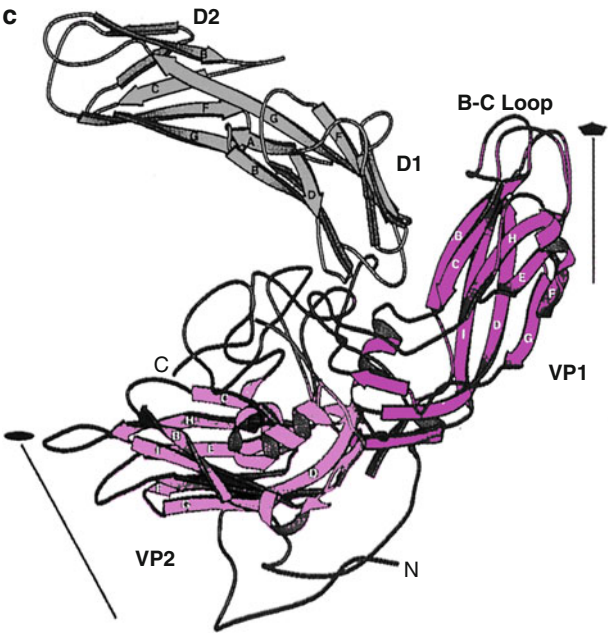


Fig. 14.1 (**a**) Structure of a picornavirus particle. The positions of capsid proteins VP1, VP2 and VP3 are shown schematically. VP4 resides on the inner side of the capsid, and is not exposed on the surface. The location of the precursor product for VP1, VP2 and VP3, the protomer, is indicated by the *grey-shaded region*. The so-called canyons, which as moat-like clefts or crevices surround the vertices of the icosahedron, are shown in *red*. (**b**) Structure of poliovirus capsid proteins VP1, VP2 and VP3. Also shown is the RNA virus capsid domain (*RVC*) of the proteins, which represents the common wedge-shaped folding pattern consisting of eight antiparallel β-sheet structures. Single β-sheets are represented by *arrows* and are indicated by *capital letters* according to their sequential order within the proteins. The α-helical regions are represented by *cylinders*. The structures of VP1, VP2 and VP3 are illustrated as ribbon models. The *numbers* refer to the amino acid positions numbered from the amino terminus of the respective capsid protein. The α-helical structures are represented by the helical folding of the ribbons. The amino- and carboxy-terminal regions are not included in the illustration for better identification of the common structural motif. (**c**) The canyon structure of human rhinovirus 14 and its interaction with the cellular receptor intercellular adhesion molecule 1 (ICAM-1). The structures of capsid proteins VP1 (*dark red*) and VP2 (*light red*) are shown as ribbon models. The antiparallel β-sheets are represented by *arrows* and are indicated by *capital letters*. The crystal structure of the amino-terminal region of CD4 was used as a basis for structure modelling of receptor domain D1 of ICAM-1 (*grey*) because of their extensive reciprocal homology (**b** From Hellen and Wimmer 1995; **c** after Olsen et al. 1993)

tight interaction must exist between the various capsid proteins within the particle of such viruses. Furthermore, these viruses also exhibit a very high resistance against detergents. Even as free virus particles, they are able to survive in the environment for relatively long periods of time.

Table 14.2 Comparison of distinct picornavirus genomes

Virus	Genome size (nucleotides)	(5′ UTR)	Polyprotein (amino acids)	(3′ UTR)
Poliovirus type 1	7,433	740	2,207	72
Human enterovirus B (coxsackievirus B3)	7,400	741	2,185	100
Hepatitis A virus	7,478	733	2,227	64
Rhinovirus (type 14)	7,209	624	2,178	47
Foot-and-mouth disease virus	8,450	1,199[a]	2,332	87

UTR untranslated region
[a]Contains a poly(C) tract of 100–170 nucleotides, depending on the virus isolate

14.1.2.2 Genome Organization and Structure

The viral nucleic acid is associated with amino acid residues on the inner side of the icosahedral particles. It consists of single-stranded RNA. The genome is between 7,212 (human rhinovirus B; type 14) and 8,450 (FMD virus) nucleotides in length (Table 14.2). The RNA has a positive sense. Accordingly, viral proteins can be directly translated from the RNA without requiring an intermediate transcription step. The 3′ end of the RNA genome is polyadenylated; the sequence of about 60 adenylate residues is coded in the viral genome. Vpg (for "viral protein genome-linked"), a small viral protein, is covalently linked to the 5′ end of the viral genome. Vpg is 22 amino acids long in poliovirus and other enteroviruses and 23 amino acids long in hepatitis A virus and rhinoviruses. It is esterified with the phosphate group of the uridine residue at the 5′ end of the genome by the OH group at position 3 in the phenol ring of a tyrosine residue.

The genome of picornaviruses contains a long single open reading frame that encodes a large precursor protein (Fig. 14.2). This experimentally not isolable protein is processed by proteolytic cleavage during its synthesis, thus leading to all the various viral components, i.e. structural and non-structural proteins as well as viral enzymes. A large untranslated sequence region (5′ UTR) is located between Vpg at the 5′ terminus of the genome and the start codon of the precursor protein; it has a length of 412 nucleotides in porcine teschovirus, 1,624 nucleotides in rhinoviruses and 1,199 nucleotides in FMD virus. The vast majority of the 5′-terminal non-coding nucleotides form intramolecular base pairings, resulting in a pronounced secondary structure (Fig. 14.3). The first 88 nucleotides immediately adjacent to the 5′ terminus form a secondary structure resembling a cloverleaf, which is involved in the initiation of genome replication for generating new positive-sense RNA molecules. The further downstream located sequence region possesses an internal ribosome entry site (IRES) that facilitates binding of ribosomes independently of the 5′-cap structure that is usually present at the 5′ end of eukaryotic mRNA molecules. Mutations in this region can strongly influence the translatability of the mRNA and virulence. Furthermore, a short untranslated sequence region is located between the stop

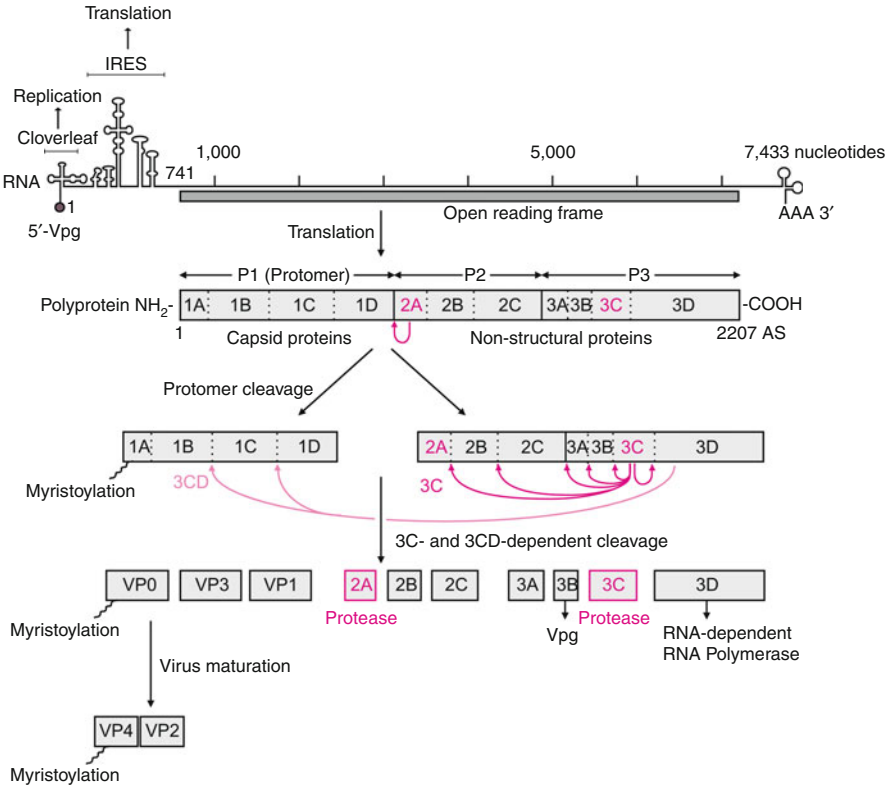


Fig. 14.2 Genome structure of picornaviruses using the example of poliovirus 1. The viral genome possesses a covalently linked protein (*Vpg*) at the 5′ terminus and is polyadenylated at its 3′ end. Adjacent to the 5′-terminal Vpg there are RNA sequences that form a cloverleaf-like secondary structure, which is essential for genome replication. *IRES* indicates the internal ribosome entry site that is necessary for binding of ribosomes to the viral RNA genome. The single open reading frame encodes a large precursor polyprotein that is processed into the distinct viral components (structural proteins, non-structural proteins and enzymes) by the proteolytic activity of 2A, 3CD and 3C proteins. The regions representing the proteases are illustrated in *red*; *red arrows* indicate the cleavage sites of the respective proteases

codon for the polyprotein and the poly(A) tail at the 3′ terminus of the genome, which comprises 47 and 100 nucleotides in rhinoviruses and coxsackieviruses, respectively.

14.1.3 Viral Proteins

14.1.3.1 Polyprotein

The open reading frame on the picornavirus genome encodes a very large continuous protein, which comprises more than 2,100 amino acids in all viruses. Its sequence contains all proteins and functions that are necessary for a successful

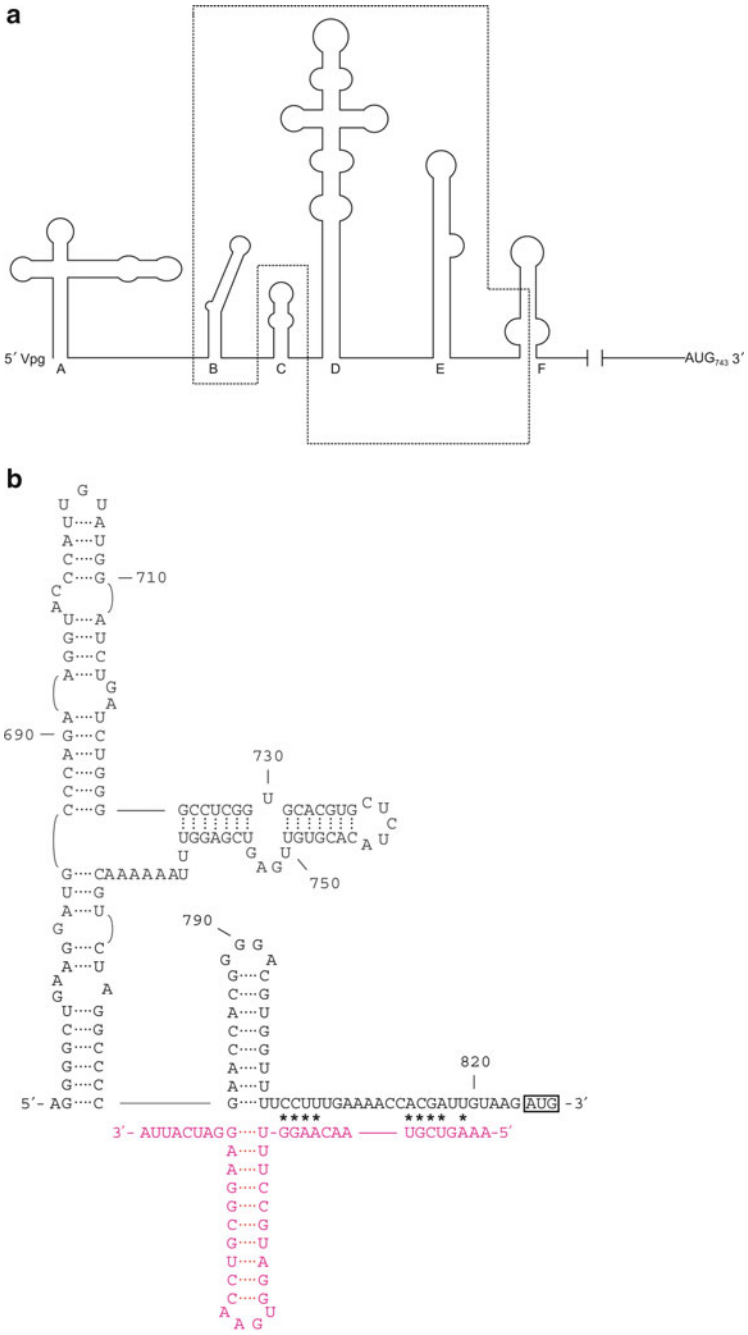


Fig. 14.3 (**a**) Computer model of the energetically favoured folding of the 5′ untranslated region (UTR) of poliovirus. The sequence elements comprising the IRES are indicated within the *frame*.

infection process. The arrangement of the proteins on the precursor polypeptide is identical in all picornaviruses (Fig. 14.2). The polyprotein is subdivided into three regions and the proteins, which arise by proteolytic cleavage, are designated with capital letters according to the respective regions of the polyprotein. The amino-terminal region contains the precursor sequences of the viral capsid proteins (1A–1D); the non-structural proteins 2A–2C, which play an important role in the adaptation of the virus to the cellular metabolism, are located in the middle of the polyprotein. Moreover, enzymatically active and further non-structural proteins (3A–3D) are generated from the carboxy-terminal regions. Usually, 11 viral proteins are generated from the precursor polyprotein. In aphthoviruses, erboviruses, kobuviruses, teschoviruses and cardioviruses, a short leader protein (L protein) is found at the amino terminus, which in mengovirus comprises 67 amino acids. In FMD virus, the L protein encompasses 205 amino acids and possesses proteolytic activity. In such cases the polyprotein does not begin with the sequences of the structural proteins, but begins with the L protein. In most picornaviruses, an amino acid sequence harbouring proteolytic activity (2A protease) is located immediately downstream of the region for the structural proteins. The 2A protease develops its proteolytic activity cotranslationally. In enteroviruses and rhinoviruses the amino acid chain between the end of the capsid proteins and the beginning of the 2A protease undergoes autocatalytic cleavage. The amino-terminal region of the precursor protein is excised from the nascent, not yet completely translated carboxy-terminal moiety, generating the protomer, the precursor of structural proteins. In aphthoviruses, erboviruses and cardioviruses, the autocatalytic cleavage reaction exerted by the 2A protease occurs not upstream but immediately downstream of its own amino acid sequences, thus leading to a polypeptide product that contains both the protomer and the 2A protease. In hepatoviruses and parechoviruses, the 2A protein does not function as a protease during the early steps of the infection cycle; instead, it develops its proteolytic activity in a late phase during the formation of new capsids. In this case, the cleavage of the structural protein moiety is performed by the 3C protease at the transition site between domains 2A and 2B. The properties and functions of all proteins that arise by cleavage of the polyprotein are summarized in Table 14.3.

14.1.3.2 Structural Proteins

The protomer encompasses the amino acids of capsid proteins, which are arranged in the order 1A, 1B, 1C and 1D; they correspond to the structural

Fig. 14.3 (continued) (**b**) IRES region of the encephalomyocarditis virus. The computer analysis prediction of the energetically favoured secondary structure of the 5′ UTR of the viral genome is shown. The *numbers* refer to the corresponding nucleotide positions starting from the 5′ terminus of the genome. By interaction with various cellular proteins, the secondary structures stabilize a nucleotide sequence located directly upstream of the start codon used for translation of the polyprotein. This sequence can form a partial double-stranded region (indicated by *stars*) with sequences at the 3′ end of the 18S ribosomal RNA (marked in *red*) within the small (49S) ribosomal subunit. The illustrated sequence ends with the start codon (AUG) of the polyprotein

Table 14.3 Comparison of the properties of picornavirus proteins

Protein	Number of amino acid residues					Modification	Function
	Poliovirus	Coxsackievirus	Hepatitis A virus	Rhinovirus	Foot-and-mouth disease virus		
Leader					205		Protease, performs the autocatalytic cleavage of the protomer, induces the degradation of eIF-4G
VP4	69	69	23	69	81	Myristoylated (lacking in hepatitis A virus)	Structural protein, on the inner side of the particle, interacts with RNA
VP2	271	261	222	262	218		Structural protein
VP3	238	238	246	236	221		Structural protein
VP1	302	284	274	290	212		Structural protein
2A	149	147	71	145	16		Enteroviruses, rhinoviruses: protease, performs protomer cleavage before of the 2A domain of polyprotein
							Aphthoviruses, erboviruses, cardioviruses: protease, performs protomer cleavage after the 2A domain of polyprotein, mediates the degradation of eIF-4G
							Hepatoviruses, parechoviruses: no protease, active during morphogenesis

2B	97	99	215	97	154		Influences host specificity
2C	329	329	335	330	317	NTP-binding	ATPase, initiation of RNA synthesis, chaperone activity forming ribonucleoprotein complexes?, helicase activity?
3A	87	89	74	85	154		Hydrophobic domain for anchoring the 3AB precursor on the membrane, influences uridylylation of Vpg
3B	22	22	23	23	23/24	Uridylylated	Vpg, covalently linked to the 5′ terminus of the viral genome
3C	182	183	219	182	214		Protease, performs all cleavage reactions, except for VP0 to VP4 and VP2 and for the protomer
3D	461	462	489	460	470		RNA-dependent RNA polymerase

The protein succession order in the table corresponds to the real sequence order in the polyprotein
NTP nucleoside triphosphate

proteins VP4, VP2, VP3 and VP1, respectively. Cellular enzymes modify the protomer by attachment of a myristic acid residue to an amino-terminal glycine. For this purpose, the previous autocatalytic removal of the L protein is necessary in aphthoviruses, erboviruses, kobuviruses, teschoviruses, and cardioviruses. In the other picornaviruses, the amino-terminal methionine is removed by cleavage. Myristoylation remains unaffected during the subsequent cleavage reactions and is detectable also in VP4. An exception is observed with hepatitis A virus as no amino-terminal fatty acid modification of the protomer has been found so far.

The virus-encoded 3C protease is responsible for processing the capsid precursor protein into the single components VP0, VP3 and VP1. This enzyme is located in the carboxy-terminal region of the polyprotein and develops its activity relatively late. The protomer is already folded into the domains that correspond to the four capsid proteins before cleavage. Nevertheless, these proteins change their conformation once more by folding during processing, thus leading to the mature proteins that are found in infectious virus particles. The final cleavage of the VP0 moiety to form VP4 and VP2 occurs during viral maturation; the proteolytic activity required for this process resides in the carboxy-terminal domain of VP0 within the sequences of the VP2 domain. A serine residue at position 10 of VP2, which is conserved among picornaviruses and is located close to the VP4/VP2 cleavage site, seems to be involved in conferring the serine protease activity that executes the autocatalytic cleavage. However, an additional tight interaction between VP0 and the viral RNA genome in the immature virus particle is essential for the proteolytic activity. It is assumed that for successful proteolytic cleavage of VP0 a base (acting as a proton donor) of the genome must interact with the amino acid sequences. Therefore, this enzyme is activated within the genome-containing but still immature virus particles, but not until they have been released from infected cells. In hepatitis A virus, the proteolytic cleavage of structural proteins is performed by a different mechanism: in this case, a protomer is formed that also contains the 2A domain in addition to the capsid proteins. The protomer is cleaved by the 3C protease at the transition sites between the VP2/VP3 and the VP3/VP1 regions; however, it does not recognize the cleavage site between the VP1 and 2A domains. The resulting protein, VP1/2A (also known as pX), is cleaved by a not yet characterized cellular protease at a later step in the infection cycle during morphogenesis of new virus particles.

VP1, VP2 and VP3 shape the faces of the icosahedral infectious virus particle. X-ray structure analyses of different picornaviruses revealed that these proteins exhibit very similar folding patterns among each other as well as among the proteins of different virus types. They consist of eight antiparallel β-sheet structures, which are connected by amino acid loops and are arranged in such a way that a wedge-shaped protein structure can be formed (Fig. 14.1b). Since this protein structure has proven to be a universally valid folding pattern for capsid proteins of small RNA viruses with particles of icosahedral symmetry, it has been designated the RNA virus capsid (RVC) domain. The eight β-sheets form the lateral sides of the conserved, wedge-shaped protein structure. The joining protein loops exhibit

considerable variability concerning length and sequence, however without influencing the basic wedge-shaped structure. The variable loop regions contain epitopes that are recognized by virus-neutralizing antibodies, which are generated during viral infection. In contrast, the amino-terminal regions of capsid proteins are situated on the inner side of the particles. They intertwine with each other forming an interconnected network which is responsible for the stability of the particles. The fact that VP1, VP2 and VP4 possess very similar structures suggests that the genes encoding these proteins have evolved from a common ancestral precursor by duplications.

In 1985, when Michael Rossmann, James Hogle and their respective co-workers published the protein structure of the rhinovirus and poliovirus capsids, they revealed not only the above-mentioned common characteristics, but also further structural features that result from folding and reciprocal interactions between the components. They found a ditch-like groove or crevice of approximately 25 Å in depth on the surface of rhinovirus B (type 14) particles which surrounds the vertices of the icosahedron and arises from the structures and interactions between different amino acid residues of VP1, VP2 and VP3. This structure, which Rossmann denominated "canyon", has been found in all picornaviruses, excepting in hepatitis A virus and FMD virus. The amino acids that line the walls of the canyon with their functional side groups facilitate binding of the respective virus particles to their specific cellular receptors (Fig. 14.1a, c). Owing to their size, neutralizing antibodies are not able to penetrate into the canyon to impede binding of the virus to its specific cellular receptor. However, they can bind to epitopes on the upper surface of the particles located near the entrances to the canyons, thus indirectly impeding attachment of the virus by steric hindrance. Because of antibody binding, surface regions are exposed to a selection pressure that leads to a certain variability of the exposed amino acids, and to the development of differing capsid variants. On the other hand, the actual receptor binding site within the canyon does not underlie such a selection mechanism and remains unaffected, thus preserving the cell specificity of the corresponding virus type.

The structural analyses also revealed the existence of a small hole-like cavity beneath the canyon floor. The cavity (pocket) is accessible from the canyon floor by a pore and has an additional connection to the inside of the particle. It contains a sphingosine-like fatty acid molecule, the "pocket factor". It is removed from the pocket during structural rearrangements of capsid proteins which are triggered upon receptor binding. During this process, VP4 is lost as an inside-associated protein, leading to destabilization of the virus particle, which facilitates the release of the RNA genome into the cytoplasm through the vertices of the receptor-associated icosahedron. Knowledge of the predominantly hydrophobic amino acids that line the cavity facilitated the development of therapeutically active and optimally adapted drugs such as pleconaril (see ▶ Chap. 9) that stabilize virus particles and inhibit the release of the RNA genome. Therefore, the use of such compounds can prevent or at least restrict viral infections. Nevertheless, it also soon became apparent that viruses are rapidly able to develop resistances.

The emergence of different serotypes that retain cell specificity is particularly pronounced in rhinoviruses, which comprise more than 100 different stable serotypes, which are especially characterized by the fact that neutralizing antibodies against them are strongly type-specific and cannot bind to the cell surface of other serotypes. Therefore, after a cold, people are protected against further infections with the same rhinovirus serotype, but not against other serotypes, which can continuously lead to recurrent colds. Such a plethora of different serotypes does not exist among enteroviruses. Poliovirus has three serotypes, and hepatitis A virus has only one serotype. The high genetic stability of enteroviruses, parechoviruses and hepatoviruses is probably correlated with the strong acid resistance of their capsids, which are able to survive in the acid environment of the stomach. Indeed, only a few amino acid sequences are capable of conferring such high acid stability, which, however, strongly limits the variability.

14.1.3.3 Enzymes

Proteases

Picornaviruses possess proteases, which become active in different phases of the infection cycle. The 2A protease is a cysteine protease and is localized immediately contiguous to the structural protein regions (Fig. 14.2). In the case of aphthoviruses, the 2A protease is very small, comprising only 16 amino acid residues; however, in poliovirus and rhinoviruses it is notably larger and has 149 and 147 amino acids, respectively. It develops its activity early during the infection cycle, and cotranslationally cleaves the protomer sequences from the nascent polyprotein between a tyrosine residue and a glycine residue. The cleavage site is located either immediately in front of (enteroviruses and rhinoviruses) or after (aphthoviruses, erboviruses, teschoviruses and cardioviruses) its own enzyme sequences. In hepatitis A virus and parechoviruses, the 2A protein does not possess proteolytic activity; instead, it is involved in the process of viral capsid formation at the end phase of the replication cycle.

Besides its role in releasing the protomer from the nascent polyprotein, the 2A protease also catalyses proteolytic cleavage reactions in cellular proteins. Best known is the indirect degradation of the protein p220: in this case, the 2A protease cleaves a cellular factor, which thereby becomes active as a protease. This enzyme induces the degradation of the cellular translation initiation factor eIF-4G (p220). This is a component of the cap-binding complex, also known as the eIF-4F complex, which comprises the cap-binding protein and translation initiation factor eIF-4A. The cap-binding complex is generally involved in the initiation of translation of eukaryotic mRNAs, as it interacts with the cap structure at the 5′ terminus of the mRNA and mediates binding of the ribosomes. Cleavage of the eIF-4G factor by the viral 2A protease leads to destruction of the functional activity of the complex in translating cellular mRNAs, resulting in a breakdown of cellular metabolism. This process has been denominated virus-host shutoff. In aphthoviruses, cleavage of the eIF-4G factor is mediated not by the 2A protease, but by the proteolytic activity of the L protein (206 amino acids), which resides at the amino terminus of the polyprotein. The L protein is also activated early during the infection cycle, and induces its own

cleavage from the polyprotein, leading to the release of the protomer. This process has not been demonstrated in other picornaviruses so far.

In hepatoviruses and parechoviruses, the activity of the 3C protease, which recognizes the amino acid sequence glutamine–glycine, is responsible for all proteolytic reactions in the precursor protein which lead to the generation of the single components from the polyprotein; in enteroviruses, rhinoviruses, cardioviruses and aphthoviruses, the 3C protease is also responsible for all proteolytic reactions except the cleavage of the protomer, which is performed by the 2A protease. Exceptions are the processing of VP0 to VP4 and VP2 during viral maturation by the enzymatic activity of VP2 and the cleavage of VP1/2A by a cellular protease in hepatitis A virus. The 3C protease domain is located at the carboxy-terminal region of the polyprotein and comprises 182 amino acids in poliovirus and rhinoviruses, and 217 residues in hepatitis A virus (Table 14.3). In a first reaction step, an intramolecular amino-terminal cleavage reaction occurs in front of the 3C moiety. The resulting intermediate product, $3CD^{pro}$, exhibits proteolytic activity. The 3D moiety present in $3CD^{pro}$ is indeed essential for processing the protomer into capsid proteins, because an efficient cleavage between VP3 and VP1 is only possible when the 3D domain is still linked with the 3C protease (Fig. 14.2). The cleavage of $3CD^{pro}$ into 3C and 3D occurs intermolecularly (in *trans*), i.e. active intermediate 3CD products interact and perform a reciprocal cleavage reaction.

The 3C protease is also involved in the specific degradation of cellular components. Its direct or indirect proteolytic effects seem to impair the RNA polymerase III dependent cellular transcription by which transfer RNA (tRNA) species and other small RNA molecules are synthesized. The degradation reaction is directed against both transcription factor TFIIIC and the TATA-box-binding protein. Furthermore, the 3C protease of FMD virus is also able to induce the cleavage of histone H3. The cleavage sites of proteases 2A and 3C are defined not only by the two amino acid residues between which the cleavage occurs, but also by the neighbouring amino acids and by the folding of the precursor proteins into secondary and tertiary structures.

RNA-Dependent RNA Polymerase

During the replication cycle of picornaviruses, the positive-sense RNA genome is transcribed into an intermediate, negative-sense RNA molecule, which, in turn, serves as a template for the synthesis of positive-sense progeny RNA genomes (for details, see Sect. 14.1.4). The synthesis of an RNA molecule by using an RNA template is a process that does not exist in cellular nucleic acid synthesis; hence, cells do not possess enzymes that can perform such (synthesis) reactions. For this reason, it is essential for picornaviruses to possess the genetic information encoding their own RNA-dependent RNA polymerase. This enzyme is located at the carboxy terminus of the polyprotein and is represented by the 3D region on the precursor molecule; the intermediate product $3CD^{pro}$, which is generated by proteolytic cleavage, possesses protease activity but no polymerase activity. The length of the 3D polymerase ($3D^{pol}$) ranges between 460 amino acids in rhinoviruses and

491 amino acids in hepatitis A virus. The error rate of RNA-dependent RNA polymerases is relatively high, as has been demonstrated for $3D^{pol}$ of poliovirus. On average, it causes one error per 2,200 polymerized nucleotides during synthesis of RNA chains. This implies that every newly synthesized RNA strand contains approximately four mutations.

14.1.3.4 Other Proteins

In addition to the structural proteins and enzymes already mentioned, further protein components are located within the sequence of the polyprotein, and are generated by the activity of the 3C protease during the infection cycle (Fig. 14.2). The very small 3B component was mentioned already; it constitutes Vpg, which is covalently linked to the 5′ terminus of the viral genome. The Vpg sequence is repeated three times in the polyprotein of aphthoviruses. The direct precursor of Vpg is 3AB. It is generally accepted that 3AB is anchored to intracellular membranes (e.g. endoplasmic reticulum, ER, membrane) by a hydrophobic amino acid sequence present in the 3A moiety. The tyrosine residue at position 3 of the 3B domain becomes uridylylated and functions as a primer for initiation of RNA synthesis during genome replication. Directly following uridylylation, the 3C protease cleaves the precursor at the cleavage site between 3A and 3B, leading to the release of Vpg from membrane anchoring. Mutations within the hydrophobic domain of the 3A protein inhibit uridylylation and RNA synthesis.

The 2C and 2BC proteins are involved in RNA replication. They are responsible for the formation of rosette-like membrane structures that are derived from the ER. The synthesis of new viral RNA occurs in the cytoplasm on the surface of such vesicles. In addition, there are partially contradictory reports in regard to their activities. The 2B protein comprises 97 amino acids in poliovirus and rhinoviruses, 154 residues in FMD virus and 215 residues in hepatitis A virus. It seems to be related to host specificity of the different viruses. It exerts its effect in *cis*, i.e. possible defects cannot be complemented by the concurrent introduction of wild-type proteins. Human rhinoviruses carrying mutations within the 2B-encoding region of the genome are also able to proliferate in murine cells. There are data indicating that mutated 2B proteins can interact with certain murine-cell-specific factors, thereby facilitating RNA replication in cells that are non-permissive for wild-type viruses. The 2B proteins of hepatitis A virus interfere with the antiviral responses mediated by retinoic acid inducible gene (RIG) I by inhibiting phosphorylation, and consequently activation of interferon regulatory factor 3 (IRF-3), thus preventing transcription of the interferon-β (IFN-β) gene (see ► Chap. 8). On the other hand, the activity of the 2B or 2BC proteins of enteroviruses and FMD virus inhibits cellular protein transport from the ER to the cell surface via Golgi vesicles. This effect causes a reduction in the concentration of MHC class I antigens on the surface of virus-infected cells, enabling them to evade the cellular immune response.

The 2C protein (length of 317 amino acids in aphthoviruses, 330 amino acids in rhinoviruses, 335 amino acids in hepatitis A virus) possesses an NTP-binding

site and ATPase activity. It appears to be involved in initiation of RNA synthesis. In poliovirus and FMD virus, mutations in the 2C gene lead to resistance against guanidine hydrochloride and benzimidazole derivatives, two effective inhibitors of viral RNA synthesis. The amino acids phenylalanine and asparagine at positions 164 and 179 (as well as their corresponding substitutions with tyrosine and glycine) seem to be important for the acquirement of resistance. Mutations within the NTP-binding site are lethal for the viruses. Furthermore, there is evidence that $2C^{ATPase}$ may act as a chaperone in the formation of ribonucleoprotein complexes, which must be formed during the replication of the viral genomes. In this connection, the 2C protein might promote the coordinated interaction of the cloverleaf structure at the 5′ terminus, with the 3′ UTR sequences, the poly(A) tail, the *cis*-responsive element and the several proteins involved in this complex.

Functions of the Protein CD155

CD155 is expressed in many cells of various tissues. The membrane-anchored variants CD155α and CD155δ are associated with proteins of the extracellular matrix, e.g. vitronectin and nectin-3. In addition, CD155α and CD155δ are associated with α_v integrins in the cytoplasmic membrane. These properties characterize CD155 as a cell–cell and cell–matrix adhesion molecule, which is linked with the cadherin adhesion system. The cytoplasmic domain of CD155α and CD155δ is associated with the protein Tctex-1, which is a subunit of the dynein motor protein complex. This protein is synthesized in all neurons, including the motor neurons of the spine. Possibly, this interaction may be important for the retrograde transport of polioviruses into the neurons, and thereby for the pathogenesis of poliomyelitis.

14.1.4 Replication

In the first step of the infection process, picornaviruses attach to specific cellular membrane proteins. On the virus side, the structures and amino acids of the canyon are the prevailing factors that mediate receptor binding. The cellular receptors of several viruses have been identified and are also molecularly well characterized; for attachment, all picornaviruses seem to use cell surface proteins that are members of the immunoglobulin superfamily (Table 14.4). In some cases, coreceptors have been identified that additionally support binding, or interact with the actual receptors.

Polioviruses bind to the glycosylated surface protein CD155, which belongs to the immunoglobulin superfamily. It possesses three immunoglobulin-like domains: an amino-terminal, variable V region, followed by two conserved C2 domains. There are four variants of CD155 that are generated by alternative splicing: CD155α and CD155δ are membrane-anchored forms and serve as receptors for polioviruses; CD155β and CD155γ are soluble isoforms. The three serotypes of poliovirus compete for the same attachment site, which is localized in the V domain

Table 14.4 Cellular receptors of various picornaviruses

Genus	Virus	Receptor	Receptor family
Enterovirus	Poliovirus	**CD155**	Ig superfamily
	Coxsackieviruses A13, 18	**CAR**	Ig superfamily
		ICAM-1	Ig superfamily
	Coxsackievirus A9	**CAR**	Ig superfamily
		Integrin $\alpha_v\beta_3$	Ig superfamily
	Coxsackieviruses A2, B1, B3; B5	**CAR**	Ig superfamily
		CD55 (DAF)	Ig superfamily
	Echovirus (types 1 and 8), Parechovirus (type 1)	**Integrin $\alpha_3\beta_1$**	Ig superfamily
	Echovirus (types 3, 6, 7, 11–13, 21, 24, 25, 29, 30, 33), Enterovirus 70	**CD55 (DAF)**	Ig superfamily
Rhinovirus	Rhinovirus (major group)	**ICAM-1**	Ig superfamily
	Rhinovirus (minor group)	**(V)LDL-receptor**	–
	Rhinoviruses A54, A89	Heparan sulphate	Heparan proteoglycan
Hepatovirus	Hepatitis A virus	**TIM-1, TIM-3**	Ig superfamily
Aphthovirus	Foot-and-mouth disease virus	**Integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$**	Ig superfamily
		Heparan sulphate	Heparan proteoglycan
Cardiovirus	Theiler's encephalomyelitis virus	**Unknown**	Sialoglycoprotein
	Subtype GDVII (neurovirulence)	Heparan sulphate	Heparan proteoglycan
	Subtype DA (low neurovirulence)	$\alpha_{2,3}$-Linked sialic acid	24 kDa Glycoprotein

The respective main receptors are highlighted in *bold*, and beneath them are the corresponding coreceptors
CAR coxsackievirus and adenovirus receptor, *ICAM-1* intercellular adhesion molecule 1, *DAF* decay-accelerating factor, *Ig* immunoglobulin *TIM* T-cell immunoglobulin- and mucin-domain-containing molecule, *VLDL* very low density lipoprotein

of the receptor. The neighbouring C2 region seems to stabilize the appropriate structures needed to facilitate receptor binding.

More than 90 % of rhinoviruses (major group) use intercellular adhesion molecule (ICAM) 1 proteins (CD54) as receptors; these glycosylated cell surface proteins also belong to the immunoglobulin superfamily and are responsible for intracellular signal transduction during inflammatory processes (Fig. 14.1c). The remaining rhinoviruses (minor group) bind to the very low density lipoprotein (LDL) receptor; however, for some viruses (human rhinoviruses 54 and 89) heparan sulphate seems to be sufficient for binding to the cell surface. In the case of hepatitis A virus, the proteins TIM-1 and TIM-3 have been identified as cellular interaction partners. The family of T-cell immunoglobulin- and mucine-domain-containing proteins (TIM proteins) represent a group of receptor proteins that also belong to

the immunoglobulin superfamily and are present in differing quantities on T_H1 and T_H2 cells and seem to regulate both the humoral and the cellular immune response.

Coxsackieviruses bind to the coxsackievirus and adenovirus receptor (CAR), also a member of the immunoglobulin superfamily, which is anchored in the cytoplasmic membrane by a hydrophobic domain in the carboxy-terminal region and exhibits two immunoglobulin-like domains. This cell surface protein, which is also used as a receptor by adenoviruses (▶ Sect. 19.4), is structurally and functionally similar to adhesion molecules. The amino-terminal domain of the CAR protein fits into the canyon on coxsackievirus capsids. Apart from the CAR protein, there are further cell surface proteins that can function as coreceptors for the various coxsackievirus types: some coxsackieviruses bind to ICAM-1 (coxsackieviruses A13, A18 and A21); coxsackievirus A9 interacts, like echovirus 9, with the vitronectin receptor, a protein that has been classified in the group of integrins ($\alpha_v\beta_3$) within the immunoglobulin superfamily. Furthermore, echoviruses 1 and 5 as well as parechovirus 1 bind to integrin $\alpha_2\beta_1$, which is also known as VLA-2. Other enteroviruses and echoviruses as well as Coxsackie B viruses use the surface protein CD55 (decay accelerating factor) as an additional interaction partner. This member of the immunoglobulin superfamily is responsible for protecting cells from lysis by the complement system. All these cell membrane proteins are expressed in different phases of leucocyte differentiation, and are involved in adhesion and recognition processes between different cell types. Another additional factor involved in the interaction between the host cell and coxsackievirus B3 is a protein with homology to nucleolin. This protein (110 kDa) is involved in the transport of ribosomal proteins from the nucleus into the cytoplasm, and is possibly also involved in attachment and penetration of adeno-associated adenovirus 2 (▶ Sect. 20.1).

However, the specific recognition of cell surface proteins is not the only feature that can account for the cell specificity of the different picornaviruses. Intracellular factors are also crucial for successful viral replication. This has been demonstrated especially in polioviruses. CD155 proteins have been found on a plethora of different cells to which polioviruses attach; however, the viral replication cycle can proceed only in a few cell types.

After attachment, structural rearrangements occur in the viruses that are bound to the cell surface. Such rearrangements are especially well studied in rhinoviruses that bind to ICAM-1 (Fig. 14.1c). A putative amphipathic α-helix within the amino-terminal region of VP1 is exposed, and interacts with lipid components in the cytoplasmic membrane. This results in a conformational change in the canyon, which leads to a tighter binding to the D1 domain of ICAM-1. VP4, which is located on the inner surface of the virions and is tightly associated with the viral genome, is released from the capsids. Additionally, the sphingosine-like molecule, the pocket factor, is also displaced during this process. The viral genome is translocated into the cytoplasm through a cylindrical orifice which is formed at the contact sites between the vertices of the icosahedron and the cell membrane. Alternatively, there are data suggesting that viral entry can also occur via endocytosis. The vesicles that are formed during this process carry abundant quantities of clathrin, a cell

membrane protein that accumulates in high concentrations at viral attachment sites. For the progression of the replication cycle, it is necessary that the viruses are released from the vesicles. An ATP-dependent proton pump located in the endocytic membrane is responsible for acidification of the interior of endocytic vesicles. This process leads to a rearrangement of the capsid in a way similar to that described above. In the next step, VP4 proteins and clathrin are released. As a result, small pores are formed at the junctions between VP1 proteins in the remodelled capsids and the vesicle membrane, through which the viral RNA is released into the cytoplasm.

Since the viral genome is a positive-sense RNA molecule that acts as mRNA, the next step consists of protein translation and synthesis of the polyprotein. The RNA does not possess the cap structure usually found at the 5′ terminus of cellular mRNAs and which is necessary for the correct binding of ribosomes to the translation initiation site. Contrarily, the viral genome contains an, in comparison with cellular mRNA species, unusual long non-coding sequence (IRES) that is located upstream of the start codon and can comprise up to 1,199 nucleotides. It possesses a strongly pronounced, stable secondary structure with intramolecular double-stranded sequence regions (Fig. 14.3). The IRES facilitates recognition of the picornavirus genome as mRNA by the ribosomal subunits, facilitating their interaction with the start codon to initiate the first steps of the translation. It is assumed that the secondary structure exerts a stabilizing effect on a nucleotide sequence located immediately upstream from the start of the translation and which is complementary to the 18S ribosomal RNA of the small ribosomal subunit. Therefore, the ribosomal RNA can hybridize with the viral genome, guiding the ribosomes to the correct start site for protein synthesis. Cellular proteins are functionally involved in initiating translation. They also bind to the IRES region, influencing thereby the cell and host specificity of the different picornaviruses. One such protein is the La protein (p52), which usually binds to the 3′ termini of transcripts, especially to tRNAs that are synthesized by RNA polymerase III, and regulates their termination. Interestingly, the La protein induces an autoimmune reaction in patients with lupus erythematosus and Sjögren syndrome. Additional IRES-binding components include polypyrimidine tract binding protein, poly (rC)-binding protein 2, poly(A)-binding protein and the Unr factor (upstream of N-ras). In addition to these proteins, also the translation initiation factor eIF-2 and other not yet characterized proteins have been identified as interaction partner of the IRES.

Following the initial translation steps, the whole polyprotein is uniformly synthesized. During translation, it is folded into the individual protein domains. In most picornaviruses (exceptions are hepatitis A virus and parechoviruses), the first proteolytic cleavage reactions occur cotranslationally during the synthesis of the polyprotein, namely immediately after translation of the sequences encoding the 2A protease. This enzyme operates autocatalytically, leading to cleavage of the protomer region at the amino terminus. The 3C and 3CD proteases are involved in processing the other viral components; owing to their carboxy-terminal location within the polyprotein, they are synthesized later. In hepatitis A virus and

parechoviruses, the protomer remains linked with the 2A domain. Owing to proteolytic cleavage, RNA-dependent RNA polymerase ($3D^{pol}$) and Vpg are available as a prerequisite for the replication of the viral genome in the infected cell. In vitro, the synthesis of the polyprotein takes approximately 15 min.

The formation of a primer molecule, Vpg-pUpU-OH, is necessary for replication of the viral genome, which leads to the generation of a complementary negative-sense RNA molecule as an intermediate product. Mediated by the hydrophobic domain of the 3AB protein, the direct precursor of Vpg, primer synthesis occurs in association with intracellular membrane compartments and is catalysed by $3D^{pol}$. The template for the uridylylation reaction is a conserved sequence motif (AAACA), which is located in the loop of a hairpin structure within the *cis*-responsive element. This *cis*-responsive element is situated in different regions of the genome among the various members of the picornaviruses: in enteroviruses, it is located within the 2C-encoding region (poliovirus 1: nucleotides 4,444–4,505); in rhinovirus A, it resides within the coding sequences for the 2A domain; in parechoviruses and rhinovirus B (rhinovirus 14), it is situated within the VP1-encoding sequences, whereas in rhinovirus C it is found within the sequences encoding VP2. In FMD virus, the *cis*-responsive element is located within the 5′ UTR upstream of the IRES sequences, whereas in hepatitis A virus, it is situated within the 3C-encoding region. Mutations in the *cis*-responsive element lead to a replication failure, which results in an inhibition of negative-strand synthesis owing to the absence of uridylylation. The Vpg-pUpU primer associates with the poly(A) tail at the 3′ terminus of the viral genome and forms the initiation structure with a free 3′-OH end as a recognition site for the RNA-dependent RNA polymerase ($3D^{pol}$), which synthesizes the complementary strand (Fig. 14.4). The initiation of negative-sense RNA synthesis is, however, also dependent on the presence of an intact cloverleaf secondary structure at the 5′ terminus of positive strands. This suggests that the positive-sense RNA genomes form a circular structure, at which the negative-strand synthesis is initiated. The circularization is mediated by the interaction of the terminal-binding proteins poly(A)-binding protein and poly(rC)-binding protein 2 as well as by the viral proteins $2C^{ATPase}$, $3CD^{pro}$ and $3C^{pol}$, which bind to the 5′ and 3′ ends of the RNA genome. A transitory intermediate double-stranded RNA molecule is formed during the RNA polymerization. The 3′ end of the newly synthesized negative strand contains two adenosine residues. These constitute a perfect structure for the complementary interaction with Vpg-pUpU primers, and initiate the synthesis of new positive-sense RNAs. The synthesis of a complete RNA molecule takes about 45 s. Besides $3D^{pol}$ and Vpg-pUpU primers, a cellular protein (67 kDa) is essential for the correct course of this process. Between five and eight positive-sense RNA strands can be synthesized from a single intermediate negative-sense RNA molecule. Only 5–10 % of all viral RNA molecules are negative-sense RNA. New viral polyproteins are translated from the newly synthesized positive-sense RNA strands. In this way, the amount of viral components can rapidly multiply within infected cells. The entire process of polyprotein synthesis and genome replication occurs in the cytoplasm in association with intracellular membrane compartments. The polyproteins become embedded in

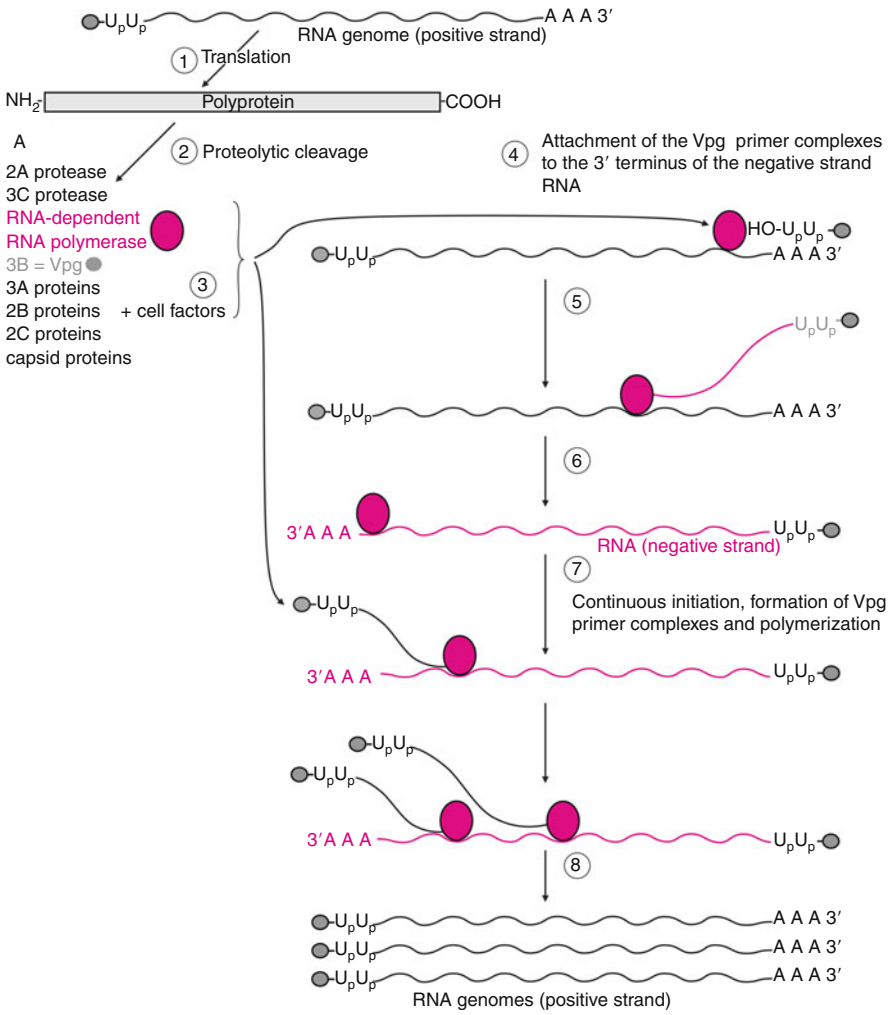


Fig. 14.4 Genome replication of picornaviruses. In a first step, the RNA genome is translated (*1*), leading to the synthesis of a polyprotein, which is processed into the individual protein components by the activity of the proteases 2A and 3C/3CD (*2*). The protein Vpg, which is subsequently uridylylated, arises during this process (*3*). Cellular proteins are also needed for this process. The Vpg-pUpU complex attaches to the 3′ terminus of the RNA genome (*4*), acting as a primer for the synthesis of the negative-sense RNA strand by the viral RNA-dependent RNA polymerase (*5*), which is also generated by proteolytic processing of the polyprotein. New Vpg-pUpU complexes attach to the 3′ terminus of the newly synthesized negative-sense RNA molecules (*6*). They function as primers for the synthesis of more RNA molecules (*7*), which now possess positive-sense polarity (*8*) and serve both as messenger RNA (mRNA) for further protein synthesis and as viral genomes

such compartments by their amino-terminal myristoylation and the hydrophobic 3AB domain.

The cellular metabolism is strongly influenced by viral replication. The large number of newly synthesized viral genomes that are present in the cell shortly after infection and the high affinity of their IRES elements for cellular ribosomes result in the cellular translation machinery being involved exclusively in the synthesis of viral proteins. In addition, the 2A protease of poliovirus and rhinoviruses induces the degradation of p220 (eIF-4G), a member of the cap-binding complex that is essential for initiation of translation of eukaryotic mRNA molecules. This is a second mechanism for inhibition of cellular protein synthesis. The poliovirus 2B and 3A proteins influence both the transport and the secretion of cellular glycoproteins. Furthermore, as described above, proteolytic degradation of TFIIIC by $3C^{pro}$ inhibits RNA polymerase III dependent transcription. Finally, even mRNA synthesis is blocked. In this case, a still unknown viral protein apparently affects the activity of RNA polymerase II. These processes are responsible for virus-host shutoff, i.e. the virus-mediated deactivation of the cellular metabolism.

As soon as sufficient amounts of viral proteins and RNA genomes are produced, the components assemble into infectious virions (self-assembly). In a first step, the myristoylated protomer, which is attached to membrane compartments, is cleaved by the activity of $3C^{pro}$ to form VP0, VP1 and VP3. These proteins remain initially bound as a complex and associate with four additional VP0/VP1/VP3 aggregates to form pentamers, the precursor structures of the vertices of the icosahedron. In hepatitis A virus, the 2A protein domain does not possess proteolytic activity and remains linked to the protomer, forming the protein pX. In this case, the 2A domain facilitates the formation of pentamers and induces the process of morphogenesis. In a further step, 12 pentameric complexes assemble and form capsid precursors. These protein shells surround the RNA genome. However, is not completely understood how the RNA genome arrives at the interior of the capsids. Sequences of the IRES region are also important for this process: they enhance the encapsulation of the RNA genome. Furthermore, considerable rearrangements of the capsid protein structure are associated with this process, which is expressed in an altered sedimentation behaviour of the particles. It is conceivable that the RNA genome reaches the interior of the so-called procapsids through an orifice, or by preliminarily binding to the outside of the particle precursors with subsequent translocation into the interior by an ample refolding process. The last step in virion formation involves the activation of the protease within the VP2 domain of VP0, which is induced by interaction between VP0 and the viral RNA. Once activated, this protease performs the maturation cleavage that leads to the mature capsid proteins VP2 and VP4. In hepatitis A virus, a still unknown cellular protease catalyses the cleavage of pX to form VP1 and 2A in this late step. The final release of the virus is triggered by alteration of membrane permeability due to infection-induced cell death. The complete replication cycle, beginning from viral attachment to the release of progeny viruses, takes approximately 8 h in poliovirus.

The intracellular processes during viral replication and apoptosis lead to morphologic alterations of the cell structure, which can be microscopically observed as a cytopathic effect: the chromatin structure is disintegrated and nucleic acid/protein complexes accumulate at the inner side of the nuclear membrane. The cytoskeleton also undergoes alterations by structural rearrangements in proteins that are associated with the microtubules. The cells take on a rounded morphology. Numerous vesicles are formed in the cytoplasm during viral replication. Finally, the cell membranes change their permeability and become more permeable because of the incorporation of increased amounts of phosphocholine.

Translation Initiation by IRES Sequences

So far, IRES sequences have been identified in the genomes of picornaviruses and pestiviruses and hepatitis C virus, which belong to the family *Flaviviridae* (Sect. 14.5). This sequence motif facilitates the translation of eukaryotic mRNA independently of both the 5′-cap structure and the cap-binding complex. Nevertheless, also a cellular gene was found to contain an IRES within the 5′ UTR of its mRNA: it encodes the binding immunoglobulin protein (BiP; immunoglobulin heavy chain binding protein; an alternative designation is 78-kDa glucose-regulated protein, GRP-78). This protein is a protein-folding enzyme (chaperone) which is active in the ER and in the Golgi apparatus. It is involved in the interaction between light and heavy chains of immunoglobulins that leads to functional antibodies.

Function of the 5′-Cap Structure

In eukaryotic protein translation, the cap-binding complex attaches to the cap region, a methylated guanosine nucleotide that is linked to the 5′ terminus of the messenger RNA via a 5′–5′ ester bond. The cap-binding complex is composed of the cap-binding protein, which interacts with the 5′ terminus, factor eIF-3 and proteins eI-4A and eI-4B, which bind to the region located up to 100 nucleotides directly upstream of the start codon, maintaining it in a single-stranded extended configuration. The small ribosomal subunit along with $tRNA^{Met}$, initiation factor eIF-2 and GTP interact with this 5′ terminally associated complex to initiate protein translation. The genome of picornaviruses functions as mRNA, but does not possess a 5′-cap structure. Therefore, it cannot perform the necessary interaction with the components of the cap-binding complex to interact with the ribosomes.

14.1.5 Human Pathogenic Picornaviruses

Some members of the picornaviruses account for various severe diseases in humans. These include, in particular, poliomyelitis and hepatitis A. However, cases with

severe clinical courses are increasingly found in connection with infections with other human enteroviruses.

14.1.5.1 Poliovirus

Epidemiology and Transmission

Poliomyelitis (infantile paralysis) was known as long ago as 1,500 BC. Major epidemics appeared especially when there were large numbers of susceptible individuals within a population, e.g. in the years between 1940 and 1950. Many of the infected children died, whereas in other cases the consequences were lifelong paralysis symptoms. Some children survived the poliovirus infection only by temporary placement in an iron lung, which allowed breathing for a limited period of time. Franklin D. Roosevelt, President of the USA from 1933 to 1945, was infected with poliovirus as an adult, and had lifelong muscular dysfunctions.

Originally, the three different types of poliovirus were distributed worldwide. They exhibit differences especially in the amino acid sequences of the variable loop regions of VP1, VP2 and VP3 and can be distinguished serologically: type 1 (Mahoney, also known as Brunhilde) has a relatively high incidence and causes severe diseases, whereas type 2 (Lansing) induced rather mild illnesses; infections with poliovirus type 3 (Leon) also have a severe clinical course. More than 150,000 cases of poliomyelitis were registered in the 1992. By virtue of the immunization programme of the World Health Organization (WHO), poliomyelitis is considered as eradicated, except for in about ten nations: as a result, Europe was declared polio-free by the WHO in June 2002. Nevertheless, serious recurrences have temporarily appeared owing to transregional dissemination: in the nations concerned (e.g. Nigeria), the immunization programme was stopped for political reasons. In 2008, infections with polioviruses 1 and 3 were reported only in a few developing countries, 98 % of them in Nigeria, India and Pakistan. Nonetheless, starting from such countries, poliovirus infections have also been imported to some regions which had already been declared as polio-free. However, the WHO has achieved successful control of such disseminations by implementing massive outbreak-control immunization measures. For this reason, acute poliovirus infections were not reported in India in 2011.

Under natural conditions, polioviruses infect only humans; however, they can be transmitted to various monkey species, and can cause epidemics in chimpanzee populations, as described by Jane Goodall. Further, poliovirus 2 could be adapted to mice as well. Polioviruses preferentially proliferate in the lymphatic tissue of the intestine, the Peyer plaques, and are excreted in the faeces of infected individuals for about 5 weeks. In the initial stage of the disease, an infection of the pharyngeal mucosa and the tonsils can frequently be observed, so the virus is present in the sputum and in the pharyngeal lavage. Polioviruses are transmitted by aerosols, via the faecal–oral route, via contact with dirt or smears and by contaminated drinking water.

Infantile Paralysis: It Became an Adult Paralysis

During past centuries, poliovirus infections were widespread and occurred mostly during the first 6 months of life under the protection of the maternal IgG antibodies, which are transferred through the placenta. Children did not become ill if they were infected in this early stage of life, but they developed an active immune protection. Later contacts with the poliovirus led to diseases only in children who had not experienced such an infection in that early period of life, because of the now lacking maternal immune protection. All in all, less than 1 % of all seronegative children became ill with poliomyelitis after contact with the virus. These data indicate that poliovirus is not highly neuroinvasive. Indeed, non-paralytic poliovirus infections were the most frequent reason for summer flu. With the increase of living standards, the first contact with poliovirus has been postponed to an advanced age; thus, the infantile paralysis became an adult paralysis.

Clinical Features

The vast majority of poliovirus infections are asymptomatic. After an incubation period of 1–2 weeks, only a few cases of poliovirus infection lead to stomach and intestinal disorders, followed by fever and flu-like symptoms, which typically resemble summer influenza. Most patients recover completely from this type of poliovirus infection (abortive polio). Approximately 1–2 % of patients subsequently develop a non-paralytic poliovirus infection; in such cases the virus infects the central nervous system, causing aseptic meningitis accompanied by muscle cramps and back pain, which may persist for about 2–10 days. In up to 2 % of patients, this illness is subsequently accompanied by symptoms of flaccid paralysis because the anterior horn cells are damaged (paralytic poliovirus infection). Approximately 10 % of these patients have a lethal outcome, e.g. due to paralysis of the respiratory muscles; about a further 10 % recover without lasting health impairments; however, in around 80 % of cases, paralyses of different magnitudes remain as permanent injury.

In recent years, the phenomenon of the so-called post-polio syndrome has been observed. The symptoms emerge 15–40 years after the occurrence of the original polio disease. It is a recurrent progressive myasthenia in association with muscle pain, joint aches and severe fatigue. It is believed that originally hardly damaged or also undamaged neural cells die owing to permanent strain, thus leading to the syndrome; many cases of such late complications are expected in the USA.

Furthermore, there were cases of vaccine-induced polio after polio immunizations with the live oral vaccine (Sabin). The attenuated vaccine virus is transiently excreted with the faeces of vaccinated patients, and can be transmitted to other members of the family; in such cases, immunosuppressed patients are particularly endangered.

Pathogenesis

After being transmitted, polioviruses infect the lymphatic tissue of the gastrointestinal tract and proliferate in the lymphatic tissue of the nasopharyngeal region. They are transported through the stomach into the intestine, where they preferentially infect the Peyer plaques, which represent the lymphatic tissue of the small intestine, and the mesenteric lymph nodes. The viruses are released directly into the bloodstream or the intestine through the lymphatic vessels. The virus infects activated monocytes expressing CD155 (the poliovirus receptor), and proliferates within them. The consequence is a mild viraemia, which elicits an initial fever attack (preliminary disease, abortive polio). Subsequently, the viruses spread throughout the organism and proliferate in the reticulohistiocytic system as well as in endothelial cells; this process is associated with a second viraemia, in which neurons can be infected, leading to a retrograde progression of the infection penetrating further into the spinal cord and brain. Especially, the large anterior horn cells and the motor neurons are infected, resulting in damage to and destruction of these cells. Poliomyelitis arises by infection of the cell-rich grey matter of the brain (*polios* is Greek for "grey"). Muscle cells and brain regions that were formerly innervated by the infected nerves are no longer innervated. The consequences are flaccid paralyses. If they are lasting, then the musculature atrophies because of inactivity. Subsequently, destroyed neurons are removed by macrophages (neuronophagy). The motor neurons remain atrophic: even years after the acute disease, generation of new glial cells (gliosis) as well as mild inflammations can be observed. Patients with paralysis symptoms of the intercostal musculature can survive the acute phase only with the aid of an iron lung. Extirpation of the tonsils, which is usually performed at the time of epidemic poliomyelitis, and irritations of the musculature (by movement, injections, etc.) frequently lead to respiratory paralysis; in such cases, the virus reaches the medulla oblongata along the damaged nerve fibres, destroying there the respective motor neurons.

Immune Response and Diagnosis

In the course of a poliovirus infection, IgM, IgA and IgG antibodies are produced against both capsid and non-structural proteins. Cross-reactivity between the three poliovirus types can only be observed by using heat-denaturated viruses. Reinfections lead to a new increase of antibody concentrations. Immunoglobulins against specific epitopes of structural proteins are neutralizing, and are measured in three discretely performed neutralization tests (polioviruses 1–3). Polioviruses can easily be cultivated in cell cultures (e.g. in primary monkey kidney cells, HeLa cells or Vero cells). The typing of isolates from faeces, pharyngeal lavage or cerebrospinal fluid is performed by neutralization tests using type-specific sera. This is the only way to reliably determine prevalence rates or increasing titres. In appropriate cases, the polymerase chain reaction (PCR) can alternatively be applied for viral genome detection and for identification of mutants. For such purposes, a pan-enterovirus PCR analysis is performed as a preliminary screening test and facilitates the identification of all human enteroviruses.

Therapy and Prophylaxis

The poliovirus vaccine (oral polio vaccine) that was developed by Albert Sabin is an attenuated live vaccine. Only 0.4–1.0 cases of reactions per million vaccinations have been observed. This vaccine induces the generation of virus-neutralizing IgG and IgA antibodies. Its efficiency has been demonstrated, for example, in the control of ongoing polio epidemics (outbreak-control immunization). It is hoped that poliovirus infections can be eradicated in the coming years by the consequent and worldwide application of this vaccine, particularly in developing countries. However, because of the possibility that polio infection symptoms can appear (vaccination-induced polio), this live vaccine must not be used in people with humoral immunodeficiencies (e.g. agammaglobulinaemia), or in patients who are immunocompromised owing to medicament usage, infections or for hereditary reasons. Since the vaccine virus is temporarily excreted in the faeces of vaccinated individuals, it can be transmitted to other members of the family; immunosuppressed individuals are particularly endangered.

The attenuated virus of the live vaccine against poliovirus 1 differs from the wild-type virus by 57 mutated nucleotides, which lead to 21 alterations in the amino acid sequence. In comparison with the wild type, the vaccine virus of poliovirus 3 exhibits ten mutations. However, the mutations that lead to the attenuation of poliovirus 2 are still unknown because the source virus for the vaccine strain has not been identified so far. Especially mutations in the nucleotide sequence of the IRES region seem to be responsible for the attenuation effect (positions 480, 481 and 472 in vaccine strains Sabin 1, 2 and 3, respectively). They influence the stability of the secondary structure of the IRES element, and consequently the association with the ribosomes, thereby affecting the effectiveness of translation. In addition, the attenuated vaccine strains exhibit mutations in genome sequences that encode for the capsid protein VP1. In this respect, vaccine polioviruses 1 and 3 exhibit 12 and two amino acid substitutions, respectively, which contribute to attenuation. In countries where poliovirus infections no longer occur, the live vaccine has not been used since 1999. The risk of acquiring a vaccine-induced poliomyelitis from the attenuated virus in such countries is higher than the risk of contracting the disease by a natural polio infection. A vaccine based on inactivated polioviruses was developed by Jonas Salk a few years before the live vaccine was developed. An improved variant of this vaccine with an enhanced antigen content is available and is now applied for basic immunizations. This inactivated vaccine is also able to induce the production of IgG antibodies. Booster vaccinations are recommended especially when travelling to developing countries (in Asia and Africa).

14.1.5.2 Human Enteroviruses and Parechoviruses

Epidemiology and Transmission

Today, human enteroviruses are subdivided into four species (A–D) and represent, together with the two serotypes of parechoviruses (originally known as echoviruses 22 and 23), a large number of infectious pathogens. Genetic recombination events between members of the same viral species are frequent and contribute to a broad range of virus variants. The viruses are distributed worldwide and their

epidemiology resembles that of polioviruses as they are primarily transmitted via the faecal–oral route and less frequently by aerosol or airborne particles. Infections with human enteroviruses occur principally in the warm months (summer flu), but in tropical regions they occur throughout the year. Seven percent of examined individuals excrete such viruses in the USA, but in tropical regions a level of up to 50 % has been reported. Therefore, recurrent epidemics are reported particularly in tropical countries of Southeast Asia, where outbreaks of enterovirus 71 are frequent. This virus type is considered among the so-called non-polio enteroviruses as the virus whose infections most frequently lead to diseases. Coxsackieviruses cause diseases only in humans; however, after experimental infections of chimpanzees, the viruses induce a clinically inapparent infection. The original classification of coxsackieviruses into subgroup A (serotypes 1–22, 24) and subgroup B (six serotypes) was based on differences concerning the histopathological lesions that they cause in experimentally infected newborn mice. Today the cultivation of coxsackieviruses is also possible in vitro (monkey kidney cells, human HeLa cells or human A549 lung carcinoma cells).

Clinical Features

The incubation period of human enterovirus infections lasts up to 2 weeks until the onset of the disease, and in rare cases more than 4 weeks. The viruses are excreted through the throat and the intestine for several weeks. The infections are characterized by a broad spectrum of different clinical manifestations. It is not possible to make unambiguous correlations between individual virus types and a particular clinical manifestation: different virus types can cause the same symptoms, whereas the same virus type can frequently be associated with many different symptoms. Patients are often simultaneously infected with several virus types; therefore, it is difficult to associate individual virus types with specific clinical pictures or symptoms. Human enteroviruses generally cause mild, cold-like illnesses, which can be associated with diarrhoea ("summer flu"); many infections are asymptomatic. Severe courses with neurological symptoms, meningitis, gastroenteritis, hand, foot and mouth disease, acute haemorrhagic conjunctivitis, myalgia, myocarditis, pleurodynia (Bornholm disease) or uveitis are only rarely observed. Patients with inherited or acquired immunological deficiencies frequently develop persistent infections, which can be associated with chronic enteritis, arthritis or meningoencephalitis. Meningitis is the predominant clinical picture in severe infections with human enteroviruses. In addition to herpesviruses, enteroviruses are considered the most frequent viral pathogens for meningitis and encephalitis. Coxsackie B viruses, particularly type B3, are believed to be the pathogen of viral myocarditis (perimyocarditis). Infantile myocarditis is frequently lethal in newborn babies. It occurs predominantly by infections acquired during delivery, when the mother has not produced antibodies against the coxsackievirus. Besides such acute forms, chronic disease types (dilatative cardiomyopathy) are also seen, especially in adults.

Some human enteroviruses (parechoviruses 1 and 2, coxsackievirus B4) are also discussed as a trigger of type 1 diabetes, which is often observed in patients with

a specific HLA type (HLA-DR and HLA-DQ) after infections with coxsackievirus B4. However, whether these infections have a causal connection to the autoimmune disorder is still controversial.

Pathogenesis

The uptake of human enteroviruses and their spread throughout the organism are similar to those for polioviruses. After initial proliferation in (the) intestinal and pharyngeal lymphatic tissues, the viruses are transported by the blood system as free virus particles or by infected lymphocytes during the second viraemic phase into the different target organs; these include the musculature, skin, meninges, myocardium, intestinal epithelia, central nervous system and respiratory system, where they settle, depending on the virus type. The molecular mechanisms that contribute to the development of the different symptoms are largely unknown. Besides hyperaemia, the following have been found in the conjunctiva of patients infected with enterovirus 70: dot-shaped bleedings (petechiae) and haemorrhages as well as infiltrations with mononuclear cells with diffuse distributed lymphocytes, which develop into striking large and swollen lymph follicles. In such cases, the cornea can be affected by epithelial opacity. Flaccid paralysis symptoms, which rarely occur during infections with enterovirus 70, are associated with degeneration of motor neurons, with haemorrhages and with neuroglial proliferation. The presence of viral proteins in microglia and neurons was demonstrated by immunofluorescence.

In the case of coxsackievirus infections, there is a primary infiltration of granulocytes, and this is followed by infiltration of mononuclear cells. The infected muscles exhibit focally necroses and the cells are disintegrated in a clod-like pattern. Necrotic areas can also be observed in neurons and glial cells of the central nervous system. After in vitro infection with coxsackievirus B3, monocytes release the cytokines TNF-α, IL-1β and IL-6. These are probably involved in the inflammation reaction. The chronic persistent myocarditis associated with coxsackievirus infections is characterized by significant lower quantities of infiltrating cells. Only single muscle cells disintegrate. Coxsackievirus RNA can be detected in heart muscle. Apparently, in this case the ratio between positive-sense and negative-sense RNA molecules is shifted to 2:1, whereas usually a larger excess of genomic RNA molecules is present in infected cells. In such cases, only a few viral genomes are synthesized and also the number of infectious coxsackieviruses is clearly lower in chronically infected regions. Type 1 diabetes mellitus, which is often diagnosed after infections with coxsackievirus B4, is probably triggered by autoimmune processes. There is evidence that similarities between viral proteins and cellular glutamate decarboxylase may be involved.

Immune Response and Diagnosis

During the course of the infection, virus-type-specific IgM, IgG and IgA antibodies are produced, and have, in part, neutralizing properties. The IgG-mediated immune reaction is possibly boosted by cross-reacting epitopes during reinfections with other virus types. In individual cases, a protective effect of IgA antibodies on the

gastrointestinal tract has been found. The IgM response plays only a minor role. Little is known about the influence of the cellular immune response on elimination of viruses; however, cytotoxic T cells seem to be responsible for the elimination of coxsackievirus B4 from the myocardium.

Human enterovirus infections are diagnosed by isolation of the virus and subsequent in vitro neutralization assays or by detection of viral RNA by PCR in faeces, urine, pharyngeal lavage, cerebrospinal fluid or eye secretions. Antibodies can be detected by virus-specific neutralization tests. Some viruses possess haemagglutination properties, which can be used for diagnosis. Because of the high prevalence, cross-reactions are frequent; therefore, ELISAs are only applicable for primary infections and often do not provide information about the virus type.

Therapy and Prophylaxis

There are neither vaccines nor therapeutic agents for prevention or treatment of human enteroviruses infections. Pleconaril, a drug that inhibits the uncoating process by hindering the release of the viral genomes, is also effective against enteroviruses. Although its efficacy against enteroviral meningitis and encephalitis has been demonstrated in clinical studies, it has not received marketing approval.

Ljungan Virus is Suspected of Causing Intrauterine Fetal Death

Human parechoviruses have a global distribution and cause, like human enteroviruses, disorders of the respiratory system and the gastrointestinal tract; severe diseases are only rarely observed. A species closely related to human parechoviruses is Ljungan virus, which is endemic in bank voles (*Clethrionomys glareolus*), especially in northern Sweden. These rodents appear in intervals of 3–4 years in large quantities and trek into barns and houses in autumn. The viruses are excreted in faeces and urine and can be transmitted to humans. Zoonotic transmissions to pregnant women are suspected of causing intrauterine fetal death in the late phase of gestation. In a Swedish study, viral genomes were detected in the placenta and in the brain of deceased fetuses in nearly 50 % of all cases examined.

14.1.5.3 Hepatitis A Virus

Epidemiology and Transmission

Hepatitis A virus was depicted by Stephen Feinstone by means of electron microscopy in 1973. It was independently isolated by Philip Provost and Gert Frösner and their respective co-workers in 1979. It has a worldwide distribution, but infections are mainly observed in tropical and subtropical regions as well as in developing countries. Infections generally occur during infancy. Six genotypes have been identified worldwide: genotypes I, II and III were isolated from infected patients, genotypes IV and VI were isolated from macaques (*Macaca fasicularis*) and genotype V was isolated from a vervet monkey (African green

monkey, *Cercopthecus aethiops*). Several antigen variants have been identified among the human genotypes, but there is only one serotype of hepatitis A virus. Whereas in central Europe and North America the seroprevalence among people older than 50 years was very high before the introduction of vaccination, acute infections with hepatitis A viruses are now relatively rare and appear in adults rather as travelling illnesses: approximately 1,000 cases are reported yearly in Germany. Hepatitis A viruses can be cultivated in primary and continuous kidney cell cultures of African green monkeys without a cytopathic effect; however, the viral reproduction cycle is very slow.

The virus is very stable against environmental influences and is excreted in large amounts in the faeces of infected individuals during the incubation period of 3–6 weeks. The virus is primarily transmitted via the faecal–oral route, by contact with dirt or smears as well as by contaminated food and drinking water. In rare cases, the infection can be transmitted by blood and saliva of ill people during viraemia. The virus is frequently transmitted among residents of asylums and retirement homes, among drug-addicted individuals, but also in kindergartens, vacation camps and in population groups with low socio-economic status.

Accumulation of Hepatitis A Virus in Mussels

The transmission of hepatitis A virus by contaminated mussels is known. In some regions of the world, untreated domestic sewage is released into rivers, lakes and seas. Since in such countries infections with hepatitis A viruses are frequent, this stable virus can reach the natural environment in this way. Mussels that grow in seas containing contaminated sewage near large towns are effectively able to filtrate the viruses from the surrounding water, thus leading to accumulation and concentration of the virus in the mussels. If such mussels are insufficiently heated during the cooking process, the virus is transmitted to the gastrointestinal tract after consumption of infected mussels.

Clinical Features

The incubation period until the appearance of the first symptoms lasts 3–6 weeks. The principal symptom of a hepatitis A virus infection is liver inflammation with jaundice (icterus), which arises by the transfer of bile salts (bilirubin) into the blood (hyperbilirubinaemia) and their elimination in the urine. The disease is, especially in children, usually inapparent, i.e. without symptoms. Jaundice symptoms begin suddenly, and are accompanied by nausea, fever and a general state of sickness that can continue for several weeks; particularly in older adults, occasionally there are also fulminant forms that can have a lethal outcome owing to cirrhosis and severe hepatic failure. The hepatitis A virus genome can be detected in the faeces by PCR even several weeks after remission of symptoms. In immunosuppressed patients, it is detectable for a considerably longer time. Persistent infections have not been observed.

Pathogenesis

Hepatitis A virus reaches the gastrointestinal tract generally by contaminated food. There is evidence that the virus infects the crypt cells of the small intestine, before it spreads via the blood system and reaches its principal target organ, the liver, and replicates there in hepatocytes. The proteins TIM-1 and TIM-3 have been identified as cellular receptors; an alternative entry pathway seems to exist for virus particles complexed with IgA molecules. These immunocomplexed viruses can bind to IgA receptors on the surface of hepatocytes. Replication of hepatitis A virus occurs in the liver 8–10 days before the onset of symptoms. The viruses spread to the intestine through the bile duct, and are excreted in the faeces. During this phase, viruses are also present in the blood, reaching concentrations of up to 10^5 virus particles per millilitre. The peak of virus release has already passed at the onset of the disease. Liver cells are destroyed by infection-induced mechanisms, viral proteins are found in the cytoplasm and even virus particles can be detected by electron microscopy. The very massive damage of liver cells leads to the release of bilirubin and transaminases into the blood. In contrast to other picornaviruses, in hepatitis A virus, functions responsible for the virus-host shutoff effect are not or are only weakly developed. Liver cells are not primarily destroyed by the viral infection. Instead, the cellular immune response is principally responsible for this process, as cytotoxic $CD8^+$ T cells have been detected in the liver of patients with acute hepatitis A. These cells release interferon-γ and give rise to the active immigration of other immunologically active cells into the liver. Infiltrating mononuclear cells are mostly located near the liver portal regions. Besides liver cells, also macrophages of the spleen and Kupffer's star cells (also known as stellate cells) contain viral proteins. They are derived from viruses. In a later infection stage, immigration of $CD4^+$ lymphocytes can be observed. In rare cases, a hepatitis A virus infection can cause a transient granulocytopenia and damage of bone marrow cells.

Initially, hepatitis A virus proliferates very slowly in infected patients; the incubation period lasts several weeks until the appearance of the first symptoms, which indicate liver cell destruction. Infected liver cells synthesize only marginal amounts of interferon-α (IFN-α) and IFN-β, with the consequence of a delayed activation of the host immune response. Phosphorylation and activation of IRF-3 is inhibited. IRF-3 activation is usually achieved by the signal transduction cascades induced either by activation of Toll-like receptor 3 (TLR3) or the RIG helicase by double-stranded RNA molecules (see ► Chaps. 7 and ► 8) which arise during the replication cycle of hepatitis A viruses. The viral non-structural protein 2B seems to block this process. Therefore, the synthesis of IFN-β is inhibited in infected cells, and the virus is able to proliferate largely uninfluenced by the immune system during the early infection.

Immune Response and Diagnosis

During hepatitis A virus infection, IgM antibodies are already present in serum at the onset of the clinical disease. IgG antibodies against capsid proteins, and to a lesser extent also against non-structural proteins, are subsequently produced, and persist lifelong. Both IgM and IgG antibodies are capable of neutralizing the virus; they account for the control of viral proliferation and for the rapid decrease of the

viral titre in blood and faeces. Neutralizing antibodies target specific domains on the capsid surface of hepatitis A virus particles. They form an immunodominant region, which involves amino acids 102–114 of VP1 and the amino acid at position 70 of VP3. Another VP1-specific epitope has been characterized around residue 221. Diagnosis of acute infection is performed by detecting viral RNA genomes in blood and stool by PCR as well as by detecting specific IgM antibodies in the serum. The presence of IgG indicates a past infection. As usual for each type of hepatitis, the transaminase and bilirubin levels are additionally determined.

Therapy and Prophylaxis

Vaccines based on in vitro cultivated and formalin-inactivated hepatitis A viruses confer very good protection after two immunizations. Whether the vaccine can last lifelong or whether subsequent booster immunizations are necessary cannot yet be definitely assessed. In fact, the vaccine against hepatitis A is considered as very effective. Current studies based on 10–12 years of vaccination experience have revealed that protecting antibodies have been detected in more than 95 % of all vaccinated individuals. Mathematical calculations predict a persistence of more than 25 years for vaccine-induced antibodies in more than 95 % of all vaccinated individuals.

By administrating virus-specific immunoglobulins, one can achieve passive immune protection lasting for 4–6 weeks. For example, such immunizations are performed as immunoprophylaxis when the time period for production of active antibodies (10–14 days) is too short before the start of the journey. There is no specific antiviral therapy.

Mini Epidemics of Hepatitis A

Because of imported single infections with hepatitis A virus which have been acquired abroad, small epidemics repeatedly emerge also in Europe; occasionally larger mini epidemics can also be observed, and these can affect more than 100 people. For example, a German butcher was infected with hepatitis A virus during his holydays in the Canary Islands. Thereafter, he infected members of his family and at least five co-workers in the butcher's shop in Germany. Apparently, there was contamination of sausage products with this very stable virus. In this way, more people were infected by foodstuff. Further hepatitis A virus infections emerged in small hospitals that were supplied by the contaminated butcher's shop. After identification of the infection chain, public health authorities initiated appropriate measures to control the mini epidemics: This was followed by simultaneous active and passive vaccinations of the workers in the butcher's shop, the exposed customers and the physicians involved. Furthermore, even effective chlorination of public swimming pools was performed. Additionally, relevant blood donor services were informed because hepatitis A viruses can also be transmitted by blood during the viraemic phase.

14.1.5.4 Rhinoviruses

Epidemiology and Transmission

Human rhinoviruses infect only humans. However, they can be transmitted to some ape species and ferrets. There are more than 100 serotypes, and these are classified into groups A and B. Another classification is based on receptor binding: 90 members of the rhinoviruses (major group) bind to ICAM-1 as a cellular receptor, whereas the rest (minor group) bind to the LDL receptor. Rhinoviruses 54 and 89 use heparan sulphate for attachment to host cells. Several serotypes exist simultaneously in a single virus population.

Rhinovirus infections appear especially in both the spring and the autumn. Presumably, they cause 40 % of all acute infections of the respiratory tract. Every person experiences one to three rhinovirus infections per year, the number decreasing with increasing age. Rhinovirus infections cause high morbidity and have great economic importance because of the associated loss of working hours. Transmission generally occurs indirectly by contact with contaminated hands or door handles and only seldom by aerosols or droplets. Rhinoviruses spread rapidly within families, kindergartens and schools.

Clinical Features

The incubation period lasts 1–3 days. Nearly 50 % of rhinovirus infections are asymptomatic; the disease begins with sneezing, coughing and a scratchy throat (flu-like infection). Fever, swelling of lymph nodes and general malaise are absent. The main syndromes are catarrh and nasal congestion. The secretion is initially aqueous and later viscous and yellowish. The symptoms last for a maximum of 1 week. If the symptoms do not subside, this is a sign that a bacterial infection of the paranasal sinuses or an inflammation of the middle ear has possibly occurred.

Pathogenesis

Rhinoviruses invade the body through the mucous membranes of the upper respiratory tract, where they bind to their cellular receptors by the canyon structures. Rhinovirus replication has adapted to the temperature of the mucosae (32–33 °C) and proceeds very fast. Progeny viruses are released by cell lysis 8–10 h after infection of epithelial cells. Maximum viral titres are found after 2–3 days. Release of the virus decreases approximately 4 days after infection. With the scanning electron microscope one can see that large quantities of cells are shed from the ciliated epithelium. This is directly caused by the cell-damaging effect of the virus, and is an ideal basis for bacterial superinfections.

Pathohistologically, hyperaemia and oedema as well as intensified production of mucous secretion with threefold to fivefold increased protein content can be observed at the onset of the disease. The secretion contains a number of inflammatory cytokines, such as IL-1β, TNF-α, IL-6 und IL-11 as well as chemokines such as IL-8, CCL5 (RANTES) and monocyte chemoattractant protein 1; particularly IL-8 induces the migration of neutrophils, monocytes and dendritic cells to the infected regions. Bradykinin, lysylbradykinin and other vasoactive substances

which increase vessel permeability are produced and secreted intensively like vascular endothelial growth factor. Altogether, the viral infection stimulates the local production of many inflammation mediators, and thus causes the typical symptoms.

Analogously to hepatitis A virus, rhinoviruses also inhibit synthesis of IFN-β. However, rhinoviruses do not inhibit phosphorylation and activation of IRF-3, but they prevent the formation of the functionally active IRF-3 dimers, which are formed in the nucleus after transport of the phosphorylated IRF-3.

Immune Response and Diagnosis

Five to 10 days after the infection, IgM, IgG und IgA antibodies against viral proteins can be found in secretions and blood. Whereas IgG is detectable for several years, IgA can be detected for only a few months; particularly IgA mediates protection against reinfections, but only against the same virus type. Most people produce $CD4^+$ T cells, which exhibit cross-reactivity with epitopes of various rhinovirus types. Nothing is known about the occurrence of cytotoxic T cells. Diagnosis is performed only clinically. Antibody determination and virus detection are not done in routine diagnostics.

Therapy and Prophylaxis

Vaccines against rhinovirus infections are not available. Because of the large number of different virus types, the development of vaccines is very difficult. It is possible to avoid a rhinovirus infection by application of interferon. However, a long-term interferon therapy should not be performed owing to the associated injuries to mucous membranes. Pleconaril, which embeds within the canyon structures of virus particles, can decrease the infection, but it also induces the fast formation of resistant viruses, so administration of this substance seems to be futile.

14.1.6 Animal Pathogenic Picornaviruses

A large number of picornaviruses have been described in various animal species and they induce a broad spectrum of diseases. FMD virus is of eminent economic importance, and can infect all ungulates (ruminants and swine). For the differential diagnosis of FMD, swine vesicular disease virus plays an important role in pigs. This virus is a member of porcine enterovirus B and causes a disease in swine that is clinically indistinguishable from FMD. Swine vesicular disease virus is acid-resistant, and is predominantly transmitted by meat products from infected swine. It remains infectious for several months in non-heated meat products such as raw sausages (e.g. salami). Diagnosis is performed by cultivation of the virus or PCR, as well as by antibody detection.

Other picornaviruses (various types of enterovirus and encephalomyocarditis virus) can induce fertility disorders or general diseases in swine, but these are relatively rare today. A formerly very important viral disease in swine is induced by porcine teschoviruses. They cause, in rare cases, a polioencephalitis in swine which largely

resembles the poliovirus-induced poliomyelitis in humans. An antigenetically very similar strain of porcine teschoviruses (Talfan virus) has a substantially lower virulence. Generally, its infection is clinically inapparent, but it induces a resilient, protective cross-immunity against the virulent techovirus. Talfan virus is spread worldwide in swine populations. Therefore, clinical cases of Teschen disease are only rarely observed owing to the high infection rate of Talfan virus.

FMD in Europe

Most countries in Europe and all countries of the European Union were free from FMD for many years. This status was essentially achieved by stringent measures in cases of epidemic outbreaks and by the extensive annual vaccination of all cattle older than 6 months from the reference date. In 1991, vaccination was stopped because the virus was eradicated within the countries of the European Union and the control of FMD was uniformly implemented throughout the European Union. From then, only sporadic outbreaks were registered until 2001, e.g. in Italy in 1993 and 1994 and in Greece in 1996. However, a massive FMD outbreak occurred in the UK in 2001. The virus (serotype 01) was probably introduced by infected pork from Asia which was used as animal food. After the initial porcine infection cycle, the virus was mainly spread by infected sheep, a species which develops only mild symptoms. Nearly 10,000 farmsteads were affected in the UK by January 2002. More than four million cloven-hoofed animals (cattle, sheep, pigs, goats and deer) were killed in the course of veterinary measures. From England, the virus was only sporadically transmitted to other countries, and the outbreaks (25 in the Netherlands, two in France and one in Ireland) were rapidly controlled by culling, and partially also by ring vaccinations. From 21 January 2002, Europe was considered to be free from FMD once more. However, on 2 August 2007, new cases of FMD emerged in southern England, and further cases were also diagnosed in the surrounding cattle farms a little later. During this outbreak, the agent was FMD virus strain BFS 1860 O1 1967 (British Field Strain 1860, serotype O, subtype 1, isolated in 1967), which was cultivated in the immediate vicinity of the Pirbright Laboratory of the Institute for Animal Health for the purpose of vaccination. Most probably, a laboratory virus escaped through old and leaky sewage systems into the environment. Owing to the rapid action of the relevant authorities, the setting up of security zones and the culling of all infected livestock, the virus was contained rapidly.

Owing to financial losses for the affected farmers as well as the ban on transport of live animals and their meat, the outbreak in England had severe financial consequences for husbandry in the UK, which had already been shocked by the BSE crisis. The outbreak in the UK has shown that there is great danger from the FMD virus and that faster diagnostics, an inexorable culling of infected animals and the consequent surveillance of animal transport are still necessary.

14.1.6.1 FMD Virus

Epidemiology and Transmission

FMD virus is one of the most important and economically most relevant animal pathogenic viruses. Although it has not played an important role in Europe for a few decades, and the countries of the European Union are considered to be FMD-free owing to systematic disease control, the FMD epidemic in the UK in 2001 has shown that infiltration of the virus is still possible at any time, and that it can induce great epidemics. Although vaccinations against FMD are no longer performed in the countries of the European Union, a reserve vaccine stock is still retained for control of potential epidemics with ring and emergency vaccinations. The measures required to prevent viral infiltration as well as production, checking and storage of the serotype-specific vaccines require huge financial expense. In Europe, FMD is a notifiable disease; veterinarians and animal owners are obliged to notify the relevant veterinary authority about every suspicion of disease. After notification, the official order to kill single animals follows, if necessary, for diagnostic purposes. If FMD is officially ascertained in the animals, killing of all cloven-hoofed animals of the livestock is ordered, and extensive epidemiological examinations are initiated to control possible dissemination of the virus. These measures concern not only FMD, but also diseases induced by viruses that cause FMD-like symptoms and which can be therefore clarified by differential diagnostics. These viruses include swine vesicular disease virus, vesicular stomatitis virus (▶ Sect. 15.1) and the now eradicated vesicular exanthema of swine virus (Sect. 14.6). FMD virus is widespread worldwide and is endemic in vast regions of Africa, South America and Asia, and also in the Asiatic part of Turkey (Anatolia) as serotypes O, A and Asia 1. From such regions, the virus is occasionally imported into the European part of Turkey and rarely also into other European countries, such as Bulgaria and the Balkan countries in 1996 (serotype O) and Greece in 1996 and 2000 (serotype Asia1).

FMD virus is considered as a prototype of the genus *Aphthovirus*. It is acid-sensitive and infects all cloven-hoofed animals; that means in Europe it infects cattle, small ruminants such as sheep and goats, including endemic and exotic ruminants (deer and also antelopes and wild cattle in zoos), and domestic swine and boars.

Epidemiologically important is the differential expression of clinical symptoms: whereas in cattle and swine FMD is a febrile disease that is accompanied by the typical formation of aphthae in the planum nasolabiale of the muzzle and the planum rostrale of the snout as well as in the coronary band of the hoofs, in sheep the infection is less apparent and can easily be overlooked. However, sheep are extremely susceptible to the highly contagious virus. Since infected sheep excrete high amounts of infectious virus, an FMD-infected sheep flock can be the origin of a fast-spreading, devastating epidemic. Epidemiologically significant is also the fact that the viruses are excreted in large amounts by swine, but also by cattle. In an epidemic wave and its spread, infection of these animal species can lead to a drastic increase in the circulating virus quantities in the animal populations.

Of further epidemiological importance is the fact that there are different serotypes of FMD virus, and against which the infected animals do not develop cross-immunity – similarly to what is found in human infections with the three different poliovirus serotypes. Besides the seven serotypes – O (Oise), A (Allemagne), C, Asia 1 and SAT (South African Territories) 1–3 – there are numerous subtypes, whose infections induce partially only a slight cross-immunity. This antigen diversity plays an important role in the control of FMD.

The virus is transmitted by droplet infections. It is relatively stable and contagious, so under favourable climatic conditions infections can be spread through the air even over long distances. This was demonstrated during an FMD outbreak on the Isle of Wight in 1981. Starting from the French mainland, the viruses were transmitted to the island over the English Channel. The distance is 250 km. Inasmuch as the virus is also excreted in milk, transmission to swine is also possible by feeding with milk products from infected cattle. The virus is acid-labile; therefore, it is inactivated in the meat of slaughtered animals after sufficiently long meat maturation. However, it remains infectious over extended periods of time in insufficiently salted and smocked raw sausages. Hence, it can be transmitted by such products that are prepared from infected animals.

Clinical Features

Infected animals excrete the viruses before the appearance of the first clinical symptoms during an incubation period of a few days. The typical aphthae appear in the planum nasolabiale of cattle as well as in the planum rostrale and in the tongue of swine, in the coronary band of the hoofs and in distinct mucosal regions of the gastrointestinal tract, e.g. in the rumina of cattle. The aphthae contain large quantities of viruses. Whereas alterations in the planum nasolabiale and in the mucous membranes of the gastrointestinal tract are the main symptoms in cattle, inflammation of the coronary band (coronitis) is usually the prevalent symptom in swine. The morbidity is high, but the mortality is low. The virus can persist in infected cattle or sheep for several weeks.

Human infections with FMD virus are extremely rare; however, single cases have occasionally been reported. These are subclinical, but they can also be associated with fever and aphthae formation in mucous membranes, like in animals.

Pathogenesis

The infection is oronasal. Initially, viruses proliferate in the mucosa of the muzzle and tongue and first small aphthae emerge (primary aphthae). Thereafter, the viruses spread through the blood system and reach all inner organs, where they replicate and cause the classic symptoms such as blistering in epithelia (accompanied by fever, drop in performance, salivation, lameness) and the typical, nearly pathognomonic (pathological) alterations such as extensive epithelial colliquative necroses and fibrosis. Infections in young cattle and swine can also affect the heart muscle by inflammation, degeneration and fibrosis. Because of its fibrillar structure, the heart muscle appears “streaked”: this condition is called tiger heart. The virus

reaches the mucosa of the muzzle via the bloodstream, Here the typical aphthae (secondary aphthae) are produced as characteristic symptoms of the disease.

It is important that the virus can persist in cattle. The virus can be isolated from throat epithelium of experimentally infected cattle (probang sample). However, it has been shown in various independent studies that these animals do not excrete viruses, and contact animals are not infected.

Immune Response and Diagnosis

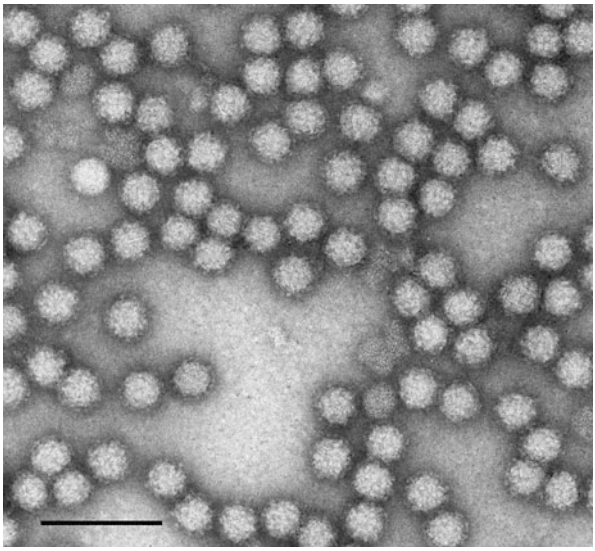
The infection confers a serotype-specific immunity. It is believed that it does not last lifelong. The diagnosis is performed by propagation of the virus in cell culture, preferentially in baby hamster kidney cells or in diverse primary and permanent bovine cell lines. Subsequently, the virus serotype can be determined by using specific antibodies in ELISAs or by means of the complement fixation reaction. Alternatively, the serotype analysis is performed by PCR followed by sequencing of the amplified fragments.

Control and Prophylaxis

FMD is a notifiable disease in many countries. In these countries each suspicion of disease has to be notified to the respective veterinary authorities. After diagnosis, the control includes killing and complete elimination of all ungulates of the livestock concerned as well as all cloven-hoofed animals in neighbouring livestock that are suspected of being infected. Quarantine areas are established in the region and extensive disinfection measures are performed. According to estimates by authorities, an FMD outbreak in a medium-sized country (e.g. Germany) would result in direct and indirect costs of several billion euros.

Recently, the non-vaccination policy regarding highly infectious epidemics such as FMD has been critically discussed within the European Union, and regulations are increasingly being relaxed. The European Commission can, in principle, allow protective vaccinations against FMD (ring vaccination) to protect not yet infected livestock, or suppressive vaccinations to reduce dissemination of the virus in already infected animal stocks or regions; in the latter case, the animals are killed later. The main problem that arises as a consequence of vaccination is the identification and distinction of vaccinated from infected animals. The development of ELISA systems that can detect antibodies against non-structural proteins, which are produced during an infection but not by vaccination, has significantly improved the acceptance of vaccination measures. The principle of distinction is as follows. During FMD virus replication, viral structural and non-structural proteins are synthesized in an animal, and are immunologically recognized; consequently, infected animals produce antibodies against both protein groups. Since vaccines are based on purified, inactivated virus particles, they solely consist of structural proteins and do not contain non-structural proteins. Therefore, vaccinated animals have only antibodies against structural proteins. Commercial ELISA systems which contain antigens of non-structural proteins 3A, 3B and 3C (also known as “3ABC ELISA”) and which can detect the presence of antibodies against such proteins have proven to be sensitive and reliable enough to distinguish between vaccinated and infected animals.

14.2 Astroviruses



Little is known about the molecular biology of the family *Astroviridae*. Like picornaviruses, caliciviruses and hepeviruses, astroviruses possess a non-enveloped capsid and a positive-sense RNA genome. During the replication cycle, they synthesize a subgenomic mRNA species which is necessary for the production of structural proteins. The name is derived from Greek *astron* (*cστpov*), which means "star", alluding to the star-like shape of a part of the astrovirus particle (Fig. 14.5).

14.2.1 Classification and Characteristic Prototypes

The family *Astroviridae* is currently subdivided into two genera, the members of which differ in regard to their host specificity (Table 14.5). The viruses of the genus *Mamastrovirus* infect humans and several mammals (swine, ovines, bovines, felines). The genus *Avastrovirus* includes viruses that infect birds (chickens, ducks, turkeys). Most astroviruses can easily be cultivated in vitro.

14.2.2 Structure

14.2.2.1 Virus Particle

Members of the astrovirus family have non-enveloped, spherical and icosahedral capsids with a diameter of about 28–30 nm (Fig. 14.5). At the capsid surface, approximately 10 % of the virions are characterized by structures which resemble a five- or six-pointed star. The particles are formed from a precursor protein, pV87,

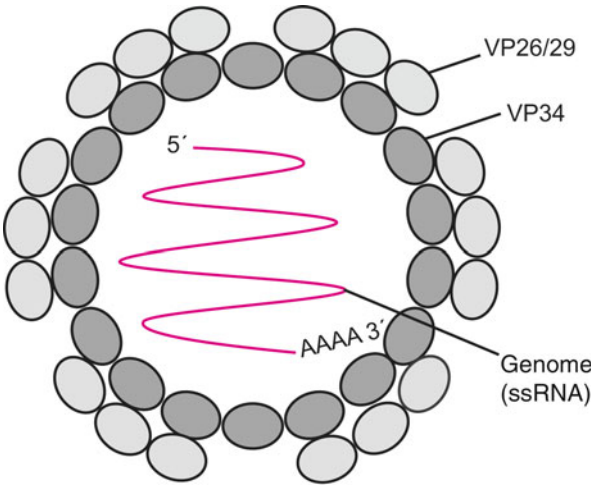


Fig. 14.5 Structure of an astrovirus particle. *ssRNA:* single-stranded RNA

Table 14.5 Characteristic representatives of astroviruses

Genus	Human virus	Animal virus
Mamastrovirus	Human astrovirus types 1-8	Feline astrovirus Bovine astrovirus types 1 and 2 Ovine astrovirus Porcine astrovirus
Avastrovirus		Avastrovirus of ducks Avastrovirus of turkeys Avastrovirus of chicken

i.e. proteolytically cleaved, thereby generating capsid proteins VP34 and VP26/VP29. The processing is necessary for astrovirus infectivity. When cultivated in CaCo-2 cells (human colonic carcinoma cell line), newly synthesized virus particles must be treated with trypsin in order to gain infectivity.

14.2.2.2 Genome Organization and Structure

The astrovirus genome consists of single-stranded, positive-sense RNA; it is polyadenylated at its 3′ terminus and has a length of about 6,800 nucleotides (6,771 and 6,813 nucleotides in human astroviruses 1 and 3, respectively). Sequence analyses revealed three open reading frames with overlapping ends (Fig. 14.6). ORF1a and ORF1b encode non-structural proteins NSP1a and NSP1a/1b. A sequence of 20 nucleotides in the region overlapping ORF1a and ORF1b is highly conserved; it forms a hairpin structure that leads to a partial translational frameshift during translation of the genomic mRNA. Thereby, the codons encoded by ORF1a are fused in frame with those of ORF1b, resulting in the

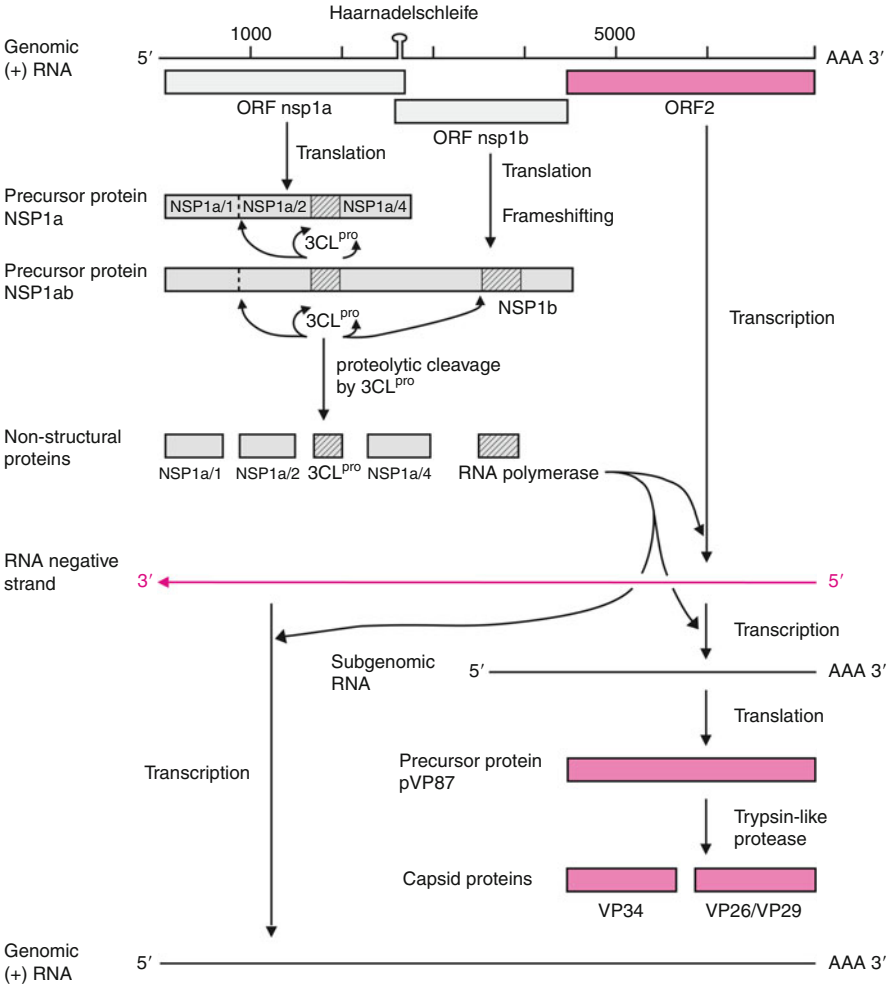


Fig. 14.6 Genome organization of astroviruses. The genome of astroviruses contains three open reading frames. The 3′ and 5′ ends of ORF1a and ORF1b overlap. A hairpin structure induces a translational frameshift leading to an in-frame fusion of ORF1a and ORF1b. The precursor protein NSP1ab contains, in addition to a protease ($3CL^{pro}$), also an RNA-dependent RNA polymerase. This enzyme is responsible for the synthesis of the negative-sense RNA molecules, from which the positive-sense RNA genomes of progeny viruses are transcribed. ORF2 encodes the precursor protein of structural proteins. It is cleaved by the activity of a trypsin-like protease as a part of the clearage product NSP1b

production of a joint precursor protein, NSP1ab. ORF2 encodes the precursor protein for the capsid proteins. At the 5′ terminus of the genomic RNA, there is a short untranslated sequence upstream of the start codon of ORF1a which comprises 10–20 and 45–85 nucleotides in avastroviruses and mamastroviruses,

respectively. Also at the 3′ end of the genomic untranslated sequences are present (130–135 nucleotides in avastroviruses and 59–85 nucleotides in mamastroviruses).

14.2.3 Viral Proteins

14.2.3.1 Non-Structural Proteins

Two precursor proteins are produced: NSP1a (103 kDa) comprises 920 amino acids and is encoded by ORF1a; NSP1ab has a molecular mass of 160 kDa and is a precursor fusion protein that arises from a hairpin-mediated translational frameshift between ORF1a and ORF1b. After translation, $3CL^{pro}$ (3C-like protease) mediates cleavage of the precursor protein NSP1a into probably four proteins (NSP1a/1, NSP1a/2, NSP1a/3 and NSP1a/4); $3CL^{pro}$ resembles the 3C protease of picornaviruses and is located in the central domain NSP1a/3 of the precursor protein. The RNA-dependent RNA polymerase is encoded by ORF1b. Few data are available concerning the function of the other cleavage products (Table 14.6).

14.2.3.2 Structural Proteins

The precursor protein (pVP87) for the production of the capsid proteins is encoded by ORF2 and comprises on average 780 amino acids; depending on the virus type, the molecular mass ranges between 70 and 90 kDa. The precursor protein

Table 14.6 Functions and properties of astrovirus proteins

Protein	Molecular mass (kDa)	Reading frame	Function/properties
NSP1a/1	20	ORF1a	????
NSP1a/2	23	ORF1a	Hydrophobic, contains putative transmembrane domains
NSP1a/3	27	ORF1a	3C-like protease $3CL^{pro}$, cleavage of precursor protein(s)
NSP1a/4	26–35	ORF1a	Contains nuclear transport signal, located in perinuclear region, associates with ER
NSP1b	≈57	ORF1b	RNA-dependent RNA polymerase, transcription, replication
pVP87	70–87	ORF2	Precursor protein of capsid proteins; different size in different virus types; cleaved by cellular typsin-like proteases
VP34	34	ORF2	Capsid protein, amino-terminal cleavage product of pVP87, conserved
VP26, VP29	26–29	ORF2	Capsid proteins, carboxy-terminal cleavage product of pVP87, variable; proteins are generated by alternative use of cleavage sites within pVP87, induction of neutralizing antibodies, substrate of cellular caspases

The protein succession order in the table corresponds to the real order of the reading frames in the genome and in the precursor proteins
ORF open reading frame, *ER* endoplasmic reticulum

aggregates to particular structures, and is subsequently cleaved by trypsin-like cellular proteases into a conserved amino-terminal domain (VP34) and a carboxy-terminal domain (VP26, VP29); the size of the latter can vary as additional trypsin cleavage sites are located within the neighbouring amino acid sequences and may be alternatively recognized and used by the enzymes. In the carboxy-terminal regions of both precursor protein pVP87 and its cleavage products VP26/VP29, a series of acidic amino acids have been described which are cleaved by cellular caspases. Both these proteolytic cleavage reactions by trypsin-like enzymes and caspases are necessary for the production of infectious virus particles.

14.2.4 Replication

The replication cycle of astroviruses has hardly been investigated. The cellular receptor to which the viruses bind on their target cells is unknown. Also, the processes that lead to penetration of virus particles and to the release of the viral genome are not well understood. The infection provokes, by still unknown mechanisms, the activation of the extracellular-signal-regulated kinase (ERK)-mediated signal pathway. This mitogen-activated signalling pathway is induced by extracellular stress signals and results in phosphorylation of ERK1/2. Thereby activated, ERK1/2 reach the cell nucleus and induce the expression of various cellular genes involved in regulating cell division and differentiation. The finding that activation of ERK-mediated signalling is important for viral replication provides evidence that astrovirus replication depends on dividing cells.

The initial step of the reproduction cycle is the translation of non-structural precursor proteins NSP1a and NSP1ab. How protein synthesis is initiated is unclear. No covalently linked protein has been found at the 5′ terminus, as observed in picornaviruses and caliciviruses; the UTR does not seem to form an IRES (see Sects. 14.1 and 14.3). Whether the 5′ end is capped is also not clear. In some translation events, a shift of the reading frame occurs in the carboxy-terminal region of NSP1a, resulting in an in-frame fusion of NSP1a and NSP1b. This process resembles the synthesis of the non-structural proteins of togaviruses (Sect. 14.6). After synthesis, the NSP1a and NSP1ab precursors are both autocatalytically cleaved by $3CL^{pro}$, whereby the functions of the diverse non-structural proteins, including RNA-dependent RNA polymerase, become available. The synthesis of complementary negative-sense RNA molecules is achieved by the activity of RNA polymerase. The negative RNA strands serve as templates for the synthesis of (1) new genomic mRNA molecules and (2) subgenomic mRNA species of about 2,000 nucleotides. The latter contain the ORF2 coding sequences and serve for translation of precursor protein pVP87 (Fig. 14.6). Apoptosis is induced in infected cells during viral replication, resulting in cell death and release of progeny viruses. However, it is unknown which of the viral proteins are responsible for this process. Caspase activities that are induced during apoptosis cleave capsid proteins VP26 and VP29, thus increasing the infectivity of released progeny viruses.

14.2.5 Human Pathogenic Astroviruses

14.2.5.1 Epidemiology and Transmission

Human astrovirus infections were first described during an outbreak of infectious gastroenteritis in a maternity clinic in the UK in 1975. Afterwards, it became evident that astroviruses are distributed worldwide and that they are, after caliciviruses, the second most frequent cause of non-bacterial diarrhoea (see Sect. 14.3). Eight serotypes have been found in human astroviruses to date, but serotype 1 is predominant. Astrovirus infections are preferentially found in children aged less than 2 years, but are also found in older people and in immunosuppressed patients. Excretion of the virus in the faeces persists for 1–2 weeks, but in immunosuppressed individuals also for considerably longer. Transmission occurs via the faecal–oral route.

14.2.5.2 Clinical Features

The incubation period is short and usually lasts 2–3 days. In some cases, astrovirus infections are asymptomatic; in general, they induce gastroenteritis accompanied by diarrhoea and occasionally also by vomiting; muscle and joint pains are rarely observed. The disease is mild and self-limiting and usually lasts 3–4 days. In immunosuppressed patients, astrovirus infections are severer and may persist for longer.

14.2.5.3 Pathogenesis

Astroviruses infect enterocytes in the small intestine and replicate in these cells. Only few signs of cell destruction and inflammation can be found histologically. The relatively low inflammatory activity can be associated with the property of capsid proteins binding to the C1q complement component, thus inhibiting the activation of the complement system as a defence reaction of the unspecific immune system (see ▶ Chap. 7). Furthermore, capsid proteins seem to exert an additional function that determines the pathogenesis of astrovirus infection: they cause an enhancement of the permeability of the epithelium of the small intestine. Because of this, the interaction of occludin, a tight-junction protein, with the cellular actin skeleton is impaired. This process is independent of active viral replication, and it may contribute to the diarrhoea symptoms.

14.2.5.4 Immune Response and Diagnosis

Astrovirus infections can be diagnosed by detection of viral proteins via antigen-capture ELISA, by electron-microscopic representation of virus particles from samples of faeces or by amplification of viral RNA by PCR. During infection, IgM antibodies and later IgG and IgA antibodies against viral structural proteins and are detectable by ELISA. The antibody concentration declines rapidly after the disease. Neither cultivation of the virus in cell cultures nor serological methods play a role in diagnostics. Nothing is known about cellular immunity.

14.2.5.5 Prophylaxis and Therapy

The best measures to avoid infections with human astroviruses are good hygiene and disinfection. Chemotherapy or vaccines are not available. In several countries, astrovirus infections have to be notified to the respective health authorities.

Mammalian Astroviruses are Veterinarily Irrelevant

Mammalian astroviruses have been isolated from swine, cattle, sheep, red deer, cats and mink; all can be propagated in vitro. They differ genetically by up to 40 %, and viruses that infect individual species form their own clusters in phylogenetic analyses. This suggests a high degree of host specificity. An exception is feline astrovirus. It is relatively closely related to human astroviruses and belongs to this cluster. Like for human astroviruses, in vitro cultivation of feline astrovirus requires the addition of trypsin to the culture medium to facilitate infection. In all cases, only mild or subclinical infections have been described; hence, the virus is without clinical relevance for these animals.

14.2.6 Animal Pathogenic Astroviruses

14.2.6.1 Epidemiology and Transmission

Astrovirus infections are frequent in poultry; there are different distinguishable serotypes. Avian nephritis virus is widespread in chickens. It causes minor growth disorders, which can become evident at the herd level. The virus is excreted and transmitted through the faeces of infected animals.

14.2.6.2 Clinical Features

Like human and mammalian astroviruses, avian astroviruses cause subclinical infections and mild diseases. Both serotypes of turkey astrovirus provoke gastroenteritis and are associated with the poultry enteritis mortality syndrome. These viruses replicate in epithelial cells of the intestine as well as in various other tissues, including thymus, bursa of Fabricius, spleen and kidney. Avian nephritis virus infects kidney epithelial cells, and is capable of inducing an interstitial nephritis. Duck astrovirus is associated with hepatitis; a mortality rate of more than 50 % has been observed in experimental transmissions.

14.2.6.3 Pathogenesis

The pathogenesis of avian astrovirus infections has barely been investigated. In contrast to infections in mammals and humans, avian astroviruses do not proliferate exclusively in epithelial cells of the intestine, they also proliferate in several other tissues; therefore, a wider range of different diseases have been observed.

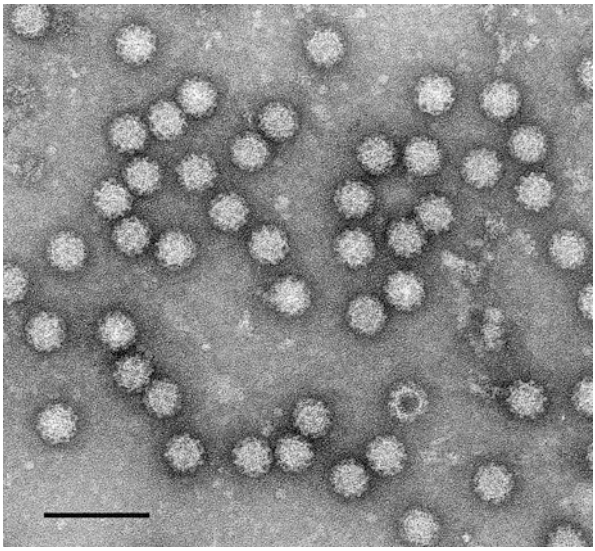
14.2.6.4 Immune Response and Diagnosis

Avian astroviruses can be propagated in cell culture. Diagnostics can be performed by detecting viral RNA genomes by PCR.

14.2.6.5 Prophylaxis and Therapy

Neither antiviral therapy nor immunoprophylaxis is available.

14.3 Caliciviruses



There is limited knowledge with regard to the molecular biology of the family *Caliciviridae*. The name is derived from the Greek word *kalyx* (meaning "goblet" or "chalice") and alludes to the cup-shaped deepened structures in the lateral surfaces of the icosahedron which can be observed in electron microscope images. Like picornaviruses, caliciviruses possess non-enveloped capsids and single-stranded, positive-sense RNA genomes. In contrast to picornaviruses, caliciviruses synthesize a subgenomic RNA species during their replication cycle. By this property, they exhibit similarities to hepeviruses and togaviruses.

14.3.1 Classification and Characteristic Prototypes

The family *Caliciviridae* is subdivided into five genera, which differ especially in the structure of their genome (Table 14.7). The genera *Vesivirus* and *Lagovirus* comprise only animal pathogens. Feline calicivirus, which belongs to the vesiviruses, is a widespread cat flu pathogen. Rabbit haemorrhagic disease virus is most prominent among the genus *Lagovirus* because it was used to combat the rabbit plague in Australia some decades ago. Human pathogenic viruses that cause diarrhoea are classified into the genera *Norovirus* and *Sapovirus*. Their members are characterized by considerable sequence differences in their genomes, and are

Table 14.7 Characteristic representatives of caliciviruses

Genus	Human virus	Animal virus
Vesivirus		Vesicular exanthema of swine virus San Miguel sea lion virus Feline calicivirus
Lagovirus		Rabbit haemorrhagic disease virus European brown hare syndrome virus
Norovirus	Norovirus; genogroups I, II, IV Norwalk virus Southhampton virus Mexico virus Desert Shield virus	Norovirus, genogroup III (bovines) Norovirus, genogroup V (mice) Porcine enteric calicivirus Jena virus Newbury agent Canine norovirus
Sapovirus	Sapovirus, genogroups I, II, IV, V Sapporo virus Parkville virus Manchester human calicivirus	Sapovirus, genogroup III (swine)
Nebovirus		Newbury-1 virus
Valovirus[a]		St-Valérien virus

[a]Proposed as a new genus

subdivided into five groups (GI–GV), which in turn contain a large number of different genotypes. They have denominations that commonly are derived from the place where they were isolated. Norovirus types GI, GII and GIV are human pathogenic viruses, whereas genogroups GIII and GV infect cattle and mice. Sapoviruses infect predominantly humans; only members of genogroup III have been isolated from swine. Until now, only one species, Newbury-1 virus, has been assigned to the genus *Nebovirus*: it infects cattle and causes diarrhoea in calves. Recently, new members of caliciviruses have also been isolated from pigs in Canada. These St-Valérien viruses cannot be assigned to any genera today; therefore, the new genus *Valovirus* has been proposed. Only a few caliciviruses can be cultivated in cell culture: feline calicivirus and San Miguel sea lion virus can be propagated largely without problems, whereas the propagation of rabbit haemorrhagic disease virus is only possible in primary hepatocytes. No cell culture system has been described for noroviruses and sapoviruses.

14.3.2 Structure

14.3.2.1 Virus Particle

Members of the family *Caliciviridae* have non-enveloped, spherical and icosahedral capsids with a diameter of about 34–39 nm and depressions in the lateral faces (Fig. 14.7). They consist of 90 units of the dimeric VP1 (60 kDa). The virus particles contain one or two molecules of VP2 (23 kDa in noroviruses), which is associated with the RNA genome, and Vpg, which is covalently linked to the 5′ terminus of the genome.

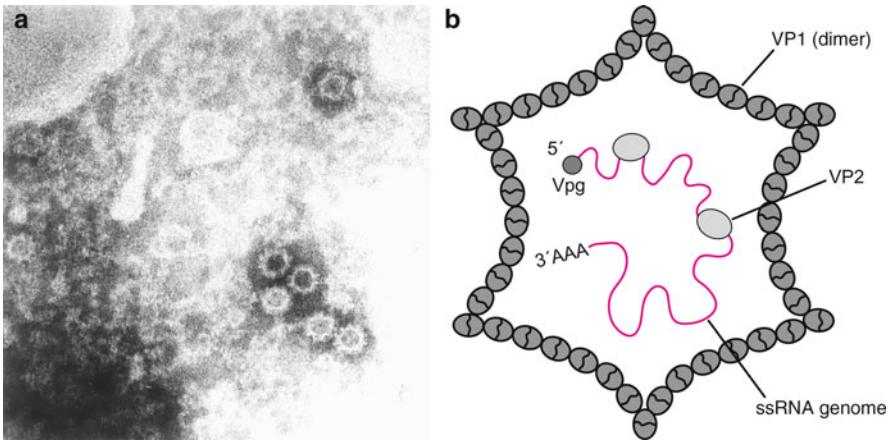


Fig. 14.7 (**a**) Electron microscope image of a calicivirus using recombinantly produced empty capsids of feline calicivirus. (**b**) The structure of a calicivirus particle

14.3.2.2 Genome Organization and Structure

The genome of caliciviruses is constituted of single-stranded, positive-sense RNA, which is polyadenylated at the 3′ terminus and has a length ranging from 7,000 to 8,000 nucleotides (7,338–7,708 nucleotides in noroviruses, 7,437 nucleotides in rabbit haemorrhagic disease virus, 7,690 nucleotides in feline calicivirus and 7,431–7,490 nucleotides in sapoviruses). A viral protein (15 kDa) is covalently linked to the 5′ terminus; it is equivalent to Vpg of picornaviruses (Sect. 14.1). Short untranslated sequences of 10–14 nucleotides in length are located in the 5′ region of the genome upstream of the start codon of ORF1. Genome sequence analyses revealed two open reading frames in sapoviruses and lagoviruses and three open reading frames in noroviruses and vesiviruses; their ends partially overlap (Fig. 14.8). ORF1 is located in the 5′ half of the genome and encodes the precursor of the non-structural proteins. ORF2 is located in the 3′ half of the genome and encodes capsid protein VP1; in sapoviruses, the VP1 open reading frame is directly interconnected with ORF1. The additional reading frame ORF3 (ORF2 in sapoviruses) is responsible for the synthesis of VP2.

14.3.3 Viral Proteins

14.3.3.1 Non-Structural Proteins

The non-structural proteins of caliciviruses are synthesized as a precursor protein of about 180–190 kDa. It is post-translationally cleaved into single components by $3CL^{pro}$, which resembles the 3C protease of picornaviruses (see Sect. 14.1). The arrangement of the individual components is similar in all caliciviruses: the

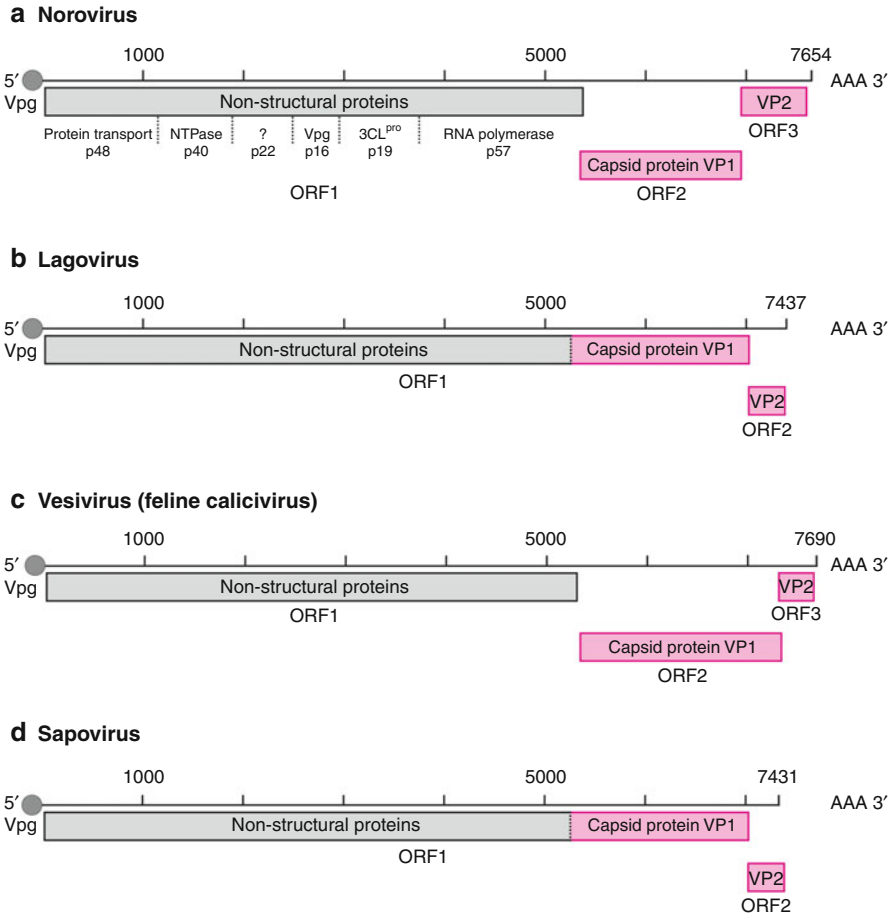


Fig. 14.8 Genome organization of caliciviruses. (**a**) Norovirus (Norwalk virus). (**b**) Lagovirus (rabbit haemorrhagic disease virus). (**c**) Vesivirus (feline calicivirus). (**d**) Sapovirus (Sapporo virus). The mRNA genome of caliciviruses is modified by a covalently linked protein (Vpg) at the 5′ end, and is polyadenylated at the 3′ terminus. It is translated in the cytoplasm. The organization of the open reading frames differs among the different genera: in noroviruses and vesiviruses there are three open reading frames. ORF1 is separated from ORF2 and is responsible for the synthesis of the precursor for the non-structural proteins. It is autocatalytically cleaved by the serine protease $3CL^{pro}$ into Vpg, an NTPase (helicase), RNA-dependent RNA polymerase and two additional non-structural proteins (p48 and p22 in noroviruses). The non-structural protein precursors of lagoviruses, sapoviruses and vesiviruses contain the respective protein functions, but they have been scarcely examined. The RNA polymerase is responsible for the synthesis of the negative-sense RNA molecules, from which the RNA genomes of progeny viruses are synthesized during the replication cycle. ORF1 encodes capsid protein VP1 and ORF3 contains the genetic information for VP2, a protein associated with the RNA genome. In lagoviruses and sapoviruses, the reading frame encoding the non-structural protein precursor is fused in frame to the open reading frame of capsid protein VP1. The small ORF2 encodes VP2

Table 14.8 Functions and properties of astrovirus proteins

Protein	Norovirus	Sapovirus	Feline calicivirus	RHDV	Function
Non-structural protein	37–48 kDa	32 kDa	32 kDa	? kDa	Associates with Golgi vesicles, influences intracellular protein transport
Non-structural protein	40–41 kDa	35 kDa	36–38 kDa	37 kDa	NTPase, helicase? essential for transcription and replication
Non-structural protein	20–22 kDa	32 kDa	30 kDa	29 kDa	Function unknown; membrane associated?
Vpg	16 kDa	14 kDa	15.5 kDa	13 kDa	Covalently linked with the 5′ terminus of RNA, translation initiation
Protease, $3CL^{pro}$	19 kDa	14 kDa	30 kDa	15 kDa	3C-like protease, cleavage of precursor proteins
Polymerase	57 kDa	57 kDa	50 kDa	58 kDa	RNA-dependent RNA polymerase, transcription, replication
Capsid protein VP1	68 kDa (ORF2)	60–62 kDa (ORF1)	60–65 kDa (ORF2)	60 kDa (ORF1)	Structural protein, dimer, major capsid protein
Structural protein VP2	23–29 kDa (ORF3)	23–29 kDa (ORF2)	12 kDa (ORF3)	10 kDa (ORF2)	Structural protein, RNA binding

The protein succession order in the table corresponds to the real order of the reading frames in the genome and in the precursor proteins
RHDV rabbit haemorrhagic disease virus

amino-terminal sequences constitute a non-structural protein (p48 in noroviruses) of variable length that is characterized by a transmembrane domain and that is associated with Golgi vesicles. It interacts with cellular vesicle-associated membrane-protein-associated protein A (VAP-A), which regulates the SNARE-mediated fusion of intracellular vesicles; the function of this viral protein is not completely resolved. The directly adjacent sequences represent an NTPase with similarity to the picornaviral 2C protein and which possibly functions as a helicase. The domain of Vpg, which is covalently linked to the 5′ terminus of the genome, is separated from the NTPase by an amino acid region of unknown function; it contains many hydrophobic amino acids and possibly induces the interaction of the precursor protein with intracellular membrane compartments, such as the 3A protein in picornaviruses. The adjoining sequences represent the domains of $3CL^{pro}$ and RNA-dependent RNA polymerase (Table 14.8).

14.3.3.2 Structural Proteins

In lagoviruses and sapoviruses, the sequences of capsid protein VP1 are fused to the carboxy-terminal end of the RNA-dependent RNA polymerase, and the separation into individual proteins is performed by the proteolytic activity of $3CL^{pro}$.

In noroviruses and vesiviruses, VP1 is encoded by separate reading frames. Vesiviruses exhibit the peculiarity that VP1 is initially synthesized as a precursor protein (73–78 kDa), which is processed by $3CL^{pro}$ into the VP1 that is part of mature virus particles. In this case, a leader sequence of 128 amino acids is removed from the amino-terminal end; its function is still unknown. If VP1 proteins are expressed in eukaryotic systems by genetic engineering methods, they can autonomously associate to form virus-like particles by a self-assembly process. The structural protein VP2 is encoded by a separate open reading frame (ORF2 in lagoviruses and sapoviruses; ORF3 in noroviruses and vesiviruses). It contains many basic amino acids and is associated in low copy numbers with the RNA genome.

14.3.4 Replication

Noroviruses and sapoviruses use the carbohydrate structures of the ABH and Lewis blood group antigens as cellular receptors, whereby distinct virus types bind to different versions of these histo-blood group antigens. Histo-blood group antigens are complex sugar structures which are present on the cell surface of erythrocytes and epithelial cells of the mucosa of the respiratory tract and the intestine. The cellular receptors of feline calicivirus are α(2,6)-linked sialic acid molecules, which are modifications of junction adhesion molecule A, a member of the immunoglobulin superfamily. After adsorption, virus particles are probably incorporated by receptor-mediated endocytosis. Nothing is known concerning the processes that lead to the release of the viral genome from capsids and endosomes.

The first step during viral replication is the translation of non-structural proteins in noroviruses and vesiviruses, as well as the translation of the fusion products of the non-structural and capsid proteins in lagoviruses and sapoviruses using the positive-sense RNA genome as mRNA. The viral genome is characterized by neither a 5′-cap structure nor an IRES element at its 5′ terminus, which otherwise mediate binding of ribosomes for cellular or picornaviral translation, respectively (see Sect. 14.1). Instead, caliciviruses have developed an alternative mechanism for translation initiation that is mediated by Vpg linked to the 5′ terminus of the RNA genome. Vpg interacts with the cellular translation initiation factor eIF-3, a component of the 40S ribosomal subunit; binding of Vpg to eIF-3 mediates attachment of the small ribosomal subunit to the 5′ end of the viral genome. This probably induces binding of the large ribosomal subunit and the translation of ORF1 from the adjacent start codon. The synthesized precursor proteins are autocatalytically cleaved by $3CL^{pro}$, whereby the functions of the different non-structural proteins, including RNA-dependent RNA polymerase, become available.

RNA-dependent RNA polymerase catalyses the synthesis of complementary negative-sense RNA molecules. The negative-sense RNA molecules are used as a template for the synthesis of new Vpg-primed genomic and subgenomic RNAs. These have a length of 2,400–2,700 nucleotides and encode the structural proteins.

This process is similar to the replication process in astroviruses (Sect. 14.2). The subgenomic RNAs are bicistronic and are responsible for the synthesis of VP1 and VP2 in lagoviruses and noroviruses. Translation initiation of VP2 occurs by an unusual mechanism. After termination of VP2 translation, the ribosomes remain bound to the mRNA. Next, translation is reinitiated, resulting in the synthesis of VP2. VP1 capsid proteins aggregate to precursor virus particles, into which the viral RNA genomes are deposited. Interestingly, subgenomic RNAs are also packaged in some virions; this has been shown in rabbit haemorrhagic disease virus, but not in feline calicivirus.

Animal Noroviruses and the Risk of Zoonotic Transmissions

Diverse animal species are infected by noroviruses; there are isolates especially from cattle and swine. Recently, norovirus infections have been observed also in dogs. In general, noroviruses cause no or only mild diseases in animals. Noroviruses of genogroup II can be isolated from swine and humans, whereas viruses of genogroup III have only been found in bovines. The direct transmission from animals to humans was hitherto considered as improbable because animal noroviruses differ substantially from human isolates. However, the isolation of a human genotype II/4 virus from swine and pork demonstrated that this assumption may not be generalized. Besides direct transmission from swine to humans, even genetic recombinations between animal and human noroviruses are conceivable in principal. However, although this has not been directly demonstrated, such a scenario does not appear impossible. It is based on the findings that human noroviruses can replicate in swine and cattle, and that genetic recombination between different genogroups occurs.

14.3.5 Human Pathogenic Caliciviruses: Noroviruses and Sapoviruses

14.3.5.1 Epidemiology and Transmission

Infections with noroviruses and sapoviruses have a global distribution. Both virus types contain several genogroups with variants that are pathogenic in humans and which can be subdivided into different genotypes. This suggests that caliciviruses have a very high mutation rate because of the high error rate of RNA-dependent RNA polymerase, which essentially contributes to the high norovirus and sapovirus variability. Infections cause gastroenteritis that is associated with severe diarrhoea and vomiting. The introduction of obligatory notification has revealed that noroviruses and sapoviruses are responsible for most intestinal infections in the countries of central Europe. The viruses are transmitted by infected individuals via stool and vomitus. Excretion of the virus lasts some days longer than the symptoms; therefore, infected individuals should not return to work not until 2 days after cessation of diarrhoea.

Faeces of immunosuppressed individuals can remain infectious for weeks or months. This causes considerable logistic problems in hospitals because patients cannot be transferred unless viruses are undetectable in stool. The non-enveloped calicivirus particles possess a high environmental resistance, which has to be considered when choosing appropriate disinfectants. Fewer than 100 virus particles are sufficient to induce an infection. Therefore, these pathogens can easily be transmitted by the faecal–oral route, but also by contaminated food and drinking water. They induce epidemics in collective facilities such as schools and homes or on cruise ships, where many people have to cohabit in a relatively confined space. Particularly affected are retirement homes and hospitals, where there is a high turnover of patients, nursing staff and visitors. In such outbreaks, the infection is also frequently transmitted by infected personnel. Whereas sapoviruses have principally been observed in gastrointestinal diseases of children, and until now have not been detected in food, noroviruses also cause food-borne infections (food poisoning); therefore, a strict code of conduct is prescribed for kitchen personnel. More recently, noroviruses have also been detected in the faeces of calves and swine. This suggests a zoonotic potential for this virus group, but this has not been demonstrated so far, however.

14.3.5.2 Clinical Features

The incubation period is very short and usually lasts 2–3 days. Noroviruses and sapoviruses cause short-lasting (2–3 days) but severe gastroenteritis with vomiting and diarrhoea. Severe general symptoms are not observed. The disease is self-limiting and does not cause long-lasting damage. Protracted courses are observed in immunocompromised patients who excrete infectious viruses for considerably longer periods. Threatening situations can emerge in older patients owing to massive fluid loss.

14.3.5.3 Pathogenesis

Noroviruses and sapoviruses infect and destroy mature intestinal enterocytes. As cellular receptors, noroviruses and sapoviruses use carbohydrate structures which are present in ABH and Lewis blood group antigens as well as on the surface of enterocytes. These saccharide structures are present not only in cell-bound form, but also in soluble form, e.g. in breast milk. Individuals belonging to the secretor-negative type are resistant because of the lack of genetic information for the enzyme $\alpha(1,2)$-fucosyltransferase. Breast-fed infants of secretor-positive mothers ingest the sugar molecules via breast milk and are temporarily protected because the saccharide molecules can complex with the viruses in the intestine of children, thereby preventing infection of enterocytes.

14.3.5.4 Immune Response and Diagnosis

Norovirus and sapovirus infections can be diagnosed by detecting virus particles via antigen ELISA or by detecting viral RNA by reverse transcription PCR (RT-PCR) from stool samples. Evidence of virus particles in stool samples by means of

electron microscopy does not play a role in diagnosis. Serological analyses to detect virus-specific antibodies do not have diagnostic significance. Nothing is known concerning cellular immunity.

14.3.5.5 Prophylaxis and Therapy

The best measure to avoid infections with human caliciviruses is systematic compliance with both personal and general hygiene measures. This also includes disinfection by using appropriate disinfectants, the strict isolation and non-transport of infected patients within the hospital, and cleaning of plants for food production and water supply. In extreme situations, entire hospital units and sections and even whole hospitals have to be closed to admission of patients. Cruise liners are temporarily quarantined. Chemotherapy or vaccines do not exist.

Genetic Differences Within Blood Group Antigens Mediate Resistance Against Viral Infections

Blood groups are characterized by the individual composition of glycolipids or proteins on the surface of erythrocytes and other somatic cells. In humans, there are a myriad of different blood group systems; the so-called histo-blood group antigens can be assigned to three families, namely the Lewis, secretor and ABO families. For example, the common feature of all groups of the ABO system is the presence of *N*-acetylglucosamine as the central sugar molecule of carbohydrate structures on cell surfaces. They are linked to galactose molecules that carry fucose residues. Together they compose the basic structure of all blood groups representing blood group 0. If one or two additional *N*-galactosamine molecules are linked to the galactose, these carbohydrate structures represent blood groups A and B, respectively. The biosynthesis of blood group antigens depends on a number of enzymes that are subject to genetic variation. If viruses use carbohydrate structures of blood group systems as receptors, the susceptibility of the respective infection is genetically determined. Therefore, people with genetic defects in the enzyme α(1,2)-fucosyltransferase are not able to synthesize the carbohydrate structures that are necessary for binding of noroviruses and sapoviruses to the cell surfaces. Hence, these "non-secretor" phenotypes cannot be infected by certain types of noroviruses and sapoviruses: they are resistant. Inasmuch as noroviruses frequently mutate, and as a result also change their receptor preferences, it must be assumed that such resistance does not apply for all virus types. Similar genetically determined resistances are also known from parvovirus B19 infections: this virus binds to blood group antigen P, a glycosphingolipid. People who do not possess the genetic information for the enzymes which are necessary for the synthesis of the corresponding carbohydrate structures cannot be infected by these types of parvoviruses (see ► Sect. 20.1)

14.3.6 Animal Pathogenic Caliciviruses

The clinical pictures caused by caliciviruses that are pathogenic in animals are clearly different from those of human pathogenic caliciviruses. Vesicular exanthema of swine virus is the prototype of the genus *Vesivirus*. Retrospective genetic analyses revealed that vesicular exanthema of swine virus is closely related to San Miguel sea lion virus, which displays different serotypes; it may therefore be of marine origin. Vesicular exanthema virus of swine exhibits a broad host spectrum, which includes various mammals, reptiles, amphibians and even fishes and nematodes. It can also be cultivated in human cell lines in vitro. Between 1930 and 1950, vesicular exanthema of swine virus caused diseases in swine in the USA which could not be distinguished by differential diagnosis from FMD. In mammals, blistering of the skin can be observed, particularly in the extremities (coronary band as well as fins), and in the mucosa of the muzzle. The last outbreak was detected in the USA in 1952; since then the virus has been deemed to be eradicated.

14.3.6.1 Feline Calicivirus

Epidemiology and Transmission

Feline calicivirus is a very important animal pathogen; it is the causative agent of cat flu. It is transmitted by direct contact and by secretions from the respiratory tract. Many infected cats develop the status of persistent infected virus carriers, which excrete the virus for weeks and months. Feline caliciviruses are antigenetically not uniform, and although distinct serotypes cannot formally be distinguished, there are many isolates whose infections induce no or a non-protecting and incomplete cross-immunity. The variability within the capsid proteins is primarily limited to a little domain of about 100 amino acids, the hypervariable E region. The nucleotide sequences of different isolates can differ in this region by up to 70 %.

Recently, feline caliciviruses have been isolated from cats with a new clinical picture. These cats show oedemas in the head and neck and ulcers in the nose, ears and paws, as well as sporadic icterus. Such isolates have been denominated as virulent systemic feline caliciviruses. This is an unfortunate designation as every feline calicivirus infection is systemic and the haemorrhagic component in the clinical picture is rare; in particular, these new isolates do not show any resemblance to rabbit haemorrhagic disease virus. Interestingly, these highly virulent isolates originate from vaccinated and mainly old cats. Genetically, they cannot be grouped together; they exhibit a similar heterogeneity like other feline caliciviruses. A genetic marker has not yet been found for these highly virulent strains.

Clinical Features

The virus induces an acute respiratory disease in cats, and it is associated with rhinitis and ulcers of the oral mucosa; polyarthritis is rarely found.

Particularly, kittens become ill within the first few months of life. Feline calicivirus can frequently be detected in old cats that suffer from chronic inflammation of the oral mucosa (stomatitis). However, an aetiological significance of the virus in this clinical picture has not been proven.

Pathogenesis

Viraemia arises during the course of infection, which also leads to the infection of synovial cells in the joints. The tonsils are a site for persistence of the virus. This virus replicates in feline cell cultures; hence, it is well investigated.

Immune Response and Diagnosis

Infected cats develop an IgG response against capsid proteins, which can be detected by ELISA and Western blotting. The diagnosis of the acute infection is done by cultivating the virus in cell culture or by detecting viral RNA by PCR.

Control and Prophylaxis

Vaccines against cat flu are available on the basis of both attenuated and inactivated viruses, and these are routinely used to vaccinate domestic cats. However, these vaccines have only limited effectiveness owing to high antigenic variability.

14.3.6.2 Rabbit Haemorrhagic Disease Virus

Epidemiology and Transmission

Around 1985, a seemingly new severe epidemic disease appeared in rabbits (*Oryctolagus cuniculus*) in China. The illness, named rabbit haemorrhagic disease, spread rapidly worldwide. It is characterized by severe haemorrhagic symptoms and fulminant hepatitis. Parvoviruses were primarily assumed as the aetiologic agent; however, calicivirus was rapidly described as the true pathogen. The origin of rabbit haemorrhagic disease virus in China has not yet been finally determined. Retrospective serological analyses have revealed that the virus was disseminated in the UK as long ago as 1950 without having shown any epidemic signs at that time.

Meanwhile, all rabbit populations are contaminated, but the initially high mortality has strongly decreased. Apparently, fast attenuation of the virulence seems to occur in rabbits, as shown by the "field study" in Australia and New Zealand. Therefore, animal health measures have been lifted. Besides direct contact between animals and coprophagy (eating faeces), the passive transmission of the virus by arthropods plays an additional role. The virus is deemed to be host-specific; it cannot be propagated in cell culture. European brown hare syndrome virus, a similar but different virus type, causes disease in the European brown hare (*Lepus europaeus*). This virus is not contagious for domestic rabbits.

Clinical Features

The virus causes a severe disease in rabbits older than 8 weeks. Irrespective of whether maternal antibodies are present, the kittens cannot be infected before that period. The disease is usually fulminant, and the animals die within hours of infection. In protracted cases, the animals bleed from all mucous membranes. Recovery is exceptional.

Pathogenesis

The virus has a tropism for hepatocytes and causes a lytic infection leading to massive liver necroses.

Immune Response and Diagnosis

Because of the fulminant course of the infection, an immune response cannot be established in the animals; the serological diagnosis is generally irrelevant. Subclinically infected animals can be identified by detecting specific antibodies in ELISA. The viral genome can be detected by RT-PCR in samples from liver, blood and faeces. Electron-microscopic verification of virus particles in the liver is also possible and is commonly used. Since the virus does not replicate in cell cultures, isolation of the virus is not possible.

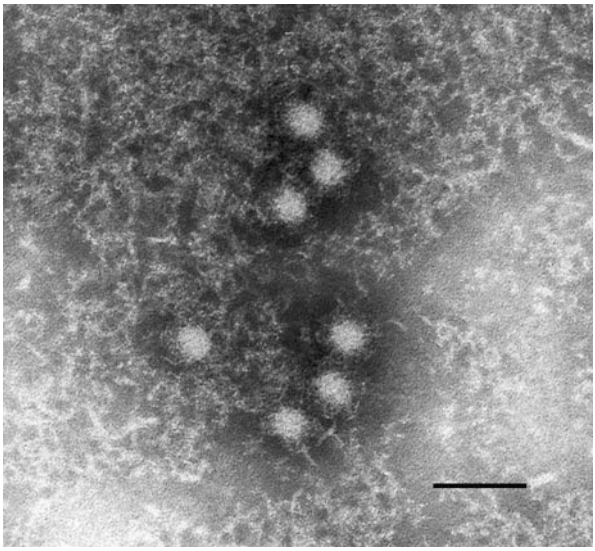
Control and Prophylaxis

For rabbits in livestock husbandry, a vaccine based on inactivated whole virus is available, and this is extracted from the liver of infected rabbits.

Rabbit Haemorrhagic Disease

Rabbit haemorrhagic disease virus gained dubious popularity when it was released for biological control of rabbit populations under the name "rabbit calicivirus" in Australia in 1996. It has been and is being controversially discussed whether other species of the unique Australian fauna may be endangered in addition to rabbits. On the other hand, the tremendous rabbit plague in Australia had considerable consequences for the flora and consequently also for the fauna. The application of the virus led to a significant reduction of the rabbit population (60 % or more) in some regions of Australia; however, in other regions it had no influence. Farmers disseminated the virus over long distances within the continent, and a lively trade in infected rabbits flourished. Furthermore, the virus was illegally introduced to and spread in New Zealand. As expected, healthy rabbits carrying infectious viruses were found in Australia and New Zealand after a short time. This is an infallible sign of natural attenuation of the virus in its host.

14.4 Hepeviruses



Hepatitis E viruses were long considered to belong to a group of unclassified viruses, the so-called non-A, non-B hepatitis viruses. Later they were classified into the family *Caliciviridae* because of electron-microscopic analyses which revealed some structural similarities. However, further investigations regarding the replication cycle and genome structure have revealed notable differences; therefore, hepatitis E viruses are now classified into their own family, the *Hepeviridae*. The family name is derived from the denomination of the hepatitis E virus.

14.4.1 Classification and Characteristic Prototypes

Hepatitis E viruses are classified into the genus *Hepevirus* (Table 14.9). They are serotypically homogenous, but are subdivided into four genotypes: genotypes 1 and 2 have only been found in humans, whereas genotypes 3 and 4 represent porcine hepatitis E viruses and are closely related to genotypes 1 and 2 and can also infect humans. Human hepatitis E virus infection is a classic zoonosis in which the pathogens are transmitted from swine to humans. Furthermore, there is also a hepatitis E virus variant which can only reproduce in chickens and other birds.

Table 14.9 Characteristic representatives of hepeviruses

Genus	Human virus	Animal virus
Hepevirus	Hepatitis E virus	Porcine hepatitis E virus Avian hepatitis E virus

14.4.2 Structure

14.4.2.1 Virus Particle

Hepatitis E viruses have non-enveloped icosahedral capsids with a diameter of 34–39 nm. In electron-microscopic images they resemble caliciviruses (Fig. 14.9) and are composed of 180 units of a capsid protein with a molecular mass of 76 kDa. The particles additionally contain various amounts of a small soluble protein of 14.5 kDa, the origin of which is still uncertain. It is possibly a cleavage product of the capsid protein.

14.4.2.2 Genome Organization and Structure

The genome of hepatitis E viruses consists of single-stranded, positive-sense RNA with a length between 7,194 and 7,232 nucleotides, depending on the different genotypes. The 5′ terminus contains a cap structure and the 3′ terminus is polyadenylated. Short non-coding sequences are located at the 5′ and 3′ ends of the genomic RNA (Fig. 14.10). Sequence analyses revealed three open reading frames with partially overlapping ends. ORF1 is situated in the 5′-oriented part of the genome half and encodes the precursor of the non-structural proteins. The second open reading frame (ORF2) directs the synthesis of the capsid protein. A further open reading frame (ORF3) encodes the small protein pORF3, which comprises 112–132 amino acids and is important for the infectivity of the virus. This open reading frame overlaps with ORF1 in most isolates. Exceptions are the Chinese isolates of hepatitis E viruses (genotype 4), in which the 3′ end of ORF1 encoding the non-structural protein precursor is separated by 28 nucleotides from the translation initiation codon of ORF3.

14.4.3 Viral Proteins

The non-structural protein precursor (186 kDa) comprises 1,692–1,709 amino acids and contains the domains of a methyltransferase/guanylyltransferase at the amino-terminal region (Fig. 14.10). It is necessary for modification of genomic and subgenomic mRNAs with a cap structure. The other non-structural proteins are a protease, which cleaves the precursor protein into the individual functional components, an NTPase/helicase and an RNA-dependent RNA polymerase (Table 14.10). The protease resembles cysteine proteases of the papain type. Whether this protease is responsible for all cleavage reactions and at which sites has not been finally elucidated. In hepatitis E viruses, the sequences encoding the capsid protein are located in a separate reading frame (ORF2) that exhibits the highest degree of conservation. The capsid protein generally has a length of 660 amino acids (about 72–76 kDa) and forms homodimers. It is characterized by a signal-peptide-like sequence followed by a signalase cleavage site at the amino terminus. Glycosylated and unglycosylated forms may be found in the cytoplasm of infected cells. Probably the capsid protein is synthesized in association with the ER membrane and translocated into the ER lumen, whereby the signal peptide is excised and the

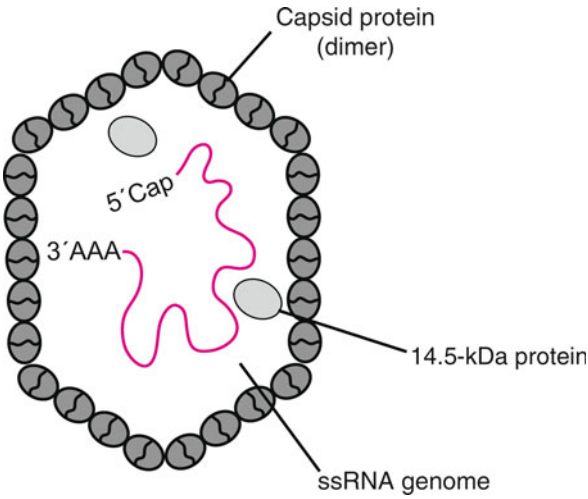


Fig. 14.9 Particle structure of a hepatitis E virus

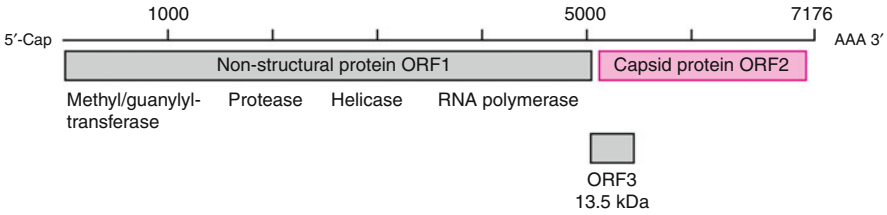


Fig. 14.10 Genome organization of hepatitis E viruses. The mRNA genome contains a cap structure at the 5′ terminus, and is polyadenylated at its 3′ end. It comprises three open reading frames: ORF1 encodes a non-structural protein containing the domains for a methyltransferase, a protease, a helicase and an RNA-dependent RNA polymerase. The protease cleaves the precursor protein into the different functional domains. The RNA-dependent RNA polymerase is responsible for the synthesis of both the antigenomes and new viral RNA genomes. ORF2 encodes the capsid protein and ORF3 encodes a non-structural protein of 13.5 kDa

amino acid chain is modified with sugar groups. Subsequently, retransport of the capsid proteins into the cytoplasm seems to occur. ORF3 encodes a small phosphoprotein, pORF3. Its function has not been conclusively resolved. Nevertheless, two-hybrid analyses have shown that it interacts with the capsid proteins; phosphorylation of the serine residue at position 80 of pORF3 is necessary for interaction. Moreover, binding to bikunin, a Kunitz-type protease inhibitor of serine proteases, and binding to haemopexin, a glycoprotein present in blood plasma that belongs to the acute phase proteins (see ▶ Chap. 7), have also been observed. To what extent these in vitro results are relevant for in vivo replication of hepatitis E viruses is not clear. New data suggest an interaction of pORF3 with dynein of microtubules. It has further been shown that pORF3 is localized in endosomes, where it interferes with protein

Table 14.10 Function and properties of hepatitis E virus proteins

Protein	Size (kDa)	Properties	Function
Capsid protein	76	Dimer; signal peptide is cleaved, partially glycosylated	Capsid protein
Capsid protein	14.5	?	Soluble protein in virions, cleavage product of the 76-kDa capsid protein?
Methyltransferase/ guanylyltransferase	110	?	Capping of genomic and subgenomic RNA molecules
Protease	?	Homology to cysteine proteases	Cleaves the precursor of non-structural proteins
RNA helicase	?	NTP-binding	Resolution of RNA secondary structures
RNA-dependent RNA polymerase	?	?	Replication Synthesis of subgenomic RNA
pORF3	13.5	Phosphorylated, homodimer	Associated with cytoskeleton

transport into the compartments of late endosomes, e.g. the transport of the epidermal growth factor receptor. Since this process is a prerequisite for the transport of phosphorylated STAT3 proteins into the nucleus and thus for initiating the non-specific immune responses (see ▶ Chaps. 7 and ▶ 8), these cannot be properly developed. By inhibiting the early immune defence, the virus may become capable of replicating without impairment.

14.4.4 Replication

The first step of hepatitis E virus reproduction is the translation of non-structural proteins using the positive-sense RNA genome as mRNA. The non-structural protein precursor is probably autocatalytically cleaved by the protease in analogy with other positive-sense RNA viruses. As a result, the functional activities of the following proteins become available in addition to the viral protease: RNA-dependent RNA polymerase, helicase and methyltransferase/guanylyltransferase. They are involved in the production of negative-sense RNA molecules, which are synthesized using the viral genome as a template. This process occurs in association with the ER membrane. The negative-sense RNA molecules are used as a template for the synthesis of new viral genomes. In addition, two subgenomic mRNAs with lengths of 2.0 and 3.7 kb are transcribed from negative-sense RNAs. The bicistronic subgenomic 2.9-kb RNA spans the region of the 3′-oriented ORF2 and ORF3. This mRNA is used for translating the capsid proteins and pORF3 (Fig. 14.10). Whether the 3.7-kb subgenomic RNA has a similar function is uncertain. The capsid proteins interact to form precursor virus particles in which the viral RNA is embedded. New viruses are released from the surface of infected cells.

14.4.5 Human and Animal Pathogenic Prototypes of Hepeviruses

14.4.5.1 Hepatitis E Virus

Epidemiology and Transmission

A new hepatitis epidemic emerged in New Delhi in 1955, and was transmitted via the faecal–oral route by contaminated drinking water; it infected 29,000 people. Further outbreaks were observed in Central America, Africa, India, China and the south of the former USSR. Occasionally, patients in non-endemic regions developed similar forms of infectious liver inflammation, e.g. also in central European countries. Owing to the mode of transmission, it was initially thought that hepatitis A viruses had provoked these diseases. They could only be identified retrospectively as hepatitis E virus epidemics. After the first isolation of the virus by Mikhail S. Balayan, whereby he inoculated himself with pathogenic material, the molecular characterization of hepatitis E viruses was performed by Daniel W. Bradley and co-workers in 1988. They were able to isolate virus particles from stool samples; these showed a positive reaction with sera from patients, and caused hepatitis in macaques after feeding. Later, it was demonstrated that the virus can be transmitted to other primates, and even to animal species such as rats, sheep and cattle. Hepatitis E viruses were isolated from swine for the first time in 1997. These viruses, as shown in subsequent investigations, are spread worldwide in both wild boar and domestic pig populations. It is assumed that adolescent animals, which are no longer suckling, are mainly infected via the faecal–oral route. At least 50 % of all animals older than 6 weeks have antibodies against the virus.

Hepatitis E viruses seem to have been disseminated in human populations for a long time. Almost 90 % of adults in Egypt have antibodies specific for hepatitis E viruses. The seroprevalence is about 40 % in India, it ranges from 4 % in southern and eastern coastal states of the USA to 30 % in the Midwest, it is approximately 4 % in Germany and it is around 16 % in southwestern France. The various genotypes are distributed in different geographical regions. Genotype 1 viruses are found in Asia and North Africa. Genotype 2 has been found in hepatitis E epidemics from Central America and Central Africa. Genotype 3 is prevalent in various European countries, in North America, South America and Japan. Genotype 4 viruses have especially been detected in China, Taiwan, Japan and Vietnam. Whereas genotypes 1 and 2 of hepatitis E virus have only been found in humans so far, genotypes 3 and 4 can infect both humans and swine; therefore, their regional distribution is coincident. Hepatitis E virus genotype 3 has been detected in 5 % of all blood samples from wild boars. In the Netherlands, hepatitis E viruses of genotype 3 have also been found in commercially available swine liver. Inoculation of swine with such viruses led to acute infections, strongly suggesting that those liver samples contained infectious hepatitis E viruses. Efficient in vitro cultivation of hepatitis E viruses is not possible today. The virus particles do not display the high particle stability of hepatitis A viruses (Sect. 14.1). Besides the usually observed faecal–oral transmission (via contaminated water) and the suspected zoonotic transmission, hepatitis E viruses are also transmitted directly from person to person by droplet and smear infections.

Clinical Features

Hepatitis E virus infections apparently do not induce clinical symptoms in swine. In humans, the severity of the disease seems to correlate with the quantity of hepatitis E virus particles transmitted upon contact. In connection with very low amounts of the virus, infections are asymptomatic, especially in children. After an average incubation period of 6–7 weeks, influenza-like symptoms appear, such as sickness, nausea, fever, itching, joint pain and headaches, which are accompanied by a steep increase in the levels of liver enzymes. The intrahepatic cholestatic jaundice which develops during the disease and is caused by disintegration of liver cells and congestion of bile can last for several weeks (light stool, dark urine and yellow eyes). The mortality rate is 1–4 %, which is considerably higher than that of infections with hepatitis A virus (Sect. 14.1). Particularly striking is the high death rate among acutely infected pregnant women, which is observed mainly in India; it is around 20 %. Apart from the mother, the unborn child may also be affected. Chronic, persistent infections can be established in immunocompromised patients; recipients of organ transplants (liver, kidney, pancreas) have been reported to develop chronic hepatitis and liver cirrhosis, which apparently are linked to persistent hepatitis E virus infections.

Pathogenesis

The hepatitis E virus enters the body predominantly through contaminated food and settles in the liver cells. How it arrives there is unknown, as is the nature of its cellular receptor. From the liver the virus is shed into the blood and through bile ducts into the intestine. Similarly to hepatitis A, both excretion of infectious hepatitis E viruses and viraemia reach their peaks before the onset of symptoms. Hepatitis E is pathohistologically characterized by cell necroses and degenerations in the liver; they can also be observed in the otherwise asymptomatic infection in swine. Immigrating granulocytes can be detected in intratubular infiltrates, whereas there are more lymphocytes than granulocytes in the portal region.

Immune Response and Diagnosis

During infection, IgM and then IgG antibodies against the viral capsid protein are initially produced, and can be detected by ELISA or Western blotting. The diagnosis of acute infections is performed by detecting IgM antibodies against capsid proteins and viral RNA in serum or stool by PCR.

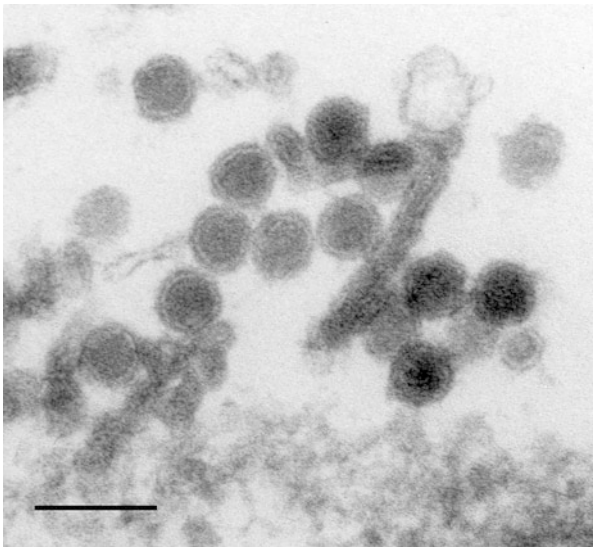
Therapy and Prophylaxis

In endemic areas, the best measure to prevent infections is maintenance of the cleanliness of water supply facilities. A vaccine based on capsid proteins produced in insect cell cultures has shown good protective results and hardly any side effects in clinical studies; however, this vaccine is not yet commercially available. An antiviral chemotherapy does not exist.

Avian Hepatitis E Virus

Avian hepatitis E virus was originally detected in the USA in tissues of a chicken which had hepatitis–splenomegaly syndrome. It was shown that this virus infects only chickens, and is spread worldwide in poultry. A high seroprevalence of about 30 % can be found in poultry. Hepatitis–splenomegaly syndrome is characterized by an increased mortality in flocks; the affected animals have an enlarged liver and spleen, as well as ovaratrophies. Histologically, bleeding and widespread or multifocal necroses can be detected in the affected livers. There is no evidence that avian hepatitis E virus can be transmitted to humans. The genome organization is identical to that of human and porcine isolates; the sequence homology is, however, relatively low (about 50–60 %). Similar to the human and porcine hepeviruses, avian hepatitis E virus cannot be propagated in cell culture Comparative analyses of genome sequences from different isolates of avian hepatitis E viruses show a significant genetic heterogeneity: the sequence identity between some isolates is only 70 %.

14.5 Flaviviruses



Flaviviruses are characterized by a single-stranded mRNA as the genome and, similarly to picornaviruses, translate it into one precursor polypeptide comprising both structural and non-structural proteins; like picornaviruses, they do not produce subgenomic mRNAs. However, flaviviruses differ from picornaviruses, astroviruses, caliciviruses and hepeviruses by an envelope which surrounds the capsids and contains viral surface proteins.

14.5.1 Classification and Characteristic Prototypes

The family *Flaviviridae* encompasses more than 70 different flavivirus types, which are classified into three genera (Table 14.11): The genus *Flavivirus* includes yellow fever virus; the jaundice that is caused by the virus was eponymous for the denomination of both the family and the genus (*flavus*, Latin for "yellow"). It was the first virus for which an insect-associated mode of transmission had been demonstrated (*Aedes* spp.). The genus *Flavivirus* includes a number of other viruses which are also transmitted by arthropods (insects and arachnids) and are pathogenic in humans. Dengue virus, which is also transmitted by *Aedes* spp., and Japanese encephalitis virus, St. Louis encephalitis virus and West Nile virus (transmitted by *Culex* mosquitoes) have been associated with febrile, haemorrhagic or neurological disorders and encephalitis, especially in tropical countries. In central Europe, the pathogen of meningoencephalitis is spread endemically in certain regions. This disease is a typical prototype of tick-borne encephalitis (TBE), which is transmitted by tick bites. On the other hand, some other flaviviruses infect only mammals; they are presumably transmitted from animal to animal (Rio Bravo virus infects only bats and Jutiapa virus infects solely rodents).

The second genus includes pestiviruses, which cause severe animal diseases such as classical swine fever (hog cholera). These viruses are not transmitted by arthropods.

Table 14.11 Characteristic prototypes of flaviviruses

Genus	Vector/carrier	Human virus	Animal virus
Flavivirus	Mosquitoes	Yellow fever virus Dengue virus types 1–4 West Nile virus Japanese encephalitis virus St. Louis encephalitis virus	Yellow fever virus (monkeys) Wesselsbron virus (ovines, bovines) West Nile virus
	Ticks	Tick-borne encephalitis virus Kyasanur forest disease virus Omsk haemorrhagic fever virus	Tick-borne encephalitis virus Kyasanur Forest disease virus Omsk haemorrhagic fever virus Louping iII virus Jutiapa virus (cotton rats) Rio Bravo virus (bats)
Pestivirus	–		Classical swine fever virus Bovine viral diarrhoea virus (mucosal disease) Ovine border disease virus
Hepacivirus	–	Hepatitis C virus	GB Virus B (New World monkeys)
Pegivirus	–	T Human pegivirus (Hepatitis G virus (GB virus C))	Theiler's disease associated virus of horses Simian pegivirus (GB virus A) bat pegivirus (GB virus D)

Because of its molecular characteristics, hepatitis C virus has been classified as a separate genus (*Hepacivirus*) within the family *Flaviviridae*. It is primarily transmitted through contaminated blood, and usually provokes a chronic infection with hepatitis in humans. It causes liver cirrhosis and primary hepatocellular carcinoma as late complications. Hepatitis C virus is similar to human pegivirus (formerly known as hepatitis G virus or GB virus C), which was isolated from a patient with liver inflammation. The virus is widespread, but contrary to initial speculations, human pegivirus infections do not cause hepatitis. Since the amino acid sequence of its precursor polyprotein exhibits only about 28 % and 20 % homology to the sequences of hepatitis C virus and yellow fever virus, respectively, it has been classified in a separate, new genus in the family *Flaviviridae*. So far, there are only very few data on the molecular biology and pathogenesis of this virus. Similar viruses have been isolted from tamavirus (GB virus A or similar pegivirus) and bat (GB virus D or bat pegivirus). Recently, Theiler's disease associated virus has been characterized to cause hepatitis in horses. In contrast, GB virus B seems to represent an additional species in the genus hepacivirus, it causes hepatitis in New World monkeys.

Arboviruses
Viruses that are transmitted by insects or arachnids are denominated arboviruses (arthropod-borne viruses). They exist mainly in tropical and subtropical climates. A prerequisite for transmission of the viruses by arthropods is that the viruses must be able to infect specific organs of the vectors, such as the epithelial cells of the gut and salivary glands. The mere uptake of virus-containing blood by mosquitoes or ticks is not enough: the viruses must be able to perform a productive infection cycle in both arthropod and mammalian cells.

14.5.2 Structure

14.5.2.1 Virus Particle

Infectious flaviviruses have a diameter of 40–50 nm. The spherical capsids consist of only one type of viral protein (C protein), and are surrounded by an envelope, in which two viral surface proteins are embedded. They are designated by the letters M and E in flaviviruses (Fig. 14.11). M protein is relatively small and has a molecular mass of 7–8 kDa; 90 dimers of the virus-type-specific E proteins are found per particle. M protein is absent in hepatitis C virus and pestiviruses; instead a smaller glycosylated surface protein, E1 (gp33), can be found in addition to the main glycoprotein, E2 (gp70). The RNA genome is contained inside the capsids and interacts strongly with the highly basic C protein.

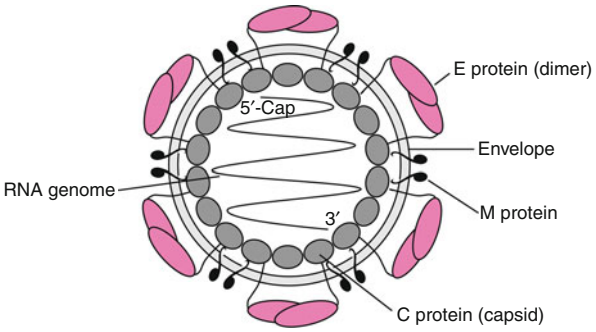


Fig. 14.11 Structure of a flavivirus particle (tick-borne encephalitis, TBE, virus). The icosahedral capsid consists of C proteins. The RNA genome is associated with the protein domains on the inner side of the capsid. The capsid is surrounded by an envelope, which is intercalated with homodimeric E and M proteins

Non-A, Non-B Hepatitis Viruses

Hepatitis C virus was assigned to the so-called non-A, non-B hepatitis viruses until its molecular identification in 1989. Initially all pathogens of liver inflammation which could not be diagnostically identified as hepatitis A virus or hepatitis B virus were classified into the group of hepatitis non-A, non-B viruses. Even after the characterization of hepatitis C virus and hepatitis E virus (the latter was characterized and classified into the family *Hepeviridae* in 1988; see Sect. 14.4), some other, hypothetical viruses are still assigned to this group. For example, there is only indirect evidence for the existence of hepatitis F virus. It has long been unclear whether hepatitis C virus should be incorporated into the family *Flaviviridae* or the family *Togaviridae*. Detailed knowledge of the molecular-biological characteristics allowed hepatitis C virus to be included as a separate genus, *Hepacivirus*, within the family *Flaviviridae*.

14.5.2.2 Genome Organization and Structure

The genome consists of single-stranded RNA and has a length of about 9,100–12,000 nucleotides: 10,862 in yellow fever virus, vaccine virus 17D; 10,664–10,723 in dengue virus; 11,141 in TBE virus (TBEV), strain Neudörfl; 9,340–9,589 in hepatitis C virus, 9,143–9,493 in human pegivirus (GB virus C), 12,308–12,573 in bovine viral diarrhoea virus (BVDV); 12,297 in classical swine fever virus. The RNA is present with positive-sense polarity and spans a large reading frame, which has a length of 10,233 nucleotides in yellow fever virus (Fig. 14.11). Like in picornaviruses, it directs the synthesis of a single common precursor protein that is cleaved into the individual components during infection. The RNA genome of flaviviruses has a cap structure at the 5′ terminus. In the case of yellow fever virus,

the reading frame is flanked at the 5′ and 3′ termini by untranslated sequences, which are 118 and 511 nucleotides in length, respectively. The 3′ terminus is not polyadenylated. However, adenosine-rich sequences of variable length are found in this region.

In contrast to members of the genus *Flavivirus*, the genomes of pestiviruses, hepaciviruses and pegiviruses do not have a cap structure at the 5′ end of the genome, but they possess – like picornaviruses – IRES elements. The IRES structure mediates binding of the ribosomal subunits and initiation of translation of the polyprotein (Sect. 14.1.4). Therefore, the 5′ UTR of hepatitis C virus is significantly longer than that of flaviviruses, it comprises about 340 nucleotides. A short sequence of uridine and adenosine residues is found at the 3′ end of the hepatitis C virus genome. Similarly to the untranslated sequences in the 3′ region of other flaviviruses, they have important functions in initiating negative-sense RNA synthesis during genome replication.

14.5.3 Viral Proteins

14.5.3.1 Polyprotein

The yellow fever virus polyprotein contains 3,411 amino acids (3,412 in TBEV). The sequences of the structural proteins are located in the amino-terminal third in the following order: capsid protein, viral envelope proteins PrM (as a precursor product of the M protein) and E (Fig. 14.11, Table 14.12), followed by the sequences of non-structural proteins NS1–NS5.

The protein arrangement is different in hepatitis C virus: the polyprotein, which comprises on average 3,000 amino acids, does not contain the sequences of the PrM protein. The E1 protein, a glycosylated envelope protein with a molecular mass of about 33 kDa, is localized after the capsid protein. Thereafter follow the sequences of a second glycoprotein (E2, gp68–72) and the small protein p7. In hepatitis C virus, NS1 is present neither as a gene nor as a protein (Fig. 14.11). The protein succession C–E1–E2–p7 is followed by non-structural proteins NS2 to NS5B. Table 14.12 provides a comparative summary of the properties of the proteins.

Like the polyproteins of hepaciviruses, the polyproteins of pestiviruses also do not contain the sequences of NS1 protein; pestiviruses also have two glycosylated envelope proteins, E1 and E2, followed by the sequences of p7 protein. Furthermore, the polyproteins of pestiviruses have some additional features: differing from flaviviruses and hepaciviruses, their genomes encode the non-structural protein N^{Pro} at the 5′ terminus of the viral genome; in the polyprotein it is located before the domains of the structural proteins (Fig. 14.11). After the sequences encoding the capsid protein C, the genetic information for an RNase (E^{rns} protein, for "envelope protein, RNase secreted") follows; this RNase is part of the virus particle and is also secreted by infected cells.

Processing of the structural protein moiety of the precursor product into the individual, functionally active components of the C, PrM and E proteins (flaviviruses), the C, E1, E2 and p7 proteins (hepaciviruses) or the C, E^{rns}, E1, E2

Table 14.12 Comparison and functions of flavivirus proteins

Protein	Yellow fever virus	TBEV	Hepatitis C virus	BVDV	Function
N^{pro}	–	–	–	23 kDa	Protease, autocatalytically cleaved from precursor protein; causes ubiquitylation and degradation of IRF-3
C	12–14 kDa	13–16 kDa	22 kDa	14 kDa	Capsid protein, interaction with RNA genome
PrM/ M	18–19 kDa 7–9 kDa	24–27 kDa 7–8 kDa	–	–	Membrane protein; cleavage by the protease furin
E	51–59 kDa	50–60 kDa	–	–	Glycosylated membrane protein, neutralizing antibodies, haemagglutinin, attachment
E^{rns}	–	–	–	44–48 kDa	RNase, secreted, glycosylated
E1	–	–	31–35 kDa	25–33 kDa	Membrane protein, glycosylated
E2	–	–	70–72 kDa	53–55 kDa	Membrane protein, glycosylated
p7	–	–	7 kDa	7 kDa	Ion channel, hydrophobic
NS1	19–25 kDa	39–41 kDa	–	–	Highly conserved, glycosylated, secreted, cell-membrane-associated, not a component of virus particles
NS2A	20–24 kDa	20 kDa	–	–	ER-membrane-associated, morphogenesis
NS2B	14 kDa	14 kDa	–	–	Zn^{2+} metalloproteinase, associates with NS3 protease (TBEV and similar viruses)
NS2	–	–	21–23 kDa	38–54 kDa	Zn^{2+}-binding
NS2/3	–	–	90–95 kDa	120–125 kDa	Zn^{2+}-binding, autocatalytic protease, it performs the cleavage into NS2 and NS3 in hepatitis C virus and in pathogenic BVDV strains
NS3	68–70 kDa	70 kDa	70 kDa	75–80 kDa	Serine protease, cleaves the non-structural proteins from the polyprotein; dsRNA helicase

(*continued*)

Table 14.12 (continued)

Protein	Yellow fever virus	TBEV	Hepatitis C virus	BVDV	Function
NS4A	16 kDa	16 kDa	8–10 kDa	7–10 kDa	Hydrophobic, associates with ER membrane, it inhibits INF-α/INF-β-mediated signalling (flaviviruses), it forms heterodimers with NS3 protease in hepatitis C virus and BVDV
NS4B	26 kDa	27 kDa	27 kDa	30 kDa	Hydrophobic, associates with ER membrane
NS5	103–104 kDs	100 kD*	–	–	Methyltransferase, RNA-dependent RNA polymerase
NS5A	–	–	56–58 kDa	58–70 kDa	Phosphorylated, membrane anchored; virus morphogenesis
NS5B	–	–	68–70 kDa	75–78 kDa	RNA-dependent RNA polymerase

The protein succession order in the table corresponds to the real order in the precursor polyprotein
BVDV bovine viral diarrhoea virus, *IRF-3* interferon regulatory factor 3, *dsRNA* doubled-stranded RNA, *INF* interferon, *TBE* tick-borne encephalitis virus

and p7 proteins (pestiviruses) is done by the cellular signalase, which is associated with the ER membrane. In cellular metabolism, this protease removes the signal peptides from the amino-terminal ends of proteins that are translated at the ER. The pestivirus N^{pro} protein is an autocatalytically active protease which cleaves itself cotranslationally from the nascent precursor protein. For all further processing reactions, viral proteases are primarily responsible: the cleavage between the NS2 and NS3 moieties is performed by the proteolytic activity of the NS2B protein; in hepatitis C virus and in non-cytopathogenic strains of BVDV, a proteolytic activity that is located in the amino-terminal domain of the NS3 protein is responsible for such reactions, but this activity is only exhibited in a fusion product of the NS2 and NS3 proteins. The NS3 protein acts as a serine protease and performs all other cleavage reactions; in hepatitis C virus, the NS4A protein is required as a cofactor for this purpose.

14.5.3.2 Structural Proteins

The capsid is constituted of C proteins. It contains a large number of basic amino acids which interact with the RNA genome, thereby forming the nucleocapsid. The carboxy terminus of the C protein is highly hydrophobic. Together with further hydrophobic domains, it mediates the interaction of the polyprotein with the ER membrane. Cleavage reactions are induced by the signalase between the following

domains: C and PrM (flaviviruses), C and E1 (hepaciviruses) and C and E^{rns} (pestiviruses). A peculiarity has been found in BVDV: mutants have been described completely lacking the sequences that encode the C protein, but which, nevertheless, exhibit the morphology of flaviviruses. Accordingly, the C protein is not essential for the formation of infectious particles. In this case, the functions of the C protein are assumed by the NS3 protein, which interacts with the RNA genome and E proteins.

The PrM protein, which is glycosylated at asparagine residues, is the precursor of the very small, non-glycosylated M protein that is anchored in the viral envelope. Exhibiting a molecular mass of approximately 19 kDa in yellow fever virus and 24–27 kDa in tick-borne flaviviruses, the PrM protein is significantly larger than the M protein in infectious particles. The amino-terminal part of the PrM protein is cleaved by the cellular protease furin at a late stage of viral morphogenesis during the passage of the immature virus through the Golgi apparatus. This cleavage reaction is essential for the infectivity of virus particles; it induces the fusogenic properties of the E protein to fuse endosomes with the viral envelope after the virus has entered the cell.

In addition, the viral envelope contains 90 dimers of glycosylated E proteins. The structure of the E protein of TBEV was elucidated by Félix A. Rey and colleagues by means of X-ray diffraction in 1995. In comparison with other structurally known viral surface proteins (► Sect. 16.3; haemagglutinin of influenza viruses), the E protein has an unusual structure: it is anchored in the membrane by a hydrophobic amino acid sequence in the carboxy-terminal region, it lies flat on the membrane and determines the size of the particle because of a bending effect caused by protein folding (Fig. 14.12). The E protein mediates attachment of the virus to target cells, and induces fusion of the viral and endosomal membranes by low pH after entry of the virus via receptor-mediated endocytosis. It is also responsible for the haemagglutination properties of flaviviruses. Virus-neutralizing antibodies directed against the E protein are induced during infection. They protect against reinfections with the same virus type.

The glycosylated E1 and E2 proteins of hepatitis C virus share sequence homologies with the corresponding proteins of pestiviruses. E1 proteins are not covalently associated with E2 proteins; they are anchored in the membrane by hydrophobic, carboxy-terminal amino acid sequences. There is a highly variable region in the C-terminal region of the E2 protein, and this differs among the different serotypes of hepatitis C virus and also among individual virus isolates.

In pestiviruses, another envelope protein has been characterized: E^{rns} protein. It is a virion component, has double-stranded RNase activity, is secreted by infected cells and induces the formation of neutralizing antibodies. The function of this protein during viral replication has not been fully clarified. In BVDV, the E^{rns} protein counteracts the immunological responses that are triggered by double-stranded RNA by its ability to degrade double-stranded RNA. These include, among others, the activation of TLR3, whereby the production of IFN-α and IFN-β is induced (see ► Chaps. 7 and ► 8) (Fig. 14.13).

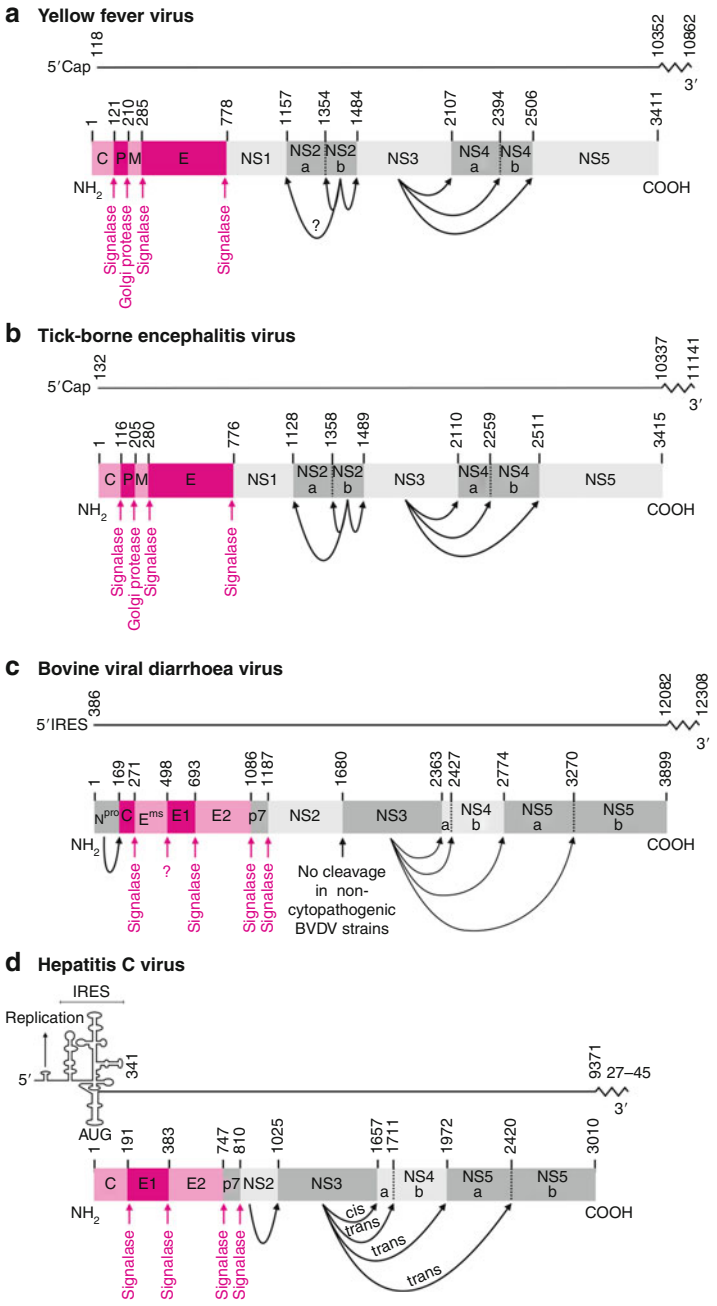


Fig. 14.12 Genome organization of flaviviruses. (**a**) Yellow fever virus, vaccine strain 17D. (**b**) TBE virus, strain Neudörfl. (**c**) Hepatitis C virus. (**d**) Bovine viral diarrhoea virus (*BVDV*), strain SD-1 (non-cytopathogenic). In hepatitis C virus and pestiviruses (BVDV), an IRES is

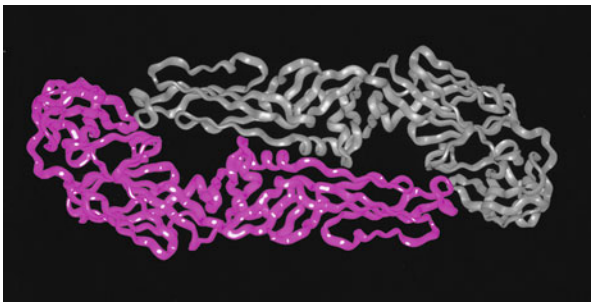


Fig. 14.13 Structure of the TBE virus E protein, represented in a ribbon model. A plan view of the homodimeric protein complex (the protein is located here on the surface of the virus) is shown. The carboxy-terminal domain, which contains the transmembrane region, was removed by proteolytic digestion (Courtesy of Franz X. Heinz, University of Vienna)

Numerous Viruses Are Able to Agglutinate Erythrocytes

Many viruses can cause haemagglutination by the respective activities of their surface proteins. That means the virus-induced agglutination and aggregation of red blood cells. Before the development of highly specific ELISA and PCR tests for detection of viral infections, the haemagglutination and haemagglutination-inhibition tests were very important diagnostic methods (▶ Chap. 13). In rare cases, they are still used today.

14.5.3.3 Non-Structural Proteins

The flavivirus NS1 protein is associated with the cell membrane. In infections of mammalian cells, but not insect cells, a soluble variant of the NS1 protein which is secreted by the cells can be observed. There is evidence that the membrane-associated NS1 protein exists as a dimer; in contrast, the secreted form is present as a hexamer. In some flaviviruses, NS1-specific antibodies seem to induce the antibody-mediated lysis of infected cells, thus exerting a protective effect. The function of the protein during the infection cycle is unknown. It is possibly involved in the replication of viral genomes, as well as in intracellular transport of viral structural proteins and in release of the virus. An immunomodulatory function has recently been found for the NS1 protein of West Nile virus: it blocks the signal transduction pathway that is mediated by TLR3 by impeding the transport of IRF-3

Fig. 14.12 (continued) located in the 5′ UTR. In members of the genus *Flavivirus* (yellow fever virus and TBE virus), the 5′ end of the genome is modified by a cap group. The genomes of flaviviruses have a continuous open reading frame. It encodes a polyprotein that is proteolytically cleaved into the different protein components (structural proteins in *colour*). Responsible for this process are enzymes which are a constitutive part of the polyprotein and autocatalytically activate themselves, as well as cellular proteases and signalases. The *numbers* refer to the amino acid positions in the polyprotein at which the cleavages occur

and nuclear factor κB into the nucleus. As a result, the production of IFN-β and proinflammatory cytokines such as IL-6 is inhibited. Furthermore, both the soluble and the membrane-associated variant of the West Nile virus NS1 protein bind to protein factor H, a regulator of complement activation. This leads to decreased accumulation of both the C3 component and the membrane attack complex (C5B to C9; see ▶ Chap. 7).

The 7-kDa protein of hepatitis C virus and pestiviruses is a small hydrophobic protein; it is believed to be present as a membrane-anchored protein, which possibly functions as an ion channel protein.

In flaviviruses, NS2 protein is cleaved into proteins NS2A and NS2B. There are few data regarding the function of NS2A. It is associated with the ER membrane and plays an important role during flavivirus morphogenesis. It also seems to inhibit the interferon-mediated antiviral immune response. The NS2B protein of flaviviruses is an essential cofactor of NS3 protease. In hepatitis C virus, the NS2 moiety fused with NS3 forms the catalytic domain of a Zn^{2+}-dependent protease which catalyses the cleavage between NS2 and NS3.

The NS3 protein of all flaviviruses is bifunctional: in the amino-terminal region, a serine protease has been localized that is responsible for all cleavage reactions in the regions of the polyprotein that follow the NS3 domain. NTP-binding sites and a helicase activity are found in the carboxy-terminal region. The latter belongs to the helicase DExH/D box superfamily, and is necessary for unwinding the highly structured double-stranded RNA intermediates that are formed during genome replication and during translation of the polyprotein. The NS3 protease of flaviviruses is a heterodimer consisting of NS2B and NS3. In hepatitis C virus, NS3 interacts with NS4A by its amino-terminal region, which contains the active centre of the serine protease, thereby forming a heterodimer that is anchored in the ER membrane by the hydrophobic domains of NS4A.

The NS4A protein of hepatitis C virus primarily has the function of interacting with the NS3 protease. Thereby the NS3/NS4A protein complex associates with the ER membranes and remains as part of the replication complex. The NS4A protein of flaviviruses is also associated with the ER membrane, but does not interact with the NS3 protein. It contributes both to the rearrangement of ER membranes and to inhibition of the signal transduction pathways that are mediated by IFN-α/IFN-β, thereby inhibiting phosphorylation of STAT1 and STAT2 proteins.

Little is known concerning the function of the membrane-anchored NS4B protein. In hepatitis C virus, it induces the formation of specific intracellular membrane compartments, where replication of the viral genomes occurs. In the case of flaviviruses, there are indications that the NS4B protein, like NS4A, impedes the interferon-mediated immune response; however, these data are controversial.

The NS5 protein of flaviviruses is a multifunctional enzyme: the amino-terminal domain possesses a methyltransferase activity that is required for capping the 5′ terminus of the genome. Since viral replication and proliferation occur in the cytoplasm of infected cells, viruses cannot rely on and use respective cellular enzymes since these are localized in the nucleus. In addition, the

amino-terminal NS5 domain also functions as an antagonist of interferon: it blocks the interferon-stimulated Jak/STAT signal transduction pathways and prevents the expression of interferon-stimulated genes. The carboxy-terminal domain of the NS5 protein has RNA-dependent RNA polymerase activity. The NS5 protein of hepatitis C virus and pestiviruses is cleaved by the NS3 protease into the moieties NS5A and NS5B. The NS5B protein constitutes the RNA-dependent RNA polymerase, which is essential for replication of the RNA genome. The membrane-anchored NS5A protein is phosphorylated and binds to RNA. The carboxy-terminal domain of NS5A has important functions in viral morphogenesis: it induces the accumulation of C proteins in intracellular membrane compartments. Deletions of the carboxy-terminal domain of NS5A suppress the formation of infectious progeny viruses.

The non-structural proteins of pestiviruses have some peculiarities: in BVDV, they are responsible for the formation of different biotypes and play an important role in the pathogenesis of mucosal disease (Sect. 14.5.6). Unique is the already mentioned N^{pro} protein, which acts as a protease and constitutes the first domain of the polyprotein. It is capable of autocatalytic cleavage from the precursor protein. Additionally, N^{pro} has another function that is important for the pathogenesis of pestivirus infections. Both in classical swine fever virus and in BVDV, it is apparent that N^{pro} binds to IRF-3 and that this interaction induces the ubiquitination and degradation of IRF-3 via proteasomes. Thus, infected cells have a significantly reduced expression of IFN-β (see also ▶ Chaps. 7 and ▶ 8).

14.5.4 Replication

The cellular receptors of many flaviviruses are known. In hepatitis C virus, receptor binding is a very complex and multifaceted process. The initial attachment to the target cells requires glycosaminoglycan and LDL receptors. Thereafter follow interactions of the viral E2 proteins with at least three entry factors, namely scavenger receptor class B type 1 (SR-B1), tetraspanin 28 (CD81) and the tight-junction protein claudin 1. The E2 protein binds to CD81 (25 kDa), a member of the tetraspanin superfamily, which spans the cytoplasmic membrane with four transmembrane regions, thereby forming two extracellular domains. CD81 has functional activities in cell adhesion, cell activation and cell motility, as well as in signal transduction pathways; it is found on the surface of many cell types, such as hepatocytes and B lymphocytes, which can be infected by hepatitis C viruses. The E2 proteins also bind to SR-B1, an 87-kDa protein found on the surface of many different cell types that are involved in cellular lipid metabolism. Serum proteins such as high-density lipoproteins are ligands for the SR-B1 receptor and enhance the infectivity of hepatitis C virus, possibly because they form complexes with the E1/E2 proteins on the surface of the virus and mediate the interaction with the SR-B1 receptor. A similar binding mechanism has also been proposed for LDLs: these serum proteins can also bind to virus particles, thereby mediating the interaction with the LDL receptor.

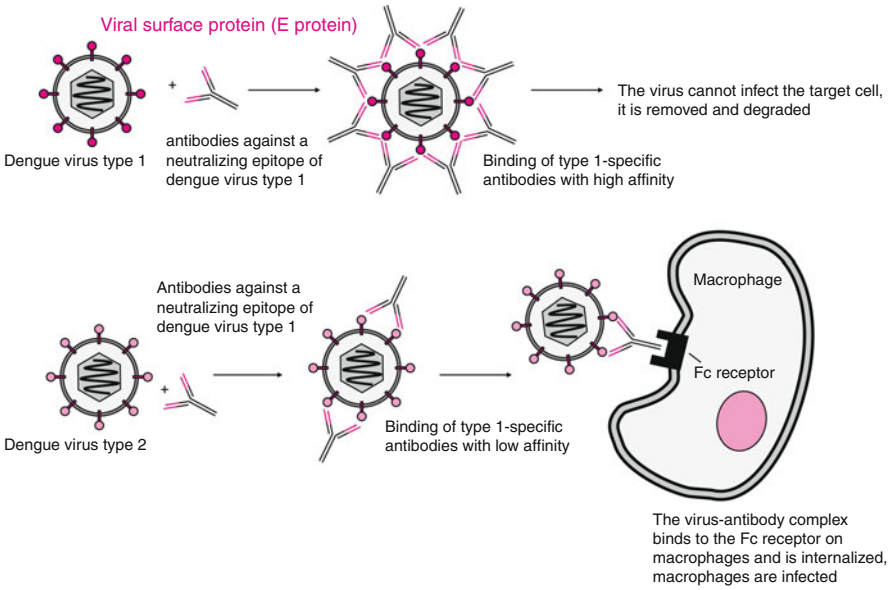


Fig. 14.14 Mode of operation of infection-enhancing antibodies. After an infection with a particular subtype of dengue virus, e.g. dengue virus type 1, subtype-specific antibodies are developed in the organism. In the case of reinfections with the same virus subtype, they can bind to the virus particles, neutralizing the virus. However, if a subsequent infection with another dengue virus subtype occurs, e.g. dengue virus type 2, the antibodies will also bind to the surface of dengue virus type 2 capsids since the amino acid sequences of both virus types are similar. Type 1-specific antibodies are not able to neutralize dengue virus type 2, however. Via the Fc region of immunoglobulins, virus–antibody complexes bind to Fc receptors present in the membrane of macrophages. This results in internalization of the complex, leading to infection of macrophages

Dengue virus and TBEV bind with low affinity to heparan sulphate, whereas integrins ($\alpha_v\beta_3$) have been described as interaction partners for West Nile virus. Besides the direct interaction of the viral envelope proteins with defined cell surface components, a second way of binding and penetration has been described for dengue virus: it depends on the presence of subneutralizing concentrations of virus-type-specific antibodies or cross-reacting immunoglobulins, which can recognize various types of related viruses. The latter is valid for dengue virus, which comprises four different serotypes. The virus-type-specific antibodies can only bind to epitopes that are specific for the respective serotype. However, there are additional domains especially in the E protein of dengue virus which are commonly shared by all serotypes. These virus-type-overlapping and cross-reacting antibodies are usually not neutralizing. If viruses are mixed in vitro with such cross-reacting antibodies or with low concentrations of type-specific antibodies that can bind to the particle surface, entry of the virus and viral infection can be induced via the Fc portion of immunoglobulins by mediating the interaction with Fc receptors on monocytes and macrophages (Fig. 14.14). This phenomenon is known as immune enhancement.

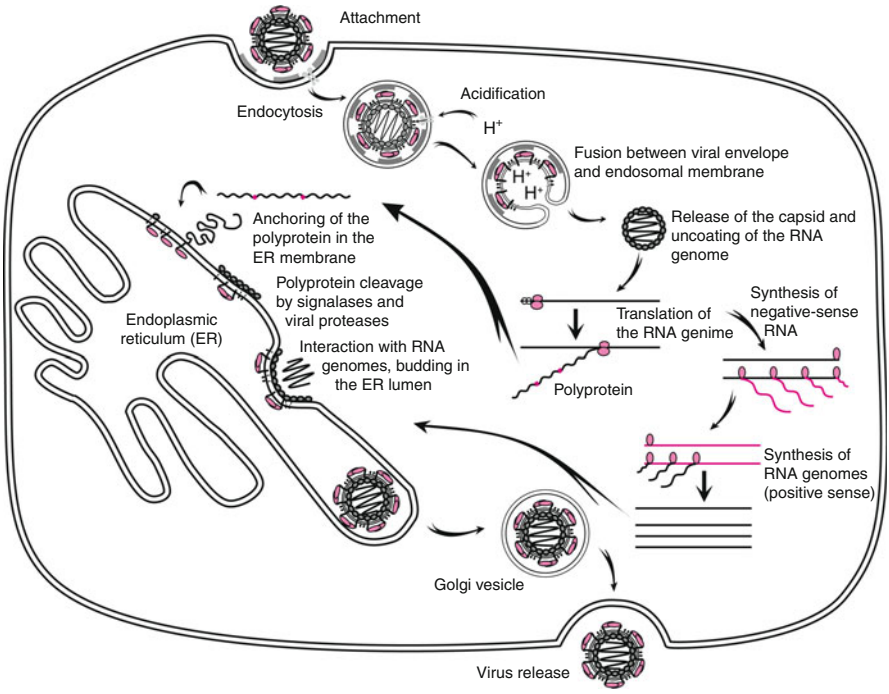


Fig. 14.15 TBE virus life cycle (the nucleus is not shown for clarity). The virus attaches to a still unknown receptor on the cytoplasmic membrane, and penetrates the cell by endocytosis. Endosomal acidification induces the fusion of the endosomal membrane with the viral envelope, whereby the capsid is released into the cytoplasm. The viral genome is used as mRNA and is translated into a polyprotein. The polyprotein is embedded in the endoplasmic reticulum (*ER*) membrane by signal-peptide-like protein domains. All subsequent steps of the infection cycle occur in close proximity to this cell compartment. The signalase, which is associated with the ER, cleaves the precursor protein in the region of structural proteins C, PrM and E. All other cleavage reactions in the region of non-structural proteins are performed by the NS3 protein, which together with the NS2B protein acts as a protease. These processing reactions give rise to the NS5 protein, which carries the RNA-dependent RNA polymerase activity and transcribes the positive-sense genome into negative-sense RNA molecules. In turn, these serve as templates for the synthesis of new viral genomes. Newly synthesized viral genomes accumulate in regions of the ER membrane that contain high concentrations of viral structural proteins. This leads to budding of virus particles into the ER lumen. In the further course, these are transported via Golgi vesicles to the cell surface and released

After attachment to the cell surface, the virus enters the cell by endocytosis (Fig. 14.15). Afterwards, it is present within the endosome in the cytoplasm and must be released from this membrane vesicle. For this purpose, the interior of the endosomes is acidified via an ATP-dependent proton pump, which is a component of the vesicular membrane. The endosomal membrane fuses with the viral envelope. The E and E1 proteins of flaviviruses and hepaciviruses/pestiviruses,

respectively, are actively involved in this membrane fusion process. During this process, the dimeric E proteins change their structure and form trimeric intermediates, in which the fusogenic domain is exposed, developing its activity. In this way, the viral capsid enters the cytoplasm; the mechanism that leads to the release of the viral nucleic acid is poorly understood.

In the next steps, the 5′ end of the genome interacts with cellular ribosomal subunits. In flaviviruses, the 5′ cap structure is responsible for this process; it binds to the components of the cap-binding complex and mediates the interaction with the ribosomes. In the case of hepatitis C virus and pestiviruses, the IRES sequence, which is located in the UTR at the 5′ terminus, is responsible for binding of ribosomes. If the C protein is present at the amino-terminal region of the polyprotein after initiation of translation, the elongation of the amino acid chain stops temporarily: the hydrophobic domain in the carboxy-terminal region of the C protein acts as a signal peptide. It interacts with the signal recognition particle, a complex of cellular polypeptides and the 5S RNA, which induces the transport of the translation complex to the ER membrane. There, the nascent amino acid chain is translocated through the ER membrane, whereby the transmembrane domains of PrM and E proteins cotranslationally anchor the polyprotein in the lipid layer (Fig. 14.15). Signalases perform the processing reactions between the C, PrM, E and NS1 moieties. Further cleavage reactions of the polyprotein are performed by NS2B/NS3 (flaviviruses) and NS3/NS4A (hepatitis C and pestiviruses) proteases (Fig. 14.12). Most of the nonstructural proteins have hydrophobic domains that contribute to their anchorage in the ER membrane. This ensures that the synthesis of the polyprotein and the subsequent steps of replication can be executed in association with this intracellular membrane compartment.

As soon as the RNA-dependent RNA polymerase and the NS5 and NS5B proteins are available in the cytoplasm, they catalyse the transcription of the positive-sense RNA genome into complementary negative-sense RNA molecules, which in turn serve as templates for the synthesis of new positive-sense RNA genomes; in hepatitis C virus, the hairpin structure in the UTR at the 5′ end of the RNA genome is essential for this process (Fig. 14.12d). It seems to have a function similar to that of the cloverleaf structure of the picornavirus genome (see Sect. 14.1). The newly synthesized positive strands are used as both genomic RNAs and mRNAs for the synthesis of further polyproteins. The details of these processes are largely unknown. In general, however, the mechanism is similar to that of picornavirus replication. Since viral replication occurs exclusively in the cytoplasm, where cellular capping enzymes are not accessible, flaviviruses have evolved their own capping enzymes. The NS5 methyltransferase is responsible for the synthesis of 5′-cap structures of positive RNA strands.

In flaviviruses, morphogenesis and formation of infectious particles is performed at the ER membrane. Analogous processes are presumed in hepatitis C virus and pestiviruses. The C, PrM and E components as well as the E1 and E2 polypeptides are inserted into the lipid layer during translation, where they accumulate and form regions containing high concentrations of viral proteins. The membrane-associated C proteins interact with both the carboxy-terminal domain of E proteins, probably

also with that of NS2A proteins, and the RNA genomes via basic amino acids. The membrane invaginates into the ER lumen, thus forming the initial budding complex, which is finally released. In this phase of viral morphogenesis, the virus particles are not yet infectious: E proteins of flaviviruses are present as a heterodimer complex with PrM polypeptides. During the following transport through the Golgi apparatus, the envelope proteins are glycosylated and PrM is processed into the M protein by the protease furin. As a consequence, the complex between E and PrM proteins is resolved and the E proteins form homodimers. Thereby the immature, non-infectious virus particles become mature virus particles. Finally, the Golgi vesicles fuse with the cytoplasmic membrane, releasing the infectious virus particles into the surrounding environment. The replication cycle of flaviviruses is illustrated schematically in Fig. 14.15.

14.5.5 Human Pathogenic Flaviviruses

The members of the genus *Flavivirus* that are pathogenic for humans are transmitted to humans by mosquito or tick bites. The tick-borne viruses are found in Europe and Asia, where they preferentially infect rodents as natural hosts; in central Europe, TBEV belongs to this group. Viruses that are transmitted by mosquito bites can be divided into two groups. Mosquitoes of the genus *Aedes* preferably bite mammals. Viruses that are transmitted by these mosquitoes, such as the pathogens of yellow fever and dengue fever or dengue shock syndrome, may cause febrile illnesses in humans, and these can be associated with haemorrhage. *Culex* mosquitoes prefer birds as hosts and transmit viruses such as the pathogens of West Nile fever, St. Louis encephalitis and Japanese encephalitis that cause neurological diseases such as meningitis and encephalitis in humans. The hepatitis C and hepatitis G viruses are primarily transmitted through contaminated blood or other body fluids; an arthropod-mediated transmission is not known for these infectious agents. As no illnesses have been observed in infections with hepatitis G virus so far, they will not be discussed in detail in the next section.

Origin of Yellow Fever Epidemics

Under natural conditions, yellow fever virus can undergo two infection cycles: jungle or savannah yellow fever and urban yellow fever. Jungle or savannah yellow fever is transmitted by mosquitoes of the species *Aedes africanus* and *Aedes haemagogus*. They breed in water accumulations in tree holes, puddles or holes in the ground. Female insects can vertically transmit the virus to their offspring. Monkeys, as intermediate hosts, also maintain the infection chain of jungle or savannah yellow fever. New World monkeys in South America become sick and may die from the infection, whereas Old World monkeys in Africa have apparently adapted well to the virus during evolution and are often infected only subclinically. As side links in the

transmission chain, the virus can be transmitted to people who stay in those regions, causing sporadic diseases. Yellow fever virus can be carried by infected individuals into urban regions where the mosquito species *Aedes aegypti* is ubiquitous; these species become infected with the virus, leading to its epidemic dissemination.

14.5.5.1 Yellow Fever Virus

Epidemiology and Transmission

Yellow fever virus is transmitted by mosquitoes of the genus *Aedes*. The first historically documented cases occurred in Mexico in 1648. Probably, the virus was originally widespread only in Africa. However, the slave trade between Africa and North America and South America by the Spanish and English conquerors led to the introduction of the mosquito/virus combination during the seventeenth and eighteenth centuries, and thereby to the epidemic dissemination of yellow fever in the tropical regions of America. Yellow fever appeared primarily in coastal cities of Africa, America and southern Europe, and it was one of the major diseases of mankind, claiming thousands of victims. There were more than 100,000 deaths from yellow fever infections during the construction of the Panama Canal. It could only be completed after the mosquitoes had been exterminated as carriers of the infection. As early as 1881, the Cuban physician Carlos Finlay presumed that the disease is transmitted by insects. This assumption was finally proved by Walter Reed in 1900. In 1902, yellow fever virus was identified as the aetiologic agent of the epidemic yellow fever; in 1929, it was transferred to monkeys, thus paving the way for further research.

Today, yellow fever is endemic in regions of Africa south of the Sahara – mainly in the tropical forests of West Africa – and in South America. Yellow fever virus can proliferate with differing efficiency in various *Aedes* species, which are well adapted to different environmental conditions in jungles, savannahs and urban centres. In Asian countries, the disease has not emerged so far. It is assumed that Asian *Aedes* species are less susceptible to the virus, and thus constitute bad carrier vectors. On the other hand, it seems conceivable that cross-immunities with dengue viruses, which are widespread in Asian countries, might prevent the occurrence of apparent yellow fever infections. It also seems possible that people in Africa may have developed a wide cross-reactive immunity owing to numerous viral infections by insects. These conditions may have prevented major epidemics of yellow fever in the population; therefore, only non-immune Europeans were affected. However, because of the advancing urbanization in recent decades, the living conditions in Africa have changed greatly, so yellow fever is now frequently observed. In recent years, epidemic outbreaks of yellow fever with more than 100,000 cases have been reported in Nigeria. The annual number of officially reported infections is approximately 2,000 in South America. Presumably, the number of unreported cases may be very high.

Yellow fever virus is genetically very stable, and there is only one serotype. It is present in the blood of infected individuals for several days during the disease.

If they are bitten by a mosquito during this time, the virus is absorbed with the blood. The virus proliferates in the intestinal epithelium as well as in body and salivary gland cells of insects. This process lasts approximately 1 week, and has been named the extrinsic incubation period. Thereafter, the mosquito is able to transmit the yellow fever virus with the salivary secretion by biting.

Clinical Features

Usually, the first symptoms of fever, nausea, headache and muscle pain appear 3–6 days after the mosquito bite; at this stage, infected people are viraemic. After a short-term improvement, in a subset of patients the symptoms may appear more pronounced, accompanied by resurging fever, vomiting of blood (signalizing haemorrhage), dehydration, hypotension, abdominal pain and signs of renal failure. In this stage of the disease, patients develop the signs of jaundice because of the destruction of liver cells and the associated increase of the level of bilirubin. The virus is then no longer present in the blood. Half of patients who enter the second phase die of severe kidney and liver failure, shock and delirium within 7–10 days. Subclinical or abortive forms of infection, in which the symptoms appear in a mitigated form or do not emerge at all, are more frequent than fulminant forms of yellow fever. The overall lethality of yellow fever is 20–50 %.

Pathogenesis

After having been introduced into the bloodstream by a mosquito bite, yellow fever viruses infect endothelial cells, lymphocytes and preferably macrophages and monocytes near the injection site. These cells transport the virus via lymphatic vessels to the lymph nodes and lymphoid tissues, where they encounter other susceptible target cells. During viraemia, high amounts of infectious viruses are produced that infect liver macrophages (Kupffer cells), which die as a result of viral replication. Next, the virus infects and destroys hepatocytes. This leads to a strong increase in the concentration of transaminases in the blood. In rare cases, infected macrophages may transport the yellow fever virus to the brain, where it may cause encephalitis. The haemorrhages, which become obvious by internal bleeding in the kidney, brain and other organs during the symptomatic phase of infection, are ascribed to a reduced production of clotting factors due to infection and subsequent destruction of liver cells.

Immune Response and Diagnosis

Yellow fever virus can be easily cultivated in human cells (HeLa and KB cell lines) and in monkey kidney cells (Vero cells) in vitro. It may also be reproduced in chicken and duck embryonic cells as well as in continuously growing rodent cell lines. In infected individuals, IgM and IgG antibodies against the E and M proteins can be detected about 1–2 weeks after infection (i.e. 5–7 days after the onset of symptoms) by ELISA, immunoblotting and immunofluorescence, haemagglutination-inhibition and virus-neutralization tests. In addition to these serological methods, detection of viral genomes by RT-PCR in blood samples is the method of choice in the early stages of infection. Neutralizing antibodies

persist lifelong, and confer durable protection against reinfections. During the viraemia, NS1-specific antibodies can induce antibody-dependent lysis of infected cells, thus making an important contribution to the control of infection and to the clearance of the virus from the organism. To what extent the cellular immune system is involved in clearance of the virus by induction of cytotoxic T cells is unclear.

Therapy and Prophylaxis

By continuous cultivation of yellow fever virus in embryonated chicken eggs, in 1937 Max Theiler was able to breed an attenuated yellow fever virus (strain 17D) which in humans does not cause any symptoms. In 1951, Theiler was awarded the Nobel Prize in Physiology or Medicine for the first development of a live vaccine. The molecular basis of attenuation is unknown. In comparison with the wild-type genome, strain 17D has a total of 68 nucleotide mutations, which lead to 32 amino acid changes in viral proteins. Most mutations are located in the gene encoding the E protein, so it is suspected that the vaccine virus may bind less efficiently to the receptors on liver cells. As a consequence, less virus is produced, which results in slower infection rates, and hence in a mild or attenuated form of infection. Low virus concentrations are found in the blood of vaccinated individuals about 3–5 days after inoculation; viraemia lasts for 1–2 days. The first immune response is detectable in 95 % of vaccinated individuals 10 days after vaccination. To maintain protection, booster vaccinations are needed in 10-year intervals. Back mutations to the wild type have never been observed; thus, the yellow fever vaccine is regarded as very successful and safe worldwide. Millions of people have been vaccinated to date. Hence, significant control and reduction of yellow fever infections has been achieved in tropical countries; large-scale epidemics are reported only very rarely today. The attenuated yellow fever vaccine is manufactured and distributed worldwide under the control of the WHO. It must be administered only in government-approved vaccination centres. Yellow fever vaccination is obligatory in many countries for travellers to or from endemic yellow fever regions.

In addition to vaccination of the population, the fight against the mosquito species involved in transmission of the virus is another important measure to control the infection, especially in endemic regions. In this relation, insecticides are just as important as the draining of breeding places for mosquito larvae.

The Attenuation of the Vaccine Virus Has Never Been Reproduced

The isolation of vaccine strain 17D of yellow fever virus by Max Theiler was serendipitous, i.e. a fortunate and accidental discovery: this strain was isolated by continuous cultivation of the wild-type virus in embryonated chicken eggs after 89–114 passages. All attempts to reproduce the result have not been successful so far.

14.5.5.2 Dengue Virus

Epidemiology and Transmission

Dengue fever has been known as a human disease for more than 200 years and was formerly called dandy fever or break-bone fever owing to the very strong joint and muscle pain. The first reports of an epidemic occurrence were from Indonesia and Egypt. A dengue fever epidemic also occurred in North America (Philadelphia) in 1780. Further outbreaks were subsequently observed periodically in almost all tropical and subtropical regions. In 1903, Harris Graham, working as a physician in Beirut, isolated a filterable pathogen from the blood of patients. Thomas L. Bancroft, an Australian physician and botanist, demonstrated its transferability by *Aedes aegypti* in 1906. In 1944, Albert Sabin and R. Walter Schlesinger identified dengue viruses as pathogens by transferring blood of infected soldiers into mice. Four different serotypes of dengue virus are known. Similarly to yellow fever, there are urban and rural forms of dengue fever. The latter is spread by *A. albopictus* and *A. scutellaris*; their natural hosts are considered to be non-human primates in tropical forests of Southeast Asia and South America. *A. aegypti* is especially involved in propagation and transmission of the infection in urban centres. Not all strains of *A. aegypti* mosquitoes are able to transmit dengue viruses. Genetic variants of the mosquitoes which do not produce receptor protein R67/R64 (molecular mass 67 kDa) in their intestinal epithelial cells are responsible for this. These mosquito strains (e.g. IBO-11) are not permissive for dengue virus infections; therefore, they cannot transmit the pathogens.

The spread of mosquitoes on the Asian continent particularly during the Second World War and the subsequent urbanization of the population led to a dramatic increase of dengue fever cases in Asia. Inasmuch as tourist traffic also increased at that time, infected mosquitoes were imported by aircraft from the Pacific regions to Central America and South America as well as to the USA. Today, dengue virus infects approximately 50 million people worldwide each year; hence, it is the most common insect-borne viral infection in humans, and is endemic in most urban areas of tropical countries. Epidemics emerge in intervals of 3–5 years. Every year, millions of people become sick from dengue fever and hundreds of thousands become sick from the associated haemorrhagic fever and the dengue shock syndrome. Whether individual dengue virus isolates differ in their virulence, and thus in determining of the severity of the disease, is uncertain. Possibly, such differences may explain the partially epidemic occurrence of dengue haemorrhagic fever, even in people who have been infected with dengue virus for the first time.

Clinical Features

The incubation period until the onset of symptoms of dengue fever lasts 3–7 days. Dengue viruses cause different disease forms. Especially in young children, the disease is a febrile illness without specific symptoms. Older children and adults develop the classic symptoms consisting of fever, skin rash and joint and muscle pain. These symptoms are associated with sensitivity to light and enlarged lymph nodes, petechial haemorrhages in the mucous membranes of the mouth, nose and

gastrointestinal tract, and thrombopenia and lymphopenia. The symptoms last for about 3–7 days, and most patients recover without any subsequent problems.

In addition to the symptoms described above, dengue haemorrhagic fever induces enhanced vascular permeability and increased internal bleeding. Blood plasma extravasates from the vessels into the surrounding tissues, leading to oedema, particularly in the abdomen and around the thoracic area. Dengue shock syndrome appears in patients with dengue haemorrhagic fever in which vascular permeability and haemorrhages are increased. The critical phase occurs when the body temperature suddenly decreases to normal levels or below (hypothermia), resulting in circulatory failure, bleeding in the gastrointestinal tract and neurological disorders. In such cases, shock conditions can occur, and are manifested by escape of blood plasma into the body cavities. Approximately 50 % of patients with dengue shock syndrome die.

The WHO has established strict criteria for the diagnosis of dengue haemorrhagic fever and dengue shock syndrome: these include severe fever, haemorrhagic symptoms, swelling of the liver and circulatory failure. The disease has been classified, depending on the severity, into four stages: stages I and II correspond to dengue haemorrhagic fever, and stages III and IV correspond to dengue shock syndrome.

Pathogenesis

Dengue viruses penetrate into the body through the bite of an infected mosquito, and infect macrophages that are located in the local environment. The infected macrophages carry the virus via lymphatic vessels to the lymph nodes, where the viruses find further target cells, in which they can replicate. After this phase, the patient is viraemic, and 10^8–10^9 infectious particles can be detected per millilitre of blood. On average, the viraemia lasts 4–5 days. In addition to macrophages, endothelial cells and possibly also bone marrow cells are susceptible to infection. Furthermore, the virus has also been detected in other organs, such as liver, lungs, kidneys and the gastrointestinal tract. To what extent it replicates in these tissues is unclear. The pathological alterations in the tissues are similar to those that can be observed in infections with yellow fever virus.

Dengue haemorrhagic fever and dengue shock syndrome are characterized by increased permeability of capillary vessel walls. Immunopathogenetic mechanisms seem to be responsible for the development of this severe disease form. The four dengue virus serotypes display homology of 63–68 % at the amino acid sequence level of their E proteins; contrarily, the homology between different variants of one dengue virus serotype is more than 90 %. Severe diseases occur especially if patients are infected with dengue virus for a second time, but now with a serotype different from that of the initial infection. Because of the primary infection, these patients possess dengue-virus-specific antibodies, which partially cross-react with the other serotypes. They can bind to the E protein on the surface of the virus. Owing to a low affinity determined by differences in the amino acid sequence of the epitopes, they are not neutralizing, but they enable the viruses, which are complexed with antibodies, to interact with Fc receptors on

macrophages, thus preferentially facilitating a more efficient penetration into the cells; therefore, the cross-reacting IgG molecules exert an infection-enhancing effect (Fig. 14.14). They contribute significantly to the development of dengue haemorrhagic fever and dengue shock syndrome.

Binding of both antibody-complexed and free dengue viruses on the surface of macrophages leads to the interaction of viral proteins with CLEC5A protein (C-type lectin domain family 5, member A, also known as myeloid DAP12-associating lectin, MDL-1). This surface protein does not act as a receptor for the interaction with dengue virus, but is involved in this process. The consequence is the induction of a signalling cascade that results in the release of large amounts of proinflammatory cytokines. If the interaction between the virus and CLEC5A is blocked by CLEC5A-specific antibodies in a mouse model, cytokine release does not occur, preventing the increase of vascular permeability.

It is uncertain to what extent the various activities of the non-structural proteins which suppress the IFN-α- and IFN-β-mediated defence strategies in vivo also influence the pathogenesis of the disease. This also applies to the property of dengue viruses to increase the expression of MHC class I antigens in infected cells in vitro. The high concentration of MHC class I proteins together with increased binding to the receptors, which are inhibitory for NK cells, lead to infected cells evading NK-cell-mediated lysis.

Immune Response and Diagnosis

IgM antibodies against viral E proteins can be detected by ELISA, immunoblotting and indirect immunofluorescence tests from the fifth day after the initial infection with dengue virus. They remain detectable for 2–3 months. The production of IgG antibodies ensues, they reach their maximum concentration about 2–3 weeks after infection and persist for a lifetime. A large percentage of antibodies against the E protein which were produced during the primary infection are not neutralizing and cross-react with other serotypes of dengue virus; only a relatively small portion of IgG molecules are type-specific and exert a neutralizing effect. In addition to the humoral immune responses, cytotoxic T cells may also be important for eradication of the virus from the organism. Clones of cytotoxic T lymphocytes which were able to lyse dengue-virus-infected cells have been detected in different people.

In secondary infections with other dengue virus serotypes, the IgM response is only of short duration. However, because there are already IgG antibodies against group-specific epitopes of E proteins, their synthesis is rapidly induced and IgG antibodies achieve more than the tenfold concentration that was detected during the initial infection.

Since dengue-virus-specific antibodies cross-react with other flaviviruses, particularly in countries where many different members of these viruses are endemic, the diagnosis of an acute infection is difficult by means of antibody detection. Therefore, explicit assertions can only be arrived at by a virus-neutralization test, by detecting viral RNA by RT-PCR or by isolation of the virus from blood samples of infected individuals; dengue viruses can be reproduced in vitro in various continuous cell lines (Vero or baby hamster kidney cells).

Therapy and Prophylaxis

So far, there are neither vaccines for prevention nor suitable antiviral drugs against dengue virus infections. The immunopathogenesis of antibody-dependent enhancement (also referred to as immune enhancement) which is related to dengue haemorrhagic fever and dengue shock syndrome renders the development of suitable vaccines very difficult. However, attenuated viruses of all four serotypes have been developed in Thailand with financial support from the Rockefeller Foundation. They are being tested as live vaccines in clinical trials. In addition, the fight against mosquitoes as the carrier of the disease and the combat against their breeding grounds is still of paramount importance.

14.5.5.3 Tick-Borne Encephalitis Virus

Epidemiology and Transmission

According to the geographical distribution, TBEV is classified into two groups: the Eastern subtypes are found particularly in the Asian part of Russia and the countries of the former Soviet Union; the Western subtypes prevail in the countries of central and eastern Europe, especially in Scandinavia and the European regions of Russia. In the literature, they are also known as Russian spring–summer encephalitis virus and central European encephalitis virus. Related virus types also exist in India (Kyasanur forest disease virus). TBEV is the only member of the group of central European encephalitis viruses which is widespread in central Europe. Louping Ill virus, which infects sheep in Great Britain and can cause encephalomyelitis, is closely related to TBEV; human disorders caused by louping Ill virus have been described only in individual cases. TBEV is endemic especially in Austria (Kärnten) and southern Germany (Danube region, Black Forest), in Slovenia, Croatia, Hungary, the Czech Republic, Slovakia, Poland, Lithuania, Latvia, Estonia and Russia. In endemic regions in central Europe the prevalence of TBEV in ticks is between 0.2 % and 0.5 %. TBEV is transmitted by tick bites, especially by *Ixodes ricinus*, the common wood tick, which is found in forests and alluvial areas. Infected ticks transmit the virus directly during sucking because TBEV accumulates in the salivary glands. Usually, people come into contact with ticks from grass and bushes when walking through infested areas. It is a mere delusion that ticks jump down from trees onto their victims. TBEV can be transmitted to humans and rodents during the period when ticks are active, particularly in the months from April to September/October. Within the tick population, TBEV can be transmitted transovarially to offspring. A rare infection route for humans is the transmission of TBEV by fresh milk and non-pasteurized raw milk products, especially from sheep and goats. These animals can be infected by ticks, and excrete the virus in the milk. However, the reservoirs of TBEV are small rodents. Infection of humans is a dead-end road because it interrupts the spread of TBEV.

Clinical Features

Infections with the central European virus type are relatively mild in comparison with infections with the eastern European virus type. About 70–90 % of individuals

infected remain asymptomatic, whereas the other 10–30 % develop a mostly gentle illness without lasting complications. The time between contact with the virus and the onset of the first symptoms is 1–2 weeks. The first signs of disease are flu-like symptoms such as fever, headache, nausea and sensitivity to light. They last about 1 week. During this time, viruses can be isolated from the blood. After this period, the health improves in most cases, but in about 10 % of patients only a transient improvement is observed for about 1 week. The second phase can range from a mild form of meningitis (inflammation of the meninx in the brain or spine; in roughly 55 % of cases) to severe forms of meningoencephalitis (inflammation of the brain; 35 % of cases) with tremor, dizziness, altered perception and paralysis. The involvement of the spinal cord is referred to as a meningomyelitis (5 %) or meningomyeloencephalitis (5 %). The mortality rate is approximately 1 % of patients with severe clinical courses. About 7 % of survivors of the second phase of infection have neurological sequelae such as paralysis, speech problems and epileptic seizures.

Pathogenesis

After inoculation by a tick bite, TBEV infects endothelial cells and macrophages at the site of the bite. The viruses are transported by them to the lymph nodes, where they find appropriate target cells for further reproduction cycles. From the lymphatic system, the viruses reach the blood. They spread in the body, and settle in the cells of the reticulohistiocytic system, where they proliferate. Infected macrophages transport the viruses into the central nervous system. In addition to specificity for infection of lymphocytes, TBEV has a marked neurotropism. As a result of the infection, the brain swells (cerebral oedema), and there is locally limited bleeding. Histopathologically, the following alterations can be recognized: inflammations in the vicinity of blood vessels, neuronal degeneration and necrosis in the brainstem region, in basal ganglia of the spinal cord and in the upper and lower cortex. The anterior horn cells in the cervical spine are particularly sensitive to the infection. This also explains the emergence of paralyses that occur preferably in the upper extremities in myelitic cases.

The TBEV E protein seems to be the key parameter for the virulence of different virus isolates: mutation of one amino acid (tyrosine to histidine at position 384) can significantly alter the virulence of infection. Apart from virus-specific features, intrinsic genetic differences between infected hosts can also affect the expression and the severity of the disease: a study in Lithuania described that serious infections associated with severe encephalitis are statistically commoner in patients with genetic defects in the gene encoding chemokine receptor CCR5 (the receptor of CCL5, also known as RANTES).

Immune Response and Diagnosis

TBEV can be cultivated in embryonated chicken eggs, in chicken embryonic cell cultures and in mammalian cells lines. However, isolation from patients is very difficult. The diagnosis of acute infection is performed by detecting virus-specific IgM antibodies in ELISA from blood and/or cerebrospinal fluid. Detection of viral

genomes by RT-PCR is possible from blood, and especially from cerebrospinal fluid; however, it is usually not successful in later infection stages. IgG antibodies are produced in the course of infection, and are virus-neutralizing and remain detectable lifelong.

Therapy and Prophylaxis

There is an inactivated vaccine made of purified and formalin-inactivated virus particles, which are cultivated, for example, in primary chicken embryonic cells. The vaccine usually contains aluminium hydroxide as an adjuvant. It exhibits a very good seroconversion rate and protective efficacy after primary immunization (three vaccinations), which lasts 3–5 years. Thereafter, booster vaccinations are required at regular intervals. Preferentially, vaccination is usually performed for people in highly endemic regions or in population groups with a high risk of being bitten by ticks because they have to remain in forests and meadows for a long time for job-related reasons. The formerly postinfection passive immunization is no longer recommended because the vaccine exerts a negative influence on the course of infection in children. A postinfection active vaccination within 3–4 days after a tick bite is currently under discussion. Effective antiviral agents for symptomatic TBEV infections are not available.

14.5.5.4 Hepatitis C Virus

Epidemiology and Transmission

Hepatitis C virus has long been classified into the so-called non-A, non-B hepatitis viruses. In 1989, Daniel W. Bradley characterized the genome of these viruses. Today, six genotypes of hepatitis C virus are known from different geographical regions; their nucleic acid sequences differ by 31–34 %. The genotypes are also subdivided into various subtypes. The most common genotype, 1b, is found in Europe, followed by genotypes 2a, 3b, 2c and 3a; however, in North America, genotype 1a is commonest, followed by genotype 1b. Genotypes 4 and 5 have only been found in Africa; on the other hand, genotype 6 prevails in some regions of Asia (China, Korea). The number of people who are chronically infected with hepatitis C virus has been estimated to be 200 million worldwide. In Germany, the prevalence is 0.5–0.6 %.

Hepatitis C virus is found only in humans. Before the introduction of appropriate test procedures, it was transmitted mostly through blood transfusions or blood products. Today, the residual risk of becoming infected by receiving a positive blood transfusion is 1:100,000. Nearly 70 % of all new infections with hepatitis C virus now occur among drug addicts, and are caused by the common use of syringes. Further methods of transmission are sexual intercourse and, in rare cases, household contact with infected patients under poor hygiene conditions. Hospital staff are endangered by injuries with needles. Even so, the source of infection is not known in approximately 30 % of cases. The virus can be transmitted vertically from mother to child during pregnancy or at birth. However, a hepatitis C virus infection of the gestating mother is not considered an indication for caesarean section.

Discovery of Hepatitis C Virus

The identification and characterization of hepatitis C virus was done using molecular-biological methods. The blood of an experimentally infected chimpanzee was the source for the isolation of the viral RNA. Representative complementary DNA clones were synthesized from the viral RNA. The encoded proteins were expressed. It was attempted to identify proteins that react with sera from patients with chronic non-A, non-B hepatitis. The respective clone was sequenced. Then, oligonucleotides were synthesized, and were used to amplify the viral RNA genome from the blood of the chimpanzee by PCR. Finally, the genome was completely sequenced. In the last step, monoclonal antibodies against the viral proteins were produced, which allowed the identification of the virus particles.

Clinical Features

A generally slight liver inflammation appears after an average incubation period of 6–8 weeks. About 75 % of infections are asymptomatic; severe clinical courses are rare. Acute infections with clinical symptoms have a favourable prognosis. Up to 80 % of all infected individuals develop a chronic persistent hepatitis or reactivated chronic hepatitis. Persistent viral RNA can be detected in the blood of such patients by modern ultrasensitive PCR methods. Chronic infections are characterized by elevated transaminase levels, which, however, can fluctuate and can also be temporarily normal: the more active the infection, the higher the values. Only a few patients who have established a chronic infection show spontaneous elimination of the pathogen. This can be observed in 0.5–0.74 % of patients. Ten percent to 20 % of patients with chronic infection develop cirrhosis over the years, and about 4 % of them develop a primary liver cell carcinoma in the course of decades. A simultaneous infection with human immunodeficiency virus promotes the emergence of cirrhosis. Additional complications include periarteritis nodosa, membrane-proliferative glomerulonephritis and idiopathic Sjögren syndrome. This is determined by circulating mixed cryoglobulins, which are considered to be a consequence of the expansion of B-cell clones, which produce pathogenic IgM with rheumatoid factor activity.

Pathogenesis

The virus directly enters the circulatory system, mainly through contaminated blood or blood products, and is transported by infected macrophages into the liver, where it infects hepatocytes. The result is liver inflammation accompanied by cell necrosis. Cellular damage seems to be primarily induced by the immune response during hepatitis C. The virus itself is only weakly cytopathogenic, as was demonstrated by continuous replication of the entire viral genome in different cell types in vitro. IFN-α is produced and secreted by liver cells. Tubular structures have been observed in the cytoplasm of infected liver cells by electron microscopy. Little is known concerning the details of the pathogenesis of the acute infection.

Antigen–antibody complexes are formed in the chronic infection form, and can be deposited in the glomeruli. They seem to be responsible for the membrane-proliferative glomerulonephritis in such patients.

The hepatitis C virus exhibits a high mutation rate and changes in patients in the course of infection. New quasispecies are formed constantly. The mutations arise during replication with a probability of 2×10^{-3}. They are attributed to the fact that the viral RNA-dependent RNA polymerase, unlike cellular DNA polymerases, does not possess an exonucleolytic proofreading mechanism to control and enhance the accuracy of RNA synthesis. The classification of genotypes and subtypes of hepatitis C virus was originally based on the sequence of the NS5 gene. However, variations are found in all regions of the genome. Only the 5′-terminal UTR containing the IRES element is highly conserved. Mutations within viral genes are not uniformly distributed. There are variable and hypervariable as well as relatively conserved sequences. Hypervariable regions are located in the amino-terminal region of the E2 protein between amino acids 1–27 and 90–97. They are recognized by antibodies, and thus are exposed to a strong immune selection pressure. The virus changes both epitopes during a chronic infection, with the result that the antibodies are no longer able to recognize them. In a similar way, mutations also alter the epitopes which are recognized by cytotoxic T cells. Presumably, this immunological selection pressure promotes the development of viral variants, which can cause a chronic infection. In addition, it has been found that the non-structural proteins of flaviviruses possess several activities that allow the pathogens – including hepatitis C virus – to avoid the defence strategies of the non-specific immune response (Sect. 14.5.3). In the case of hepatitis C virus, the NS3/4A protease cleaves the cellular factors Cardif (CARD-adapter-inducing IFN-β) and TRIF (Toll/IL-1-receptor-domain-containing adapter), which activate the interferon regulatory factor and initiate the synthesis of IFN-α and IFN-β. In vitro, the NS5A protein inhibits protein kinase R, which is activated by IFN-α and inhibits translation. However, whether this mechanism also occurs in vivo to counteract the effect of interferon has not been demonstrated; the same also applies to the other examples mentioned. It is also not known whether specific mutations are important for the virulence of the different quasispecies. Some subtypes appear to differ in their sensitivity to IFN-α, whereby genotypes 1 and 4 are particularly resistant.

It has not been conclusively resolved in which way hepatitis C virus promotes carcinogenesis. There is evidence that specific sequences of the C protein interact with cellular Ras proteins and that this interaction induces transformation. The time span between infection and the formation of a primary hepatocellular carcinoma is approximately 20–40 years. The origin is a chronic infection in adolescents and adults. It is thought that persistent inflammation processes are responsible for the development of cancer over the years. Because of the infiltration of immunologically active cells and their secretion of cytokines, liver cells are destroyed. This cell-damaging process can be intensified by certain cofactors, such as alcohol

consumption. In individual cases, mutations can arise in the cellular genome during such processes, which then stimulate liver cells to proliferate continuously, and contribute to the development of hepatocellular carcinoma.

The perinatal transmission of the virus from infected mothers to their newborn children plays only a marginal role in carcinogenesis associated with hepatitis C virus, unlike carcinogenesis associated with hepatitis B virus (▶ Sect. 19.1). Double infections with hepatitis B and hepatitis C viruses are found in Japan in up to 18 % of primary hepatocellular carcinomas. Simultaneous infection with hepatitis B, hepatitis C and hepatitis D viruses cause a shortening of the incubation period until the emergence of the carcinoma.

Immune Response and Diagnosis

Increased transaminase levels may be elucidative for the diagnosis of hepatitis C virus infections, although this does not allow any further assignment of the pathogen. The main approach for diagnosis of hepatitis C virus infections is ELISA, which is used for screening. If the test findings are positive, it can be inferred that a fresh, chronic or past hepatitis C virus infection has occurred. A more exact serological differentiation is not possible. Owing to the high sensitivity of these screening tests, immunoblotting or analogous methods have additionally been introduced as confirmatory tests to exclude non-specific results. Quantitative RT-PCR for detection of viral RNA genomes is the most important method used today, and is usually applied as a confirmatory test, especially since this method immediately provides the level of the viral load. In general, serum or plasma is used as the source material; liver biopsies are used only in exceptional cases. In addition, the genotype is usually determined by PCR and hybridization tests, as this is crucial for determining the duration of therapy. Since the phase in which an acute hepatitis C infection cannot be serologically diagnosed with certainty lasts several months, automated tests have been introduced for the detection of viral C proteins in order to shorten this phase.

In ELISA or in immunoblotting, recombinant viral proteins are used to detect specific antibodies. IgM antibodies against the NS4 and C proteins can be found in acute infections. However, since these may persist or correlate with the level of liver injury and also with the genotype, IgM diagnostic tests are not of great importance. This is further indication that both viral gene expression and protein synthesis are constantly occurring in chronic infections. IgG antibodies against the C protein can be detected a few days to a few weeks after the onset of symptoms; IgG antibodies against non-structural proteins (NS3, NS4, NS5) are detectable later. Immunoglobulins against envelope proteins E1 and E2 are detected at an early stage in only about 10 % of acute infections. It is unknown whether these antibodies are not formed or whether they cannot be detected owing to the variability of the amino acid sequence and the lack of sensitivity of the test systems. Cytotoxic T lymphocytes can be detected in the blood of patients after stimulation by peptides that are derived from viral proteins.

Therapy and Prophylaxis

There is no vaccine against hepatitis C virus because of the quasispecies problem. The use of IFN-α, and especially the use of IFN-α compounds which act as a depot (pegylated interferon), in combination with ribavirin has proved to be successful for the treatment of chronic infections. In acute infections, curing rates above 90 % can be achieved even with IFN-α alone. In many cases, treatment of chronic infections leads to a significant reduction of the viral load in the peripheral blood, where it is no longer detectable even with ultrasensitive methods. However, there are many treatment failures: especially infections with genotypes 1 and 4 have proven to be largely resistant. Therefore, patients infected with genotypes 1 and 4 are currently treated for 48 weeks, with testing for treatment success after the 12th week (corresponding decrease of viral load in quantitative PCR). If the expected decrease of viral load does not occur, the therapy is stopped. In contrast, in cases of infection with other genotypes, patients are treated for 24 weeks. Inhibitors of the viral protease NS3 have recently been introduced as alternative drugs, e.g. boceprevir, a substance derived from peptides. Despite these advances in drug therapy, the effects of chronic hepatitis C virus infections are responsible for about 20 % of all liver transplants.

Hepatitis G Virus (human pegivirus, GB virus C)

Hepatitis G virus was originally isolated by Friedrich Deinhard. In 1967, he inoculated marmosets with the serum of a surgeon who had hepatitis (G. Barker, according to his initials the virus is sometimes also referred to as GB virus), and was able to isolate a virus from the infected monkeys. In 1995, the genome of this virus was sequenced by Scott Muerhoff and co-workers; it was classified into the family *Flaviviridae* owing to the arrangement of its genes. Since an IRES sequence was identified at the 5′ end of the RNA genome, and genes are present coding for two glycoproteins (E1 and E2), it is related to hepatitis C virus. Subsequently, infections with this pathogen, which had been designated hepatitis G virus and is named human pegivirus today, were identified in many people who responded to the infection with the formation of specific antibodies. Meanwhile, different subtypes of this virus have been identified. The initial presumption that hepatitis G viruses cause liver inflammation in humans has not been verified. Occasionally, these viruses were also isolated from patients with hepatitis because of the high prevalence rates – up to 4 % of blood donors are viraemic. Both the acute and the persistent infection are apparently asymptomatic. There is even evidence that in patients who are infected with human immunodeficiency virus and human pegivirus, the human immunodeficiency virus infection shows an attenuated clinical course. This effect may be a result of the properties of the E2 protein of human pegivirus, which inhibits the replication cycle of human immunodeficiency virus, possibly on the basis of similar epitopes, and leads to cross-reacting immune responses.

14.5.6 Human and Animal Pathogenic Flaviviruses

14.5.6.1 West Nile Virus

West Nile virus is a virus with zoonotic potential. It was originally widespread only in the Old World (Asian and African countries, Romania). In 1999, it also appeared on the American continent, and spread throughout the entire North American continent in subsequent years. West Nile virus falls into the Japanese encephalitis complex, which includes Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus and Kunjin virus. The latter are widespread on the Australian continent.

Epidemiology and Transmission

West Nile virus is transmitted to birds by ornithophilous mosquito species, particularly *Culex univittatus* and *C. pipiens*. It is believed that different bird species exhibit significant differences in their susceptibility for infection with West Nile virus; corvids seem to be particularly susceptible. It has been shown that the pathogen can also be directly transmitted among mosquitoes, at least under laboratory conditions, when two mosquitoes (an infected and an uninfected mosquito) simultaneously suck the blood of the same bird. In addition, the virus can also be transmitted by aerosols among birds. The virus proliferates in susceptible birds and is present in the blood at concentrations that allow transmission by mosquitoes, which occasionally transmit the virus to other hosts, such as horses and humans. Whether birds can establish an infection is unknown. The intra- and intercontinental spread of the virus is done by infected migratory birds. People who are infected with West Nile virus can transmit the pathogen through blood and organ donations, as well as via human milk.

Clinical Features

Humans After an incubation period of 3 days to 2 weeks, patients develop flu-like symptoms such as fever, headaches and back, joint and muscle pains. Nausea, diarrhoea and general lymph node swelling are also observed in some cases; there are also signs of a skin rash especially among children. In severe clinical courses, which are frequently found in elderly patients, the initial symptoms are followed by liver and heart muscle inflammations as well as encephalitis. About 5–10 % of patients with neurological symptoms die.

Animals Avian infections are systemic and also result in encephalomyelitis; extraneuronal lesions are very common. Therefore, myocarditis, muscle degeneration and lymphocytic infiltrations are found in various organs, such as pancreas, lung and liver. An atrophy of the bursa of Fabricius is also frequent. In addition, clinically asymptomatic infections are typical. Among birds, there are considerable differences in susceptibility. Corvids and raptors are considered to be highly susceptible and often have severe disease patterns; thus, monitoring programmes should especially include these birds.

Although in the USA there are a large number of infected horses, clinically inapparent infection is the rule in this species. Experimental infections with

mosquitoes infected with West Nile virus resulted in clinical symptoms in only about 10 % of the exposed and infected horses. In contrast to infections in birds, almost exclusively neurological symptoms are found in horses as a result of poliomeningoencephalitis; other manifestations are practically not observed.

Pathogenesis

During infection of humans, West Nile virus is introduced into the bloodstream through a mosquito bite, and binds to integrins ($\alpha_v\beta_3$) on the surface of monocytes, macrophages and endothelial cells, and invades the whole organism; furthermore, interaction with the proteins ICAM-3 and dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) has also been described. There are only a few data concerning the details of the pathogenesis. Similarly to TBEV, for West Nile virus, patients with genetic defects in the chemokine receptor CCR5 have increased risk of developing neurological symptoms and thus severe diseases from infection. Possibly, this receptor and its interaction with the ligand are responsible for the regulation of migration of macrophages and T lymphocytes into the infected tissues.

The Receptor CCR5: Friend or Foe in Viral Infections?

The chemokine receptor CCR5 attracted attention a few years ago: it was identified as a coreceptor, to which human immunodeficiency virus binds on the surface of monocytes and macrophages to initiate the infection (see ► Sect. 18.1). The importance was additionally underscored by the finding that people with genetic defects in the CCR5 gene cannot be infected by human immunodeficiency virus; they are largely resistant. These data constitute the basis for the development of new therapeutics which are aimed at blocking the interaction between human immunodeficiency virus and the CCR5 protein on the cell surface, and should prevent infections. However, the use of such inhibitors could increase susceptibility for developing severe infections with West Nile virus. In using them, we would possibly combat one evil, while accepting another.

Immune Response and Diagnosis

The diagnosis is done by RT-PCR by cultivating the pathogen in chicken eggs or in cell cultures and subsequent isolation of the virus. The virus agglutinates goose erythrocytes, and thus can be detected by haemagglutination or haemagglutination-inhibition tests. IgM and IgG antibodies can only be detected by ELISA.

Control and Prophylaxis

An immunoprophylaxis is not available. In large cities, water accumulations are treated with pesticides to reduce the numbers of mosquitoes. However, the virtue of this measure is controversially debated. Because of deaths, donated blood is examined for the presence of West Nile virus in the USA.

West Nile Virus as a "New Virus" on the American Continent

West Nile virus caused a series of fatal encephalitis cases in humans in New York in 1999. This attracted much attention at that time, especially because the virus was considered as a classic pathogen of the Old World and it was entirely unknown on the American continent and in the urban area of New York. West Nile virus usually infects birds (songbirds, crows), which constitute the reservoir for the pathogen. They can transport the virus over long distances. The virus can be transmitted to horses and humans by mosquitoes of the genus *Culex*. The virus spread in just 1 year throughout the eastern USA. Diseases and deaths due to West Nile virus infections were reported especially in the eastern USA in the years after 1999. In 2002, the pathogen had spread into more than 39 states and 4,156 infections were documented in humans, 248 of whom died. The deaths occurred primarily in people of advanced age (the average age of the people who died was 79 years). Furthermore, during this period more than 3,400 confirmed infection cases were recorded in horses, which develop a clinical picture similar to that in humans. The infection wave reached its climax in 2003, when West Nile virus infections were diagnosed in almost 10,000 patients in all states of the USA. Since then, dead birds are extensively examined to determine whether they are infected with West Nile virus, especially the highly susceptible crows. In subsequent years, the number of infections decreased to 3,000–4,000 per year. To minimize the risk of infection, a very intensive and costly monitoring programme has been implemented, which includes five levels: mosquitoes, sentinel chicken flocks, diseased birds, other sick animals and diseased humans. Because of the infection wave and the risk of transmission of infection through contaminated blood donations, all blood donations have been tested by PCR for the presence of West Nile virus in the USA since 2003; along with the decrease in the number of new infections, the number of detections of West Nile virus in blood donations decreased from 818 in 2003 to below 200 in 2008. In European blood donors, West Nile virus is detected much less frequently; in Germany, for example, it was found with an antibody prevalence of only 0.03 % without evidence of viral RNA in blood donations.

14.5.7 Animal Pathogenic Flaviviruses

The animal pathogenic flaviviruses are divided into two groups: one group is composed of viruses that are transmitted by arthropods (West Nile virus, louping ill virus and TBEV); infections by viruses of the second group occur independently of arthropods (pestiviruses). As animal pathogens, pestiviruses are economically significant, particularly classical swine fever virus and BVDV. West Nile virus can cause fatal infections in humans (see Sect. 14.5.6). TBEV, in rare cases, can also infect ruminants or dogs, causing clinical pictures similar to those of human

infections (Sect. 14.5.5). The distribution of louping ill virus is confined to Great Britain. Louping ill virus, like TBEV, is transmitted by ticks and causes encephalitis in sheep. Humans can also be infected by this virus and become ill, although this occurs extremely rarely. Because of the minor importance of louping ill virus, a more extensive description is not given here.

14.5.7.1 Classical Swine Fever Virus

There are a number of pathogenic flaviviruses within the genus *Pestivirus* which cause economically important diseases in swine and ruminants. These include primarily classical swine fever virus, whose infections cause the classical ("European") swine fever in pigs. Clinically, it cannot be distinguished from the similar African swine fever. The latter is induced by African swine fever virus, a DNA virus from the new family *Asfarviridae* (► Sect. 19.7).

Epidemiology and Transmission

The clinical picture of classical swine fever is characterized by severe haemorrhagic and general symptoms. In addition to this severe form, clinically atypical infections occur very frequently (nowadays almost exclusively), and exhibit only few clear and partially mild symptoms. This complicates the rapid clinical diagnosis and can contribute to fast dissemination, considering the high contagiousness of the virus.

Transmission occurs primarily by direct animal contact, particularly as a result of the purchase of pigs in fattening farms which have a subclinical persistent infection or exhibit only attenuated symptoms. A further infection source is contaminated animal food. Frequently, infection occurs via kitchen waste containing the meat of infected animals; therefore, feeding of kitchen waste to pigs is strictly prohibited. Recently, outbreaks of swine fever have occurred in which the virus was transmitted by wild boars. In those cases two factors played a crucial role: direct contact (between pasture or grazing pigs and wild boars) and feeding of pigs with wild boar meat (swine-holding hunters and poachers).

Of particular epidemiological importance are piglets, which develop persistent infections and contain the pathogens in blood after intrauterine transmission by infected mother pigs. Such animals excrete the virus permanently (chronic carrier), and can remain asymptomatic for several months. However, they eventually develop clinical symptoms, and do not reach the age of more than 16 months.

Clinical Features

Classical swine fever is characterized by a peracute to acute disease pattern, which may be accompanied by respiratory or gastrointestinal disorders. Central nervous system symptoms have also been described, such as tremor, paralysis and convulsions. The morbidity can be up to 100 % in livestock. The atypical or chronic infections are less dramatic, and thus can be easily overlooked. In pregnant sows, classical swine fever can cause miscarriages of mummified piglets, or farrowing of weak piglets.

Pathogenesis

Infections with the pathogen of classical swine fever usually occur by oral transmission. During a viraemia, viruses replicate primarily in the tonsils and from there reach nearly all endothelial cells and lymphatic organs, including the bone marrow. Viral replication is associated with significant cellular destruction, which is manifested in multiple haemorrhages, a massive lymphopenia and thrombocytopenia, and disseminated intravascular coagulopathy. Characteristic are multiple splenic infarctions and a severe atrophy of lymphoid organs, which progresses with increasing duration of the disease. Frequently, an encephalitis can also develop. The viruses are transmitted in the intrauterine way. Depending on the gestational age at the time of infection, premature re-entry or return to heat (re-entering oestrus), abortion or birth of malformed piglets can occur. Live-born piglets of infected sows develop a persistent viraemia, like calves during BVDV infections in cattle. They excrete the virus permanently and play a major epidemiological role.

Immune Response and Diagnosis

The diagnosis of classical swine fever is initiated by the veterinary authority after notification. Diagnosis is possible by detection of antibodies using ELISA or neutralization tests. In this context, it is important to distinguish between antibodies against classical swine fever virus and antibodies against BVDV, which can also infect swine, but does not induce any disease, and hence is not subject to compulsory supervision for animal health purposes. Differentiation requires the parallel titration of sera which contain the proteins of BVDV or classical swine fever virus as antigens. However, more important is the direct detection of the virus, which can be achieved by isolating the pathogen in permanent porcine kidney cell cultures, by serological characterization by monoclonal antibodies in immunofluorescence tests (if necessary by flow cytometry) or by genetic characterization by means of PCR.

Control and Prophylaxis

In most countries the regimen that is used to control classical swine fever is rather strict. If cases occur, all pigs on the farm are culled, and extensive epidemiological investigations are implemented to determine the spread of the virus.

Recently, genetically engineered vaccines against swine fever have been developed. They contain glycoprotein E2, which is produced by means of recombinant baculoviruses. These marker vaccines induce an immune reaction exclusively against glycoprotein E2, which allows differentiation between vaccinated and infected swine. However, they are not suitable for emergency vaccinations because a robust protection can be developed only a few days after vaccination.

Interestingly, a highly effective live vaccine has long been available, and has been used in many European countries for many years. It is based on a virus that was attenuated by numerous passages in rabbits (C strain, “lapinized virus”). This virus is now used only for oral immunization of wild boars with bait vaccines in the wild. It is no longer used for immunization of the well-controlled pig populations

because trade of meat from vaccinated animals is accompanied by strict requirements, which make its application economically (currently) unattractive.

Controlling Classical Swine Fever in the European Union
The countries of the European Union work together to control economically important animal diseases, and to adopt binding regulations for all member states. These include the eradication of classical swine fever. Infections at the flock level can be confirmed simply and quickly by antibody detection. Vaccination is not permitted because its application would make the simple serological detection of infected pig herds impossible. Therefore, all animals of an affected herd will be killed in cases of classical swine fever outbreaks owing to the high contagiousness of the virus. Their carcasses have to be destroyed and disposed of, possible movements of animals from the herd must be followed in comprehensive epidemiological surveys, and animals suspected of being contaminated have to be put into quarantine and under official veterinarian surveillance. This expensive method of sanitation is extremely effective and ultimately more cost-efficient than vaccination, which would only save individual pigs within a farm for a short time, but would entail trade restrictions on pigs and pork within and outside the European Union. Restricted trade would result in enormous economic losses. However, the killing of large numbers of animals, especially the killing of non-infected animals, is a subject of debate regarding ethical aspects. The European Union is considering reversing the non-vaccination policy, which would allow vaccination of non-infected flocks when there are outbreaks. The sale of meat from vaccinated animals is currently a problem because it is not internationally accepted.

14.5.7.2 Bovine Viral Diarrhoea Virus

Epidemiology and Transmission

BVDV is an economically important animal pathogen. For years, there has been discussion of whether there are two separate pestiviruses, namely BVDV-1 and BVDV-2, or whether they are different genotypes of a single species of virus. Although they cause the same symptoms, they can be distinguished by their genome sequences and the antibody response induced in cattle. The virus is excreted in the faeces and via mucosal secretions, and is ingested orally.

Clinical Features

In adult animals, BVDV causes a subclinical infection or mild, self-limiting diarrhoeas. Peracute haemorrhagic symptoms are rarely observed. Whether in such cases particular strains or biotypes of BVDV are responsible is being discussed. After infection, the animals develop lifelong immunity. Economic damage is caused by the virus if susceptible, i.e. immunologically unprotected, pregnant cows are infected. Depending on the time of infection, infection leads to abortion,

birth of malformed calves or birth of persistently infected, viraemic calves. These animals can develop mucosal disease, a fatal disease form.

Mucosal disease is a generalized disorder of chronically infected cattle. The virus proliferates lytically in the cells of all mucous membranes and endothelial tissues. The infected animals develop the severe clinical picture of haemorrhagic fever, which invariably ends fatally. There is bleeding in all mucous membranes, and the virus is found in almost every organ.

Pathogenesis

The viral persistence is based on a BVDV-type-specific central immune tolerance, which is established in the embryo against a non-cytopathogenic biotype in the course of infection. These calves are completely immunocompetent against other viral infections, even against BVDV infections with a serologically distinct virus type. During persistence of BVDV, mutations occur in the viral genome, which lead to the development of a virus with a new biotype. This cytopathogenic virus then causes the lethal clinical picture of mucosal disease.

The molecular basis for the emergence of the cytopathogenic virus resides in genetic mutations that lead to different processing of the non-structural protein NS3. Whereas it is produced as the NS2–NS3 fusion protein in non-cytopathogenic BVDV, NS3 proteins do not possess the NS2 fusion part in the corresponding cytopathogenic viruses. The altered processing occurs when cellular genome sequences are integrated into the NS2 coding region within the viral genome. This process changes the pattern of proteolytic cleavage in the precursor protein. It has been shown by homologous RNA recombination that a number of cellular genes have been integrated into the viral genome (Fig. 14.16). Besides the integration of cellular gene sequences, e.g. by incorporating ubiquitin-coding sequences, rearrangements or deletions of viral genome segments have also been described. All these processes lead to the production of free NS3 proteins, which are generated by cleavage of the precursor protein by the cellular ubiquitin hydrolase or the viral protease N^{pro} (Fig. 14.16). The underlying mechanisms that lead to the enhanced virulence are unclear. Classical swine fever virus and border disease virus of sheep (another pestivirus) also produce free NS3 proteins during infection, but without inducing the severe symptoms which are developed in mucosal disease.

Immune Response and Diagnosis

BVDV infections are usually diagnosed by isolating the virus in cell culture, by RT-PCR or by detection of the BVDV antigen in peripheral blood lymphocytes using immunofluorescence tests (flow cytometry) or ELISA. Maternal antibodies can interfere with these tests, and their usage yields reliable results only from the sixth month of life. Therefore, the detection of the viral E^{rns} protein in serum is usual today. It is secreted by virus-infected cells. Maternal antibodies against the E^{rns} protein are hardly present in calves, so verification of infection is possible from the 20th day of life by detection of E^{rns} proteins using ELISA.

The infection produces long-lasting immunity, which probably lasts for life.

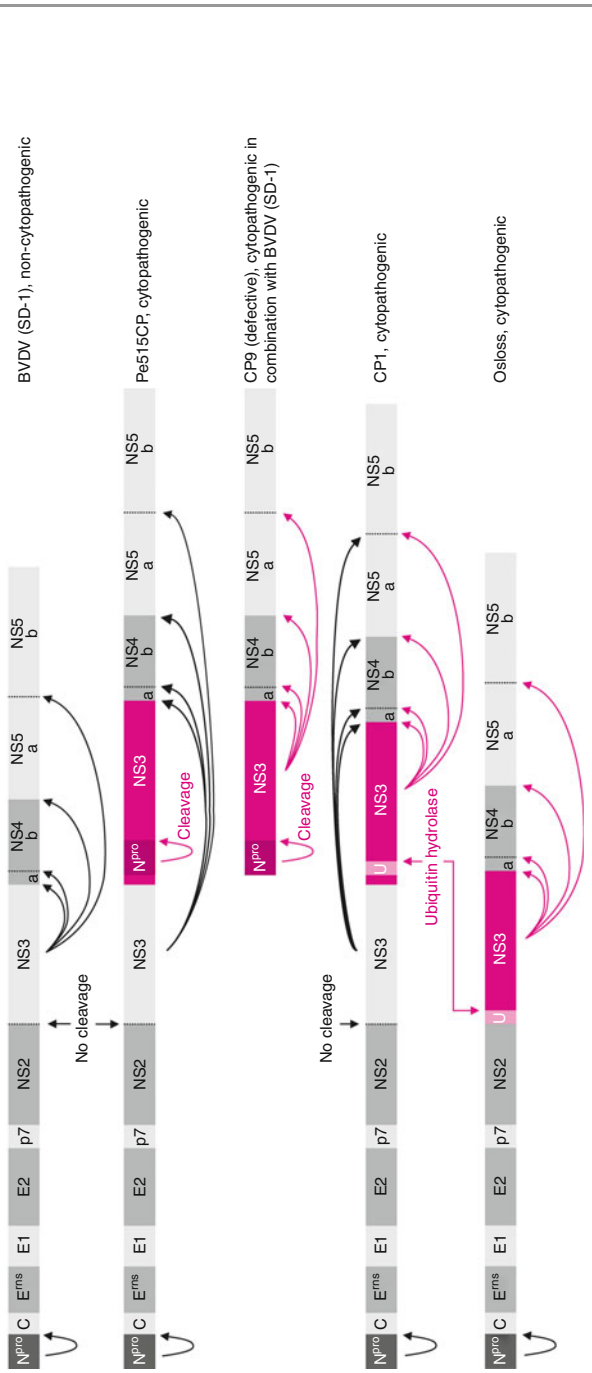


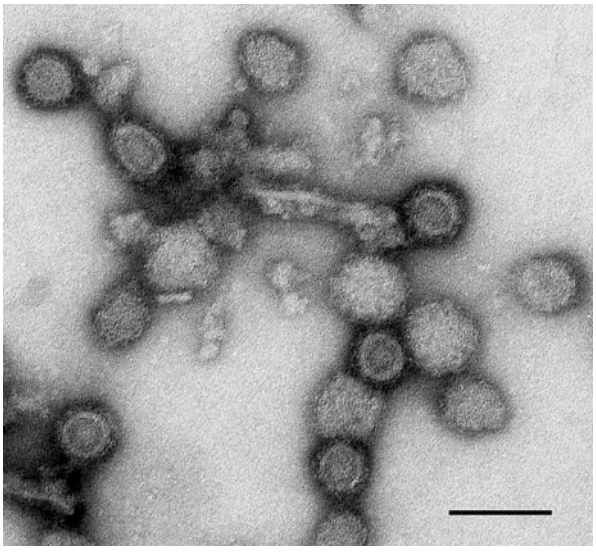
Fig. 14.16 Recombination events in the genome of BVDV. In the non-cytopathogenic strains (BVDV SD-1), no cleavage occurs between proteins NS2 and NS3, and both remain fused and act as a protease to cleave the non-structural part of the precursor protein. In cytopathogenic strains, various recombinations with cellular DNA are found. In such cases, ubiquitin sequences (*U*) are integrated into the viral RNA genomes upstream of the NS3 region (strains CP1 and Osloss). Cleavage and release of NS3 proteins is performed by the cellular ubiquitin hydrolase. Alternatively, recombination events may occur in the cytopathogenic strains, which rearrange the N^{pro} sequences amino-terminally to the NS3 region. The proteolytic activity of N^{pro} leads subsequently to autocatalytic cleavage and the formation of an NS3 protein in addition to the NS2–NS3 fusion product (strain Pe515CP). Furthermore, there are defective viral genomes lacking the regions for the structural and NS2–NS3 proteins but which have rearranged the N^{pro} region upstream of the sequences of NS3 (strain CP9) similarly to strain Pe515CP. These defective viruses become pathogenic when they are present in animals in combination with non-cytopathogenic, infectious strains (SD-1)

Control and Prophylaxis

The principal objective is to avoid the birth of calves with persistent viraemia. In principle, this is possible through two measures. The first measure is that all female offspring are vaccinated before sexual maturity. This prevents the animals being susceptible to a viral infection during pregnancy. Transplacental transmissions of the virus and, ultimately, to new calves with persistent infections are avoided. The other measure is the early identification of animals with persistent viraemia, and their removal from the livestock. This is only possible by individualized virus detection. The serological analysis of representative samples from young animals of a herd to be examined provides, however, good indications for the presence of persistent virus carriers. Owing to nationwide measures, the number of persistently infected cattle is declining. It is assumed that less than 1 % of all cattle exhibit a persistent BVDV infection today.

A series of vaccines are available. They contain either inactivated or attenuated viruses with reproduction capacity. The former are generally not able to prevent intrauterine transmission of the virus. Live vaccines, however, are based on a cytopathogenic BVDV strain. In persistently infected animals, the live vaccine triggers mucosal disease immediately after vaccination or after recombination between the wild type and the vaccine strain. These vaccines are therefore problematic. Furthermore, persistently infected calves can be produced after inoculation of pregnant animals with the live vaccine. It is hence desirable to develop potent inactivated vaccines which reliably prevent intrauterine transmission and protect against the known BVDV genotypes.

14.6 Togaviruses



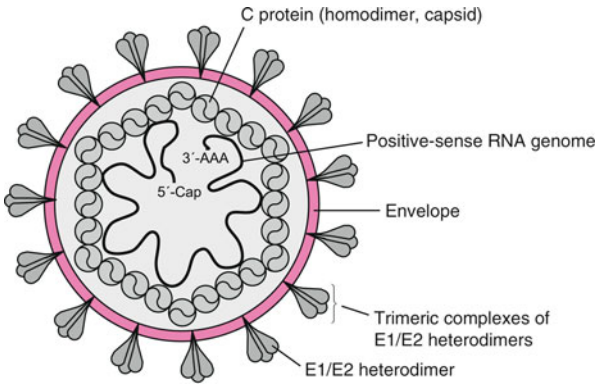


Fig. 14.17 Structure of a togavirus particle. The icosahedral capsid is composed of the C protein; the viral RNA genome is attached to the inner side of the capsid. The capsid is enclosed by an envelope, in which the viral surface proteins are embedded

Togaviruses were originally classified into a common virus family along with flaviviruses owing to their morphological similarity. However, as details about the replication mechanisms became known, significant differences were revealed that led to the current classification into two separate families. With regard to evolution, flaviviruses can be considered as the precursor of togaviruses because they are also enveloped viruses which synthesize polyproteins during their replication cycle. However, togaviruses have developed the synthesis of a subgenomic RNA for translation of structural proteins, which confer the capability of adjusting the quantity of different proteins to the respective requirements, thus resembling astroviruses, caliciviruses and hepeviruses. The name of this virus family is derived from the Latin term *toga* (meaning "mantle" or "shell"): the first electron micrographs revealed the image of a capsid that is surrounded by a wide envelope (Fig. 14.17).

14.6.1 Classification and Characteristic Prototypes

The togavirus family (*Togaviridae*) comprises two genera (Table 14.13). The alphaviruses, which are transmitted by insects, are especially known as pathogens of animal encephalitis and arthritis in America, Africa and Asia. They are classified according to their antigenic similarity in different complexes that are not host-specific and are transmitted by different mosquito species between animal species (horses, rodents, various birds such as pheasants and cranes) and humans, where they proliferate. In humans, they occasionally cause symptomatic infections; infections with chikungunya virus have been occasionally described during the summer months in southern Europe (Italy) since 2005. On the other hand, the members of the second genus, *Rubivirus*, are distributed worldwide. The pathogen of rubella infection belongs to this genus. These viruses are not transmitted by insects.

Table 14.13 Characteristic prototypes of togaviruses

Genus	Human virus	Animal virus	Vector/carrier
Alphavirus	Semliki Forest complex Chikungunya virus	Semliki Forest complex Semliki Forest virus (rodents)	*Aedes* spp.
		Chikungunya virus (non-human primates)	*Aedes* spp.
	O'nyong-nyong virus Ross River virus	O'nyong-nyong virus (animal host unknown)	*Anopheles funestus, A. gambiae*
		Ross River virus (marsupials)	*Culex annulirostris*
		Western equine encephalitis complex Western equine encephalitis virus (birds, horses) Sindbis virus (rodents)	*Culex tarsalis, C. quinuefasciatus*
		Eastern equine encephalitis complex Eastern equine encephalitis virus (bird horses)	*Culex* spp.
		Venezuelan equine encephalitis complex Venezuelan equine encephalitis virus (rodents, horses) Everglades virus	 *Culex* spp., *Aedes* spp. *Culex* spp.
Rubivirus	Rubella virus	–	–

Semliki Forest Virus and Sindbis Virus: Two Well-Studied Prototypes of Alphaviruses

Semliki Forest virus and Sindbis virus are the best studied representatives of togaviruses with regard to their molecular biology and replication mechanisms; therefore, they have long been considered as prototypes of this virus family. Sindbis virus is related to western equine encephalitis virus, which is common on the American continent (Sect. 14.6.6). Both species of virus can be easily reproduced in cell cultures and show a pronounced cytopathic effect. Sindbis virus is widespread in Africa, eastern Europe and Asia. It is transmitted by mosquitoes of the genus *Culex*, and only in rare cases causes a febrile illness with rash and joint pain, which is similar in terms of clinical features and pathogenesis to a disease caused by some flaviviruses in terms of the clinical picture and pathogenesis. Neurotropic isolates have previously been described only in individual cases. Semliki Forest virus is endemic in Africa, India and Southeast Asia. It is transmitted by *Aedes* spp. and is largely non-pathogenic in humans. Therefore, it is now frequently used as a genetic engineering vector for heterologous gene expression in eukaryotic cell cultures.

14.6.2 Structure

14.6.2.1 Virus Particle

The infectious particles of togaviruses have a diameter of 60–80 nm and consist of icosahedral or spherical capsids (diameter 40 nm), which are enclosed by an envelope. Glycoproteins E1 and E2 are embedded in the envelope. They are present as E1/E2 heterodimers, which further associate to form trimeric protein complexes. There are about 80 trimers per virion. They form spike-like protrusions of 6–8 nm in length on the surface of the virus (Fig. 14.17). The trimers mediate attachment to cellular receptors and are binding targets of virus-neutralizing antibodies. In rubella virus, there are also E1 homodimers on the particles. The capsid is composed of 240 dimers of the C protein. It contains the RNA genome, and is associated with amino acids on the inner side of the capsid.

14.6.2.2 Genome Organization and Structure

The togavirus genome consists of a single-stranded, positive-sense RNA molecule, which is capped at the 5′ terminus and polyadenylated at the 3′ end. It has a length of 9,762 (rubella virus, strain Therien), 11,703 (Sindbis virus), 11,675 (eastern equine encephalitis virus) or 11,442 (Semliki Forest virus) nucleotides. The genome encompasses two open reading frames: the open reading frame beginning at the 5′ terminus encodes the precursor protein of the four non-structural proteins NSP1–NSP4 in alphaviruses, as well as p150 and p90 in rubiviruses; the open reading frame in the 3′-terminal half of the genome contains the genetic information encoding the structural proteins, C, E1 and E2 (Fig. 14.18). The two open reading frames are separated by a few nucleotides in Sindbis virus and Semliki Forest virus, whereas the intergenic region of rubella virus has a length of 124 nucleotides. The polyprotein of the structural proteins is translated from another open reading frame. There is a short UTR at the 5′ end of the genome (41 nucleotides in rubella virus, 60–80 nucleotides in Sindbis virus). Between the stop codon of the second open reading frame and the poly(A) tail at the 3′ terminus of rubella virus there are 61 nucleotides (264 nucleotides in Semliki Forest virus, 322 nucleotides in Sindbis virus), and these are folded into defined secondary structures.

14.6.3 Viral Proteins

14.6.3.1 Polyprotein of Non-Structural Proteins

The precursor protein of the up to four non-structural proteins NSP1–NSP4 differs among the various togaviruses. Two different precursors are synthesized in Sindbis virus; one includes proteins NSP1 – NSP3, and ends at an opal stop codon (UGA), which is located between coding regions NSP3 and NSP4 (Fig. 14.18a). However, this termination signal for translation is skipped in 20 % of cases, and the translation is continued until the end of the NSP4 protein. In contrast, only one full-length non-structural polyprotein is synthesized in Semliki Forest virus and in rubella virus

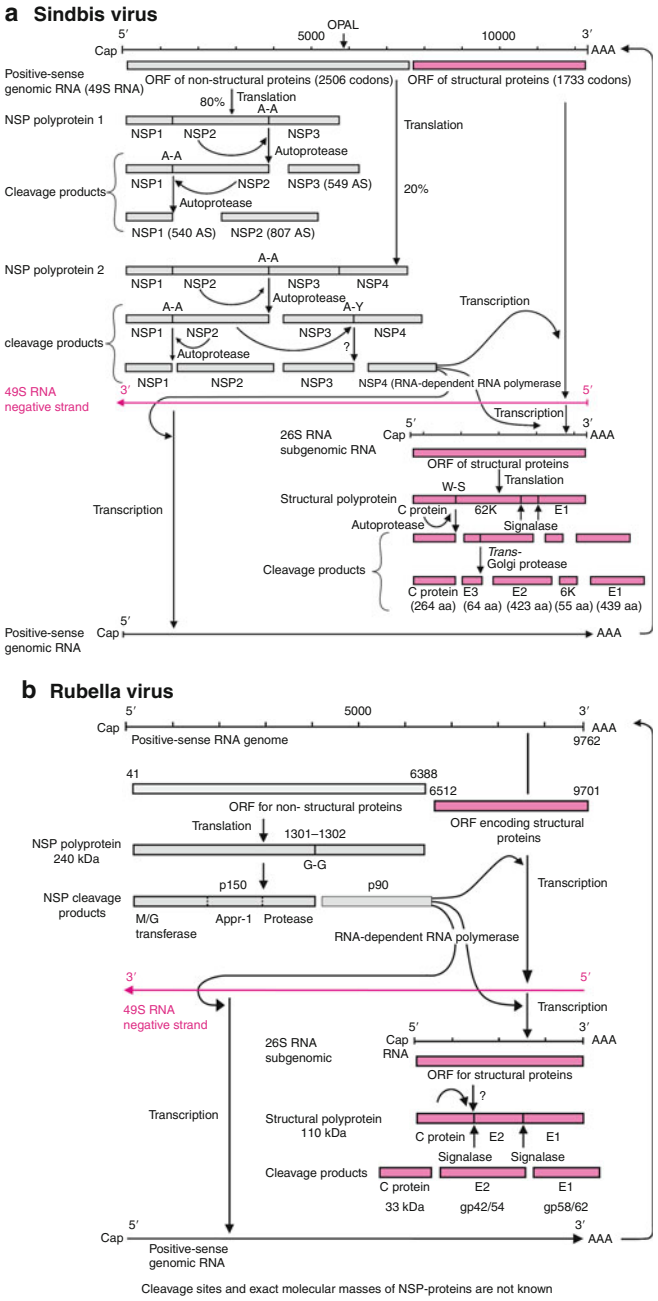


Fig. 14.18 Genome organization and replication of togaviruses. (**a**) Sindbis virus. (**b**) Rubella virus. The RNA genomes encode a single version (rubella virus) or two versions of the non-structural polyprotein as well as one structural polyprotein. First, the precursor of the

(240 kDa in rubella virus; Fig. 14.18b). Whether similar two-stage translation processes play a role in other togaviruses is not known. Table 14.14 provides a summary of the size and function of togavirus proteins.

14.6.3.2 Non-Structural Proteins

Alphaviruses

The data on the cleavage reactions of the precursor which generate non-structural proteins NSP1–NSP4 have principally emerged from studies in Sindbis virus and Semliki Forest virus. It is expected that the non-structural proteins in the other togavirus species fulfil identical functions in the infection cycle. Their activities play an important role in viral transcription and replication.

The NSP1 protein is a methyltransferase/guanylyltransferase involved in the formation of methylated 5′-cap structures in viral RNA molecules. Togaviruses need to encode this enzyme function, because they can not use the corresponding cellular functions that are localized in the nucleus, as their replication cycle occurs exclusively in the cytoplasm. The sequences of the NSP1 protein are highly conserved among the different togaviruses. The protein is modified with palmitic acid at cysteine residues 418–421 (in Semliki Forest virus). This determines, together with an amphipathic α-helix in the centre of the protein, the association with intracellular membrane compartments.

The amino-terminal region of the NSP2 protein displays RNA helicase activity, which is necessary for transcription and genome replication. A proteolytic activity is localized in the carboxy-terminal region and autocatalytically cleaves the precursor protein between the NSP2 and NSP3 moieties. This proteolytic processing step generates the products NSP1–NSP2 and NSP3–NSP4 (or NSP3 in Sindbis virus). Two consecutive alanine residues serve as a recognition sequence in Sindbis virus. The NSP1–NSP2 protein is further cleaved into NSP1 and NSP2 by the NSP2 protease. Whether the NSP2 protease is also involved in the processing of the other precursor protein (NSP3–NSP4), whose cleavage at the amino acid sequence alanine–tyrosine proceeds only very slowly, has not been elucidated yet.

The NSP3 protein is required for viral genome replication; however, it is not known how it works. It has a short half-life, is present in a phosphorylated form and is partially linked to intracellular membranes in the cell.

Fig. 14.18 (continued) non-structural proteins is synthesized and autocatalytically cleaved into the individual components by the intrinsic protease activity of the polyprotein. As a result, RNA-dependent RNA polymerase is generated, which uses the positive-sense RNA genome as a template and synthesizes a negative-sense RNA. This in turn serves as a template for the synthesis of both new positive-sense RNA genomes and subgenomic RNA molecules. The latter is used as mRNA for translation of the polyprotein for the structural components and which is processed into the various individual components by the activity of cellular signalases. *Appr-1* protein domain with homology to the cellular protein ADP-ribose 1-monophosphate processing enzyme, *aa* amino acids, *M/G transferase* methyltransferase/guanylyltransferase, *NSP* non-structural protein

Table 14.14 Function and size of togavirus proteins

Protein	Sindbisvirus	Semliki Forest virus	Rubella virus	Function
NSP polyprotein	2,506 aa	2,431 aa	2,116–2,205 aa 240 KDa	Precursor of non-structural proteins
NSP1	540 aa	537		Methyltransferase/ guanylyltransferase, 5′-capping enzyme; palmitoylated
NSP2	807 aa	798 aa		Protease, helicase (nucleotidase)
NSP3	549 aa	482 aa		Active during replication
NSP4	610 aa	614 aa		RNA-dependent RNA polymerase
p150	–	–	1,300/1,301 aa 150 kDa	Methyltransferase/ guanylyltransferase, 5′-capping enzyme; cysteine protease
p90	–	–	815–905 aa 90 kDa	Helicase, RNA-dependent RNA polymerase
Structural polyprotein	1,733 aa	1,739 aa	1,063 aa 110 kDa	Precursor of structural proteins
C	264 aa	267 aa	260–300 aa 33 kDa	Capsid protein, dimeric, protease
E3	64 aa	64 aa	–	Cleavage product, N-terminal domain of E2
E2	423 aa	418 aa	42–54 kDa	Glycosylated, palmitoylated, neutralization in Sindbis virus and Semliki Forest virus; haemagglutination and fusion in rubella virus
6K	55 aa	60 aa	–	Cleavage product, signal sequence at the N-terminus; ion channel protein?
E1	439 aa	438 aa	58–62 kDa	Glycosylated, palmitoylated, neutralization in rubella virus, haemagglutination and fusion in Sindbis virus and Semliki Forest virus

The protein succession order in the table corresponds to the real localization in the corresponding polyprotein
aa amino acids

The NSP4 protein is an RNA-dependent RNA polymerase. It is active in the synthesis of both negative-sense RNA molecules and genomic and subgenomic RNA species.

Rubiviruses

In the case of rubella virus, only two cleavage products are generated by proteolytic processing of the precursor of the non-structural proteins: p150 corresponds to the

amino-terminal region; p90 is equivalent to the carboxy-terminal region. The proteolytic activity of a Zn^{2+}-dependent, papain-like cysteine protease resides in the carboxy-terminal domain of p150, which cleaves the precursor polyprotein between two glycine residues at positions 1301 and 1302. The amino-terminal region of p150 is active as a methyltransferase/guanylyltransferase, which is necessary for 5′-capping of viral RNAs. The amino acid sequence between both of these enzyme domains is homologous to the cellular protein ADP-ribose 1-monophosphate processing enzyme (Appr-1). Whether this function is necessary for viral infection is still not clear. The protein p90 possesses both RNA helicase and RNA-dependent RNA polymerase activity.

14.6.3.3 Polyprotein of Structural Proteins

In all togaviruses the structural polyprotein is significantly smaller than that of the non-structural proteins. In rubella virus, it has a molecular mass of 110 kDa (Fig. 14.18b). It contains the sequences of the C, E2 and E1 proteins. In alphaviruses, between the respective protein segments there are connecting amino acid sequences which are removed during polyprotein processing and viral maturation. The synthesis of the structural polyproteins occurs in the ER membrane. Amino acid sequences that are analogous to signal peptides have been found in alphaviruses directly after the carboxy-terminal end of the C protein (i.e. at the amino terminus of p62 protein, from which E2 arises) and within the 6K protein upstream of the E1 domain (Fig. 14.18a). In rubella virus, they are located at the carboxy-terminal ends of the C and E2 proteins, respectively. These regions are responsible for the transport of the nascent polyprotein and its anchoring in the ER membrane. Membrane-associated proteases (signalases) cleave the precursor proteins after the signal-peptide-like sequences, thus leading to the generation of the individual components. In alphaviruses, an additional autocatalytic protease activity has been identified within the C protein. It contributes to the cleavage of the capsid protein from the precursor and resembles a serine protease. The cleavage occurs at the amino acid sequence tryptophan–serine. Similar functions have also been postulated for the C protein of rubella virus.

14.6.3.4 Capsid Protein (C Protein)

Depending on the type of virus, the C protein has a length of 260–300 amino acids and a molecular mass of approximately 33 kDa. After cleavage from the nascent polyprotein chain by its autoproteolytic activity in alphaviruses or by the signalase-mediated release in rubella virus, C protein dimerizes and associates with the viral RNA genome to form nucleocapsids. The RNA-binding protein domain resides between amino acid residues 28 and 56. This interaction is very strong because only very few free C proteins can be found in the cytoplasm of infected cells. After their synthesis, C proteins are subject to complex phosphorylation and dephosphorylation processes. The degree of modification seems to influence the interaction with the RNA genome: non-phosphorylated C proteins bind to the genomes much more strongly than phosphorylated C proteins. The dephosphorylation of C proteins in the late stage of the replication cycle, presumably catalysed by the cellular protein

phosphatase 1A, seems to promote the interaction with the RNA genomes and their packaging. This dephosphorylation reaction may prevent premature interactions of RNA genomes and C proteins.

14.6.3.5 Glycoprotein E2

In alphaviruses, the E2 protein is formed by cleavage of a precursor protein of molecular mass 62 kDa. This protein, p62, is anchored in the ER membrane by hydrophobic sequences in its carboxy-terminal domain, and is transported to the cell membrane by the Golgi apparatus. Thus, the carboxy-terminal end is oriented towards the cytoplasm. It possesses amino acids that specifically interact with the C proteins of the nucleocapsid. As a result, the assembly process is induced in the late phase of the infection cycle, during which the ER membrane envelops the preformed capsids. On the way to the cell surface, p62 is modified with sugar and fatty acid groups and cleaved by a trypsin-like protease into the amino-terminal E3 moiety and the E2 protein in the *trans*-Golgi region. In Sindbis virus, E3 is released from the cell surface, whereas in Semliki Forest virus, it remains associated with the E2 protein and is detectable in various amounts in the virion. In alphaviruses, the vast majority of neutralizing antibodies are directed against the E2 protein, which is present in the virion as a heterodimer with E1. Three important epitopes have been characterized.

The E2 protein (gp42–54) of rubella virus is also glycosylated, modified with fatty acids and anchored in the membrane by its carboxy-terminal amino acids. An E3 moiety, which is similar to that of alphaviruses, has not been identified in rubella virus. The E2 protein of rubella virus has haemagglutination and membrane-fusion activities, and is present predominantly as a heterodimer with E1. However, in this case, most of the neutralizing antibodies are not directed against the E2 protein, but are directed against the E1 protein.

14.6.3.6 Glycoprotein E1

In alphaviruses, a short, hydrophobic region of 55–60 amino acids is located between the carboxy terminus of the E2 protein and the beginning of the E1 sequences. It is referred to as 6K protein owing to its size of about 6 kDa. It contains signal-peptide-like sequences, which are recognized and cleaved by signalases, and mediates translocation of the amino acid chain of the E1 protein through the ER membrane during translation. It is also found in small quantities in infectious particles. However, it has also been ascertained that 6K protein has independent functions: it seems to be a pore-forming ion channel protein, which affects the membrane permeability of infected cells. It belongs to the viroporin protein family, which also includes the 7K proteins of hepaciviruses and pestiviruses (Sect. 14.5). The E1 protein, which is anchored to the membrane by a hydrophobic transmembrane region at the carboxy terminus, is glycosylated and modified by fatty acids. In alphaviruses, it appears to be associated with the haemagglutination und fusion activities. In contrast to the E2 protein, only a few E1-specific antibodies with virus-neutralizing function have been found.

In rubella virus, neutralizing antibodies are predominantly directed against the E1 protein. Two protein domains which are recognized by protecting antibodies

have been identified. The monomeric protein has a molecular mass of 58–62 kDa, it is glycosylated and is modified with palmitic acid. The E1 protein of rubella virus is responsible for attachment of the particle to cellular receptors. Mutations in the regions of the genome which code for the hydrophobic regions of the E1 protein reduce the infectivity of the virus.

14.6.4 Replication

The cellular receptors of rubella virus and most alphaviruses are not known. In Sindbis virus, laminin and the laminin receptor precursor have been identified as receptors on the surface of chicken fibroblasts, but other cellular proteins also bind the virions; in the case of rubella virus, specific phospholipids on the cell surface seem to be involved in binding the virus. The virus particles penetrate into the cells by receptor-mediated endocytosis. The interior of endocytotic vesicles (endosomes) is acidified in an energy-dependent process through the import of H^+ ions. This generates conformational changes of the viral envelope proteins which lead to the fusion of the viral envelope with the endosomal membrane, resulting in the release of the capsid. How the tight interaction of C proteins with the RNA genome is abolished is not known. However, the polarity of the positive-sense RNA allows binding of the cellular cap-binding complex via the cap structure at the 5′ terminus and the association with ribosomal subunits, which initiate translation of the sequences encoding the non-structural polyprotein. This polypeptide is synthesized on ribosomes in the cytoplasm, it associates with the ER membrane by hydrophobic amino acid sequences and the palmitoylation in the NSP1 moiety, and is cleaved by the cysteine protease present in NSP2 (alphaviruses) or p150 (rubella virus) into the individual components. As soon as the activity of the RNA-dependent RNA polymerase is present in the form of functionally active NSP4 protein, the negative-sense RNA is synthesized. In Sindbis virus, the NSP4 protein forms a complex with the uncleaved NSP polyprotein (Fig. 14.18a). The initiation occurs at the 3′ end in a highly conserved nucleotide sequence, which is located directly upstream of the poly(A) tail. In addition, nucleotides from the untranslated sequences at the 5′ terminus seem to be involved. These are partially complementary to sequences at the 3′ end and can form a partial double-stranded RNA, thus leading to circularization of the genome. Cellular proteins influence the initiation of RNA synthesis at the 3′ end: phosphorylated forms of the cellular protein calreticulin bind to the 3′ end of the rubella virus genome. The details of initiation of RNA synthesis are unknown; however, it is clear that in the further course of the replication process an RNA product which is complementary to the whole genome is generated. This process occurs on the ER membrane.

From the negative-sense RNA, new full-length complementary RNA genomes and an additional subgenomic RNA molecule are synthesized. The latter is initiated at the transition region between the two reading frames. It contains the sequences encoding the structural polyprotein. RNA synthesis is catalysed by the RNA-dependent RNA polymerase of the NSP4 protein and the other cleavage products

of the non-structural proteins. The subgenomic RNA is capped and methylated at the 5′ end, thus facilitating translation of the structural proteins. It is also referred to as 26S RNA according to its sedimentation behaviour – in contrast to the genomic 49S RNA (Fig. 14.18). In infected cells, many more subgenomic RNA molecules than genomic RNA molecules are synthesized. Similar replication processes that lead to the synthesis of a subgenomic mRNA have also been found in astroviruses, caliciviruses and hepeviruses (Sects. 14.2–14.4).

If sufficient amounts of dephosphorylated C proteins are present, they associate with nucleotide sequences in the 5′ region of the newly synthesized genomic positive-sense 49S RNA molecules and assemble into nucleocapsid precursors. These initial packaging steps also prevent interaction with the cap-binding complex and ribosomal subunits and that genomic RNA is translated, thus interrupting the synthesis of further NSP polyproteins. This relatively simple regulatory mechanism ensures that viral structural components are preferentially produced in the late phase of infection because they are required in much larger quantities for the formation of virus particles than the enzymatically active non-structural proteins at this time.

Subsequently, the preformed nucleocapsids associate with the carboxy-terminal regions of E2 proteins, and are then surrounded by the membrane and the viral glycoproteins embedded in it. These budding complexes can arise on the membranes of the ER and the Golgi apparatus as well as on the plasma membrane. The enveloped virions are either transported through Golgi vesicles to the cell surface or directly released there. During infection, apoptosis is also induced in the cells. This process, which results in cell death, has been found particularly in infections with rubella virus, whose C proteins also act proapoptotically.

14.6.5 Human Pathogenic Togaviruses

14.6.5.1 Rubella Virus

Epidemiology and Transmission

Rubella (German measles), which was frequently an epidemic disease, was described in detail by the German physician George de Maton in 1814. The virus was transmitted by ultrafiltrates to humans and monkeys for the first time in 1938. During an epidemic in Australia in 1940, the ophthalmologist Sir Norman Gregg discovered that the mothers of children with congenital cataracts, hearing loss and heart malformations (Gregg syndrome) had experienced a rubella infection during pregnancy (Table 14.15). Therefore, the virus causes not only the harmless rubella, but also severe embryopathies. The virus was cultivated for the first time in 1962. It generates a cytopathic effect in kidney cell lines of rabbits (RK-13) or monkeys (Vero cells). After a major epidemic in the USA in 1964, an attenuated live vaccine was developed in 1967. Its application led to the result that rubella occurs only very rarely today; since 2001, it has been considered as eradicated in the USA. Acute rubella infections are also rare in Europe owing to vaccination; there are estimated to be 150–400 cases per year in Germany, for example.

Table 14.15 Rubella embryopathy and its symptoms

Syndrome	Organ	Symptoms
Gregg syndrome	Heart	Persistent duct of Botallo Aortic stenosis
	Eyes	Cataract Glaucoma Retionopathy
	Ears	Inner ear defects
Expanded rubella syndrome		Mental retardation Low birth weight Dwarfism, osteopathy Encephalitis Hepatosplenomegaly Pneumonia Thrombocytopenia Purpura
Late rubella syndrome		Chronic exanthem Growth arrest Interstitial pneumonia IgG and IgA Hypogammaglobulinaemia Persistence of IgM
Late manifestation		Ear lesions Diabetes mellitus Progressive panencephalitis Convulsion disorder

The rubella virus is serologically uniform. With respect to its nucleic acid sequences, all isolates can be classified into two strains (clades 1 and 2), which can also be subdivided into ten genotypes (1a–1g, 2a–2c); the rubella virus of clade 2 has been isolated only in the Eurasian region. Rubella virus is found only in humans, but can be transmitted to some monkey species. Transmissions occur by droplet infection, and lead to infections during fleeting contact in about 20 % of cases. Carriers are infected individuals in the viraemic phase, which starts just 6 days before the onset of the exanthem and lasts 1–2 weeks (Fig. 14.19), infected infants who were born with congential rubella syndrome, and rarely adults with asymptomatic reinfections, who, nevertheless, can still transmit virus. Viruses are also found in the lacrimal fluid, in urine, in cervical secretions, in the stool, in the lungs, in cerebrospinal fluid and in synovial fluid.

Clinical Features

Postnatal Infections

Rubella is a relatively harmless, not very febrile illness. About half of infections are asymptomatic in children. The symptoms associated with the disease, mottled and not confluent exanthem, appear approximately 1–2 weeks after contact with the rubella virus and remain for up to 5 days (Fig. 14.19). The disease is often uncharacteristic and therefore indistinguishable from other viral diseases. Cold-like symptoms are absent; swollen neck lymph nodes are frequently observed. Especially in young women, the infection is associated in part with arthralgia of

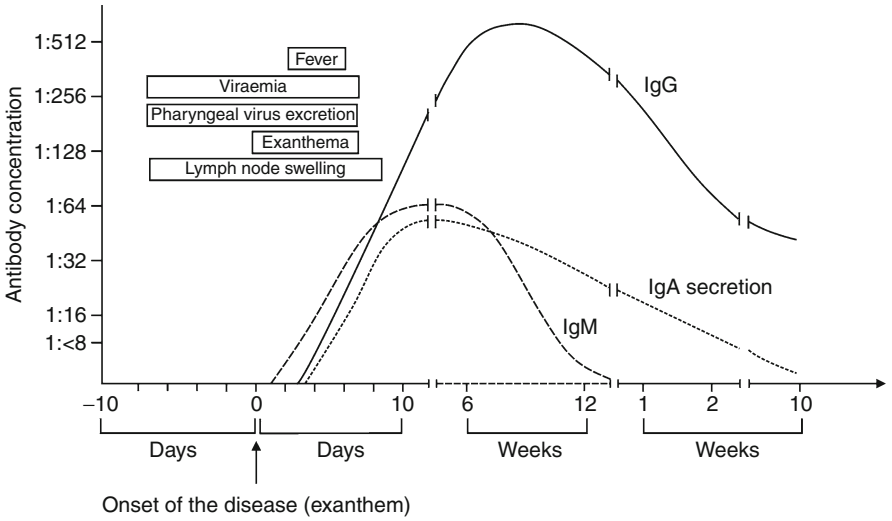


Fig. 14.19 Time course of antibody formation during rubella virus infection. Day 0 on the scale indicates the time at which the first appearance of the exanthem is observed. The incubation period lasts up to 10 days. As early as before the appearance of the exanthem, swollen lymph nodes are found, and rubella virus is detectable in the blood and pharyngeal lavage. Immediately after the onset of symptoms, IgM can be detected in the blood; its concentration decreases within 3–6 months. IgG antibodies appear after IgM antibodies and remain detectable for life

small joints, which usually subsides within a few weeks. A thrombocytopenia is rarely observed and its pathogenesis is still unclear. Encephalitis occurs with an incidence of 1:6,000. About 20 % of rubella infections with postinfectious encephalitis are fatal.

Prenatal Infections

If infections with rubella virus occur during the first trimester of pregnancy, abortion, stillbirth and malformations of the embryo can occur. By contrast, the mother shows no or only mild symptoms. A large number of embryos are particularly damaged during the first 20 weeks of pregnancy, the time of organ differentiation. Multiple defects occur primarily after an infection in the first 2 months of gestation; however, the complications are reduced to nearly zero after the third month of pregnancy. During the viraemic phase, the placenta is infected in 80–90 % of cases, whereas the embryo is infected with a probability of 60–70 %. Cardinal symptoms include eye damage, heart defects and inner-ear defects. Hearing defects, panencephalitis, diabetes and epilepsy or convulsion disorder are observed as long-term effects (Table 14.15).

Pathogenesis

The virus is transmitted by droplet infections. It penetrates into the organism through the mucous membranes of the upper respiratory tract. The primary replication occurs in the epithelium of this region. There, macrophages and lymphocytes

are infected, and carry the virus to local lymph nodes. There, the virus finds further target cells, where it replicates. Subsequently, the lymph nodes become extremely swollen. The viraemia probably originates from the infected lymph nodes, and rubella viruses are present in the blood (as free particles and in cell-bound form). They spread throughout the organism and can be detected in lacrimal fluid, in the nasal–pharyngeal region, in cervical secretions, in cerebrospinal fluid and in synovial fluid. The exanthem appears together with the first virus-specific antibodies. Immune complexes, i.e. antibody–virus complexes, are responsible for this response. They attach to the endothelium of blood capillaries in the skin and cause local inflammations.

Even the acute arthritis which is associated with the infection has been attributed to virus–antibody complexes that are present in synovial fluid. In cells of the synovial membranes, IL-1 is synthesized in large quantities, an indication that inflammation processes are occurring. Evidence that rubella virus can proliferate and persist in synovial cells has been found in infants with congenital rubella syndrome, i.e. in children who were infected during the embryonic stage. In such infants, rubella virus can be detected in the growth areas of the bones, the epiphyses and diaphyses. It is presumed that in these children persistent viruses trigger the production of interferons, which inhibit the proliferation of bone cells, resulting in retardation of growth of the extremities. In the rare cases of postinfectious encephalitis, viral proteins can occasionally be detected in brain tissue. It is suspected that a cellular autoimmune response is triggered against basic myelin protein in the spinal cord and nerve sheaths because lymphocytes of such patients proliferate after addition of this protein.

In infections during pregnancy, the viruses are transported into the placenta and the chorionic villi by blood. They proliferate there, invade the endothelium of placental blood vessels and reach the circulatory system of the child. The infected and detached endothelial cells form so-called emboli, which spread the virus in the organism. After birth, production and excretion of the virus last a long time (up to 1 year). How injury to the differentiating organs and impairment of embryonic cell division is achieved is unknown. It is thought that interferons and possibly other cytokines with cytotoxic properties or apoptosis mechanisms may play an important role.

Immune Response and Diagnosis

Postnatal Rubella

During infection, IgM, IgA and IgG antibodies are produced against the viral E1, E2 and C proteins. E1-specific immunoglobulins are neutralizing. Antibodies are detected by haemagglutination-inhibition tests or ELISA. Virus-specific IgM is detectable for about 4–6 months after infection; IgG is detectable lifelong (Fig. 14.19). Acute rubella infections can be diagnosed by the common presence of virus-specific IgM and IgG as well as by detection of viral RNA by PCR. The sole presence of IgG antibodies indicates a previous infection. In immune individuals, cytotoxic T cells can be detected, and these preferentially recognize epitopes of the C protein.

Prenatal Rubella

Embryonal infections can be diagnosed early by detecting viral RNA by PCR analyses of chorionic villi samples. Virus-specific IgM cannot be detected in umbilical cord blood before the 22nd and 23rd weeks of pregnancy. However, the mere detection of IgM and viral RNA does not provide evidence for whether there is an embryopathy. Rubella-virus-specific IgM antibodies and viral RNA remain detectable after birth for a long time. A peripheral tolerance, possibly in combination with a disturbance in switching the synthesis of antibody classes from IgM to IgG, may be responsible for the persistence of rubella infection. The stimulation of lymphocytes by viral proteins is significantly reduced.

Prenatal rubella is a medical indication for abortion. Because of the currently applicable regulations for maternity guidelines in many countries, immunity against rubella must be examined and documented in the maternity record book. In the case of suspected rubella exposure of a non-immune pregnant woman, the antibody titre has to be determined. If IgG antibodies are present at levels of 10 IU/mL and above, the patient is protected against the infection and there is no danger of damage to the embryo. If the level is below 10 IU/mL, and there is also no clear reactivity in alternative tests, rubella-virus-specific IgG should be administered within 3 days after exposure because a fresh infection is possible. Subsequently, it must be ascertained by further antibody tests whether an infection is actually ongoing or has finished in the pregnant woman, and whether the embryo has been infected. A further complication is that IgM reactivity in the ELISA may be unspecific during gestation. If rubella-virus-specific IgM antibodies are present in the umbilical cord blood and viral RNA is detected by PCR, this is an indication for termination of pregnancy because of the high risk of possible harm to the unborn child by the infection. The retrospective determination of the probable date of infection of the mother is of crucial importance since the risk of embryopathy decreases significantly in the course of the first trimester of pregnancy. This includes immunoblot analysis of avidity for and reactivity against individual proteins.

Therapy and Prophylaxis

There is an attenuated live vaccine against rubella virus infections. Strain RA 27/3 has been used since 1979. This vaccine induces high antibody titres, and its application has led to a dramatic decrease in the incidence of rubella in Europe and North America, where it occurs only sporadically. Today, the rubella vaccine is administered as a combined vaccine with attenuated live vaccine strains against measles, mumps and varicella at the beginning of the second year of life; a two-time application is considered as protective. Since the introduction of vaccination, both the number of infections and the number of congenital rubella embryopathies have substantially decreased. In most countries both congenital and acute postnatal rubella are notifiable.

In cases of proven exposure of non-immune pregnant women to rubella virus, a passive immunization with virus-specific IgG may be performed. The protective effect is the greater, the sooner the passive immunization is performed; even 3 days

after exposure, there is only a slight protection. The use of the live vaccine is not allowed during pregnancy. An antiviral chemotherapy does not exist.

Chikungunya Virus Has Recently Caused Human Diseases in Europe
Chikungunya virus was isolated from a patient with a febrile illness in the former Tanganyika (now part of Tanzania) in 1953. Infections with this virus have likely been noticed since the late eighteenth century but incorrectly documented as epidemic outbreaks of dengue fever. Between 1960 and 2003, chikungunya virus was repeatedly ascertained to be the cause of regionally restricted epidemics in the countries of eastern, southern and western Africa as well as in Southeast Asia (among others, in India, Pakistan, Malaysia, Thailand, Indonesia and Vietnam). Since 2004, the virus has repeatedly been found also in patients in different European countries, particularly in Italy, but also in Germany, Belgium, France, Spain, the UK and Norway. Infections induce severe fever, arthralgia and rash, and have a high morbidity. In particular, joint swelling and inflammations can last for months – they gave the virus its name: *chikungunya* means "that which bends up". The natural hosts and reservoirs for the pathogens are non-human primates in Africa and Southeast Asia. The virus is transmitted to a number of different *Aedes* mosquito species, which further spread it to and also between humans. The tiger mosquito (*A. albopictus*) is considered to be particularly responsible for the chikungunya virus infections that have been observed in Europe in the last few years, as these mosquitoes can also exist in subtropical and cooler regions. Presumably the virus was originally introduced to European countries by tourists or mosquitoes, resulting in local outbreaks. In particular, further warming of the climate could have the effect that the *Aedes* mosquitoes increasingly feel at home in Europe, which could thus lead to increased infections with chikungunya virus.

14.6.6 Animal Pathogenic Togaviruses

14.6.6.1 The Various Equine Encephalitis Viruses

Epidemiology and Transmission

The genus *Alphavirus* encompasses pathogens that are transmitted by arthropods, essentially by mosquitoes of the genera *Culex* and *Aedes*, and can cause various diseases in humans and animals. In addition to the neurotropic alphaviruses that can cause encephalitis, in particular the different types of equine encephalitis viruses and some other alphaviruses of the New World, alphaviruses play a role in Europe, Africa and Asia, and usually provoke mild symptoms or moderate arthritis.

Besides their significance as zoonotic pathogens, equine encephalitis viruses are also important because of their epidemiology and molecular evolution. All have an enzootic cycle that includes small rodents and birds. They are transmitted between

their hosts by mosquitoes, and the host specificity of the mosquitoes determines the nature of the infection. Horses and humans are merely accidental hosts from which the virus cannot spread further. Only certain epizootic subtypes of Venezuelan equine encephalitis virus are able to cause viraemia in horses or humans, during which they proliferate in the large quantities that are necessary to infect a sucking mosquito. Infection of horses and humans contributes to dissemination of the viruses solely in such cases.

Eastern equine encephalitis virus is endemic in the southern USA and in many countries of South America. It asymptomatically infects different bird species, including various songbirds and wading birds. In other introduced bird species such as pheasant and emu, it causes lethal infections and severe economic losses. Certain mosquitoes that suck blood only in birds, particularly *Culiseta melanura*, maintain this endemic bird cycle. If blood of infected birds is sucked by other mosquito species which can also bite mammals and humans, they can cause epidemics which include mammals as hosts. In regions with a temperate climate, there is a seasonal accumulation of diseases in the late summer, whereas in tropical climates, infections and transmissions of the reservoir hosts occur throughout the year.

Western equine encephalitis virus is also widespread on the American continent, and is transmitted by various mosquito species. Whereas in North America the enzootic cycle is largely maintained by infection of various songbirds, in South America, especially rodents play a prominent role as a reservoir. The infection of horses and humans is also accidental.

Unlike the eastern and western equine encephalitis viruses, the switch from an enzootic to an epizootic cycle in Venezuelan equine encephalitis virus depends on a mutation in the coding sequences for the E2 protein of the prevalent enzootic virus. Whereas the enzootic subtypes I-D to I-F and II–VI induce only limited infections in small rodents and are transmitted by mosquitoes of the genus *Culex*, the transmission of the epizootic subtypes I-AB and I-C is performed by mosquitoes of the genera *Aedes* and *Psoropha* and involves the infection of a variety of mammals, including horses and humans. The pathogenetic basis for the epizootic types lies in their ability to cause a high-titre viraemia in their hosts and to facilitate transmission. Phylogenetic studies have revealed that the epizootic subtypes possibly arise from enzootic viruses before each epizootic outbreak. Thus, the epizootic biotype can be generated by replacement of the E2 gene from an enzootic I-D virus by that of an I-AB virus.

In addition to the epizootiology of these viruses, their evolution is also interesting. It has been shown that western equine encephalitis virus emerged from a recombination between eastern equine encephalitis virus and a similar precursor virus with similarity to Sindbis virus (► Chap. 12). This recombination resulted in the replacement of the genes encoding the glycoproteins of eastern equine encephalitis virus by those of the Sindbis-like virus.

Clinical Features

Infections with the alphaviruses that are widespread in eastern Europe, Asia and Africa are often asymptomatic (Semliki Forest virus) or are associated with slight

fever, rash and arthritis (Sindbis virus). The American equine encephalitis viruses, however, frequently induce encephalitis in the infected organism.

Pathogenesis

Alphaviruses are transmitted by insect bites directly into the bloodstream and attach, using envelope proteins, to unknown receptors on endothelial and lymphatic cells. They then proliferate in these cells. They are transported through the bloodstream to other target organs. The damaged endothelium allows the transfer of the virus into the central nervous system, where it proliferates in neurons. The mechanisms of neuronal injury by induction of apoptosis have been studied in detail in Sindbis virus infections in mice. Sindbis virus shows an age-dependent pathogenicity in mice. Newborn mice become lethally ill after an intracerebral infection, whereas mice do not become sick at the age of 4 weeks and eliminate the virus 1 week after inoculation. The virus is eliminated by virus-specific antibodies. This phenomenon is based on the fact that the viral infection induces apoptosis in immature neurons, whereas mature neurons can prevent this process by an unknown factor. However, the resistance is not absolute and can be broken by extremely virulent virus strains. Obviously, the viral glycoprotein E2 is of extraordinary importance: the study of mutant viruses that were produced by site-directed mutagenesis revealed a special role in virulence of the amino acid histidine at position 55.

Immune Response and Diagnosis

Equine encephalitis virus infections lead to a stable, long-lasting immunity. The diagnosis can be ascertained by the direct detection of the virus in the blood during the viraemic phase, or after death in brain tissue. Indirect evidence of infection by the examination of serum pairs that are obtained at the time of acute infection and after a further 3 weeks is commonly used. The antibody titres are determined in haemagglutination-inhibition or in virus-neutralization tests.

Control and Prophylaxis

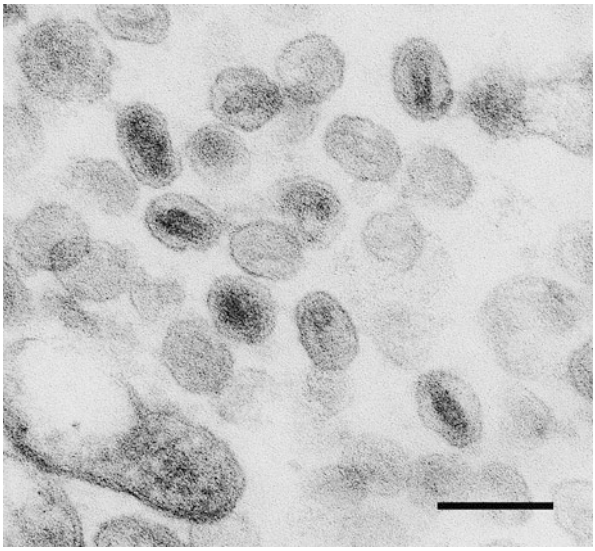
Vaccines are available against infections with all members of equine encephalitis viruses, and are used in humans, horses and birds such as the threatened whooping crane (*Grus americana*). They are based on inactivated viruses that were propagated in cell culture. A consequent vaccination can prevent the emergence of epizootic diseases, or favourably influence its development after an outbreak.

Enzootic and Epizootic Cycles Determine the Epidemiology of Equine Encephalitis Viruses

Eastern equine encephalitis virus, western equine encephalitis virus and Venezuelan equine encephalitis virus are arboviruses (*ar*thropod-*bo*rne), which usually replicate asymptomatically in their hosts, especially songbirds and rodents, and establish a sufficiently high viraemia to be transmitted by mosquitoes among these hosts. This is referred to as an enzootic cycle.

Various factors such as a massive proliferation of not strictly host-specific mosquito species, which serve as reservoir for the virus, can lead to sucking of other species, thus contributing to the transmission to humans, horses and other mammals. This is referred to as an epizootic cycle.

14.7 Arteriviruses



Arteriviruses constitute along with coronaviruses (Sect. 14.8) and roniviruses the order *Nidovirales*. This classification is based on the genome organization, on the use of polycistronic mRNA transcripts for viral gene expression and on the transcription and translation strategies, i.e. on features and processes which are similar in the members of these virus families. The name of the order has its origin in the Latin word *nidus* meaning "nest". It refers to the unique transcriptional strategy of the members of the order *Nidovirales*. During mRNA synthesis, transcripts are synthesized with the same 5′ and 3′ termini, but in which the 5′ terminal sequence region is combined with different RNA segments that are localized farther downstream; therefore, sets of polycistronic (nested) mRNA molecules are generated. On the other hand, the differences in the size of the viral genomes and particles as well as in the sequence and nature of the structural proteins are so clear that they have been classified into separate families. Compared with the particles and the genomes of coronaviruses and roniviruses, those of arteriviruses are much smaller. Members of the Roniviridae and the Mesoniviridae, a recently designed virus family, infect only invertebrates and insects; therefore, they will not be discussed in more detail in this book.

Table 14.16 Characteristic prototypes of arteriviruses

Genus	Animal virus
Arterivirus	Equine arteritis virus
	Lactate dehydrogenase elevating virus of mice
	Porcine reproductive and respiratory syndrome virus
	Simian haemorrhagic fever virus

14.7.1 Classification and Characteristic Prototypes

In the family of arteriviruses, which comprises exclusively animal pathogens, there is only one genus (Table 14.16). The characteristic prototype is equine arteritis virus, which induces persistent asymptomatic infections in horses and donkeys; however, it can also cause miscarriages or haemorrhagic fever in these animals. Lactate dehydrogenase elevating virus (LDV) and simian haemorrhagic fever virus infect mice and various African and Asian monkeys, respectively. These pathogens were described for the first time in the nineteenth century. By contrast, infections of swine with porcine reproductive and respiratory syndrome virus (PRRSV) occurred for the first time in Europe and the USA nearly simultaneously between 1983 and 1988.

14.7.2 Structure

14.7.2.1 Virus Particle

The infectious particles of arteriviruses have a diameter of 40–60 nm and consist of spherical or icosahedral nucleocapsids (diameter 25–35 nm), which are surrounded by an envelope (Fig. 14.20). In contrast to coronaviruses, arteriviruses possess envelope proteins protruding only slightly from the surface of the particle (10–14 nm). Four viral glycoproteins are inserted in the envelope: GP_2, GP_3, GP_4 and GP_5; in the case of LDV, GP_2, GP_3, GP_4 and GP_7. In addition, the M and E proteins are associated with the viral membrane. The nucleocapsid inside the envelope is composed of N proteins and the single-stranded RNA genome.

14.7.2.2 Genome Organization and Structure

The genomes of arteriviruses have an organization similar to that of coronaviruses; however, the succession of coding regions is much more densely packed than in the latter. Therefore, they are shorter and comprise between 12,704 nucleotides in equine arteritis virus and approximately 15,000 and 15,700 nucleotides in PRRSV and simian haemorrhagic fever virus, respectively. They consist of single-stranded, positive-sense RNA containing a methylated cap structure at the 5′ terminus and a poly(A) tail the 3′ end. The coding regions of the genome are flanked by 156–221 and 59–117 untranslated nucleotides at the 5′ and 3′ termini, respectively. Two large reading frames (1a and 1b), of which 1a begins at the 5′ terminus, have overlapping ends and cover approximately two thirds of

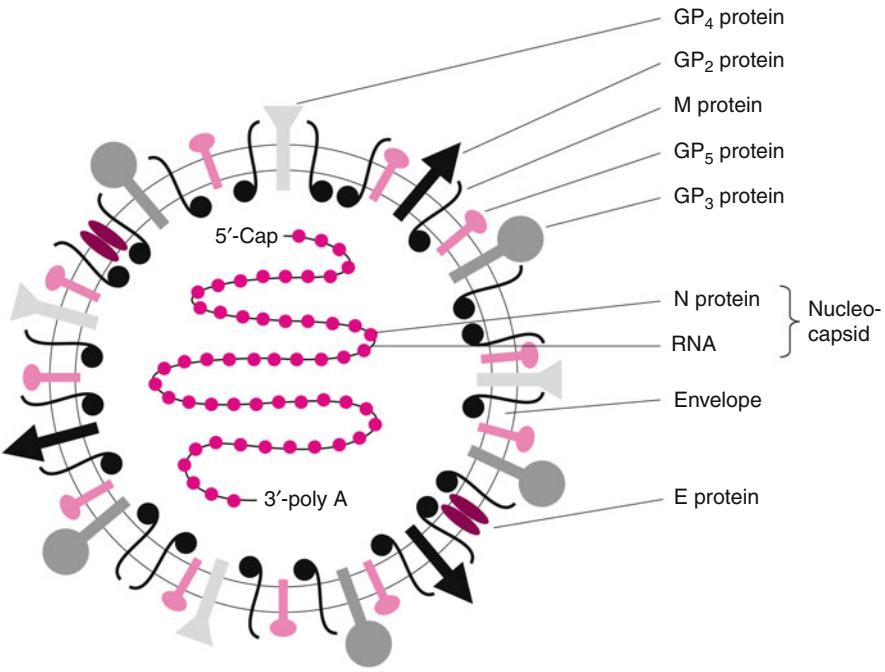


Fig. 14.20 Structure of an arterivirus particle represented by porcine reproductive and respiratory syndrome virus. Inside the particle, the RNA genome is associated with N proteins in a helical nucleocapsid, which is enclosed by a membrane envelope, in which four viral glycoproteins (GP_2–GP_5) and the non-glycosylated M protein are embedded

the genome (Fig. 14.21). They contain the genetic information for the synthesis of two polyproteins (1a and 1ab), from which the non-structural proteins are generated by proteolytic cleavage. A ribosomal frameshift event during translation leads to skipping the stop codon at the end of reading frame 1a, resulting in the synthesis of the larger protein 1ab. Autocatalytic cleavage leads to the generation of three proteases (NSP1, NSP2, NSP4), RNA-dependent RNA polymerase, a helicase and several other non-structural proteins of unknown function. Reading frames 2–7 (or 9 in PRRSV) encoding the structural proteins are located downstream of the genes of non-structural proteins. Towards the 3′ end, they encode the different glycoproteins and the M and N proteins; the E protein is encoded by an internal reading frame located on the second longest mRNA.

14.7.3 Viral Proteins

14.7.3.1 Non-Structural Proteins

Two major precursor products of the non-structural proteins are synthesized during translation of the genomic mRNA: protein 1a has a molecular mass of 187–260 kDa

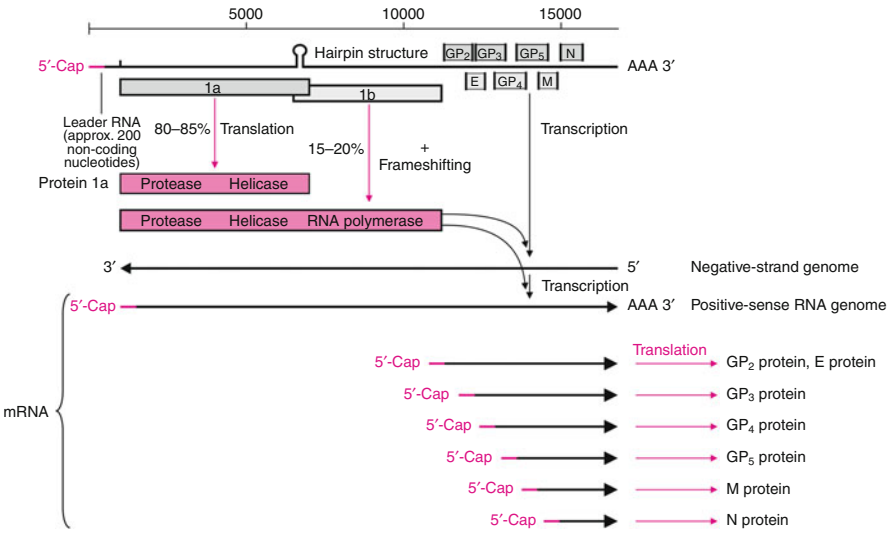


Fig. 14.21 Genome organization and replication in arteriviruses. The viral RNA genome functions as mRNA, and is translated in the cytoplasm. The two overlapping reading frames encode non-structural polyproteins 1a and 1ab. A hairpin structure induces a ribosomal frameshift during translation that leads in approximately 15–20 % of all translation processes to the synthesis of non-structural polyprotein 1ab, which contains the RNA-dependent RNA polymerase domain in its carboxy-terminal region. Both polyproteins are cleaved by the autocatalytic activity of two cysteine proteases and a serine protease that are located in the amino-terminal regions of the precursor proteins. The RNA-dependent RNA polymerase transcribes the positive-sense RNA genome into a negative-sense RNA strand. This serves both as a template for the synthesis of new positive-sense RNA genomes and for transcription of a series of subgenomic mRNA species, which are modified at the 5′ end with a cap group and contain identical leader sequences in all mRNA molecules. The structural proteins are translated from the different subgenomic mRNAs, whose reading frames are localized in the 3′-terminal third of the positive-sense RNA genome. They partially overlap

among the different members of arteriviruses, the amino-terminal half of protein 1ab (345–421 kDa) is identical to that of protein 1a. The three proteases (NSP1, NSP2, NSP4) that reside in the amino-terminal region of both proteins cleave the precursor proteins into a total of 12 non-structural proteins (NSP1–NSP12), whose functions are in some cases not resolved.

The NSP1 and NSP2 proteins are cysteine proteases which are autocatalytically cleaved from the polyproteins (Table 14.17). NSP4 is a serine protease and is similar to the 3C protease of picornaviruses (Sect. 14.1). Its activity is required for subsequent processing of the precursor proteins 1a and 1ab at up to eight cleavage sites. This proteolytic processing generates, among others, RNA-dependent RNA polymerase, metal ion binding protein MP and a helicase (NSP10). The latter has a Zn^{2+} ion binding domain, which is needed for the synthesis of viral mRNAs, but is not necessary for genome replication. NSP11 constitutes the endoribonuclease

Table 14.17 Functions and properties of arterivirus proteins

Protein	Size (kDa)	Properties	Function
GP_5/GP_7 (LDV)	24–44	Glycosylated	Membrane protein; major structural protein neutralizing antibodies, forms heterodimers with M protein, apoptosis induction?
GP_2	20–35	Glycosylated	Membrane protein, minor structural protein, heterotrimer with GP_3 and GP_4
GP_3	27	Glycosylated	Membrane protein; minor structural protein, heterotrimer with GP_2 und GP_4
GP_4	20	Glycosylated	Membrane protein; minor structural protein, heterotrimer with GP_2 und GP_3
M	16–20	–	Membrane protein; major structural protein, forms heterodimers with GP_5 and GP_7
N	12–15	Phosphorylated	Nucleocapsid protein; major structural protein, homodimer
E	7–8	Very hydrophobic	Ion channel protein? Homooligomers
NSP1	29		Papain-like cysteine protease
NSP2	61		Cysteine protease
NSP4	21		Serine protease, homology to chymotrypsin and 3C proteases of picornaviruses, main protease
NSP9	?		RNA-dependent RNA polymerase
NSP10	?		RNA helicase
NSP11	?		Endoribonuclease (NendoU)
MP	?	Zn^{2+}-binding	?

(nidoviral uridylate-specific endoribonuclease, NendoU) which is present in all members of the order *Nidovirales*. Its activity seems to be especially necessary for the synthesis of subgenomic RNA molecules. In most cases, the functional assignments have been made by comparative sequence analysis and not by direct purification and biochemical characterization of protein activities.

XendoU and NendoU

The cellular endoribonuclease XendoU, originally discovered in *Xenopus laevis*, is responsible for processing of nucleic RNA species and belongs to a small protein family, into which the nidoviral endoribonuclease NendoU is also classified. The enzymatic activity of NendoU is dependent on Mn^{2+} ions. It preferentially cleaves double-stranded RNA before or after uridine residues in the sequences GUU and GU, generating molecules with 2′-3′-cyclic phosphate ends. Furthermore, NendoU enzymes, which have only been found in nidoviruses so far, seem to possess some additional, but not yet characterized activities.

14.7.3.2 Structural Proteins

The structural proteins of arteriviruses can be subdivided into major and minor proteins. Major proteins are GP_5 (GP_7 in LDV), M and N. The other group includes GP_2, GP_3, GP_4 and E proteins. The function of the structural E protein has not been definitively resolved, but it is essential for infectivity, and there are indications that it might be an ion channel protein. The glycoproteins are present in complex arrangements, GP_5/GP_7 and M proteins form heterodimers, whereas GP_2, GP_3 and GP_4 form heterotrimers. In addition, intermediates of the GP_2 and GP_4 heterodimers can be observed, and associate with GP_3 by cysteine bonds in the subsequent step. This complex formation with GP_3 is a prerequisite for their installation in the viral envelope. The neutralizing epitopes are located primarily in the GP_5 or GP_7 proteins. However, the presence of heterodimeric structures with the M protein seems to be essential for their correct conformation.

The M protein is the structural protein of arteriviruses with the highest degree of conservation. It resembles the M protein of coronaviruses. The carboxy-terminal domain is located inside the particle, whereas the short amino-terminal region is localized on the viral surface and is flanked by hydrophobic sequences that anchor the protein in the envelope. Through a cysteine residue in the amino-terminal domain, the M protein forms a disulphide bond with glycoprotein GP_5, or GP_7 in LDV.

The phosphorylated N protein (12–15 kDa) is bound to the RNA genome and forms the nucleocapsid.

14.7.4 Replication

Arteriviruses preferably infect macrophages and gain access to the cell through receptor-mediated endocytosis. The receptors used by the virus for attachment are not definitively known. PRRSV appears to interact with heparan sulphate on the surface of macrophages. In addition, it has also been found that the virus binds to CD163 and to the amino-terminal variable immunoglobulin-like domain of sialoadhesin, a member of the immunoglobulin superfamily on the surface of macrophages. Which viral glycoprotein mediates this interaction is not clear. The replication cycle is very similar to that of coronavirus; also in arteriviruses, all replication steps occur in the cytoplasm of the cell. As already mentioned, non-structural polyproteins 1a and 1ab are primarily translated from the genomic RNA by induction of a ribosomal frameshifting mechanism. Protein 1ab comprises the RNA-dependent RNA polymerase (Fig. 14.21).

In the next step, the complementary strand is synthesized by the polymerase activity of the enzyme using the genomic RNA as a template. It encompasses the entire genome and has a negative polarity. Later, it has two functions in the replication cycle: it serves as a template for the synthesis of new viral genomes and for the production of subgenomic mRNA species, from which the various structural proteins of arteriviruses are translated. All subgenomic mRNAs have

the same 5′ and 3′ termini, which correspond to those of the viral genome. At the 5′ ends there is a uniform sequence, the leader RNA. It is capped at the 5′ terminus and corresponds to the sequences in the 5′ UTR of the genome. The leader sequence serves as a primer for the synthesis of subgenomic mRNA species. Near its 3′ end, the leader RNA exhibits a conserved sequence (UCAAC in equine arteritis virus). Sequences complementary to this motif are found in the negative-sense RNA at different regions. They are located upstream of the different initiation sites for the synthesis of subgenomic mRNA species in the region of the genome between the end of the reading frame encoding polyprotein 1ab and the 3′ end of the genome: they can hybridize with the leader RNA, thus providing a small double-stranded region with a free 3′-OH end to continue polymerization. As in coronaviruses, the RNA polymerase can itself probably not initiate the synthesis of subgenomic mRNA species at the different sites. Only the reading frames adjacent to the 5′ end of these nested transcripts are usually translated into proteins; transcription of an internal reading frame has only been observed in the case of the E protein.

The N protein interacts with genomic RNA to form the nucleocapsids and binds to the carboxy-terminal domain of the M protein, which is embedded in the ER membrane. This interaction triggers the budding process, during which the nucleocapsid is surrounded by the membrane containing M protein and glycoproteins. The particles formed are released into the ER lumen, and are further transported via Golgi vesicles to the cell surface, where they are released into the environment.

14.7.5 Animal Pathogenic Arteriviruses

No arteriviruses that are able to infect or to cause disease in humans are known. The most important animal pathogenic arteriviruses include equine arteritis virus and PRRSV.

14.7.5.1 Equine Arteritis Virus

Epidemiology and Transmission

Equine arteritis virus has a worldwide distribution. It induces a well-known disorder that is now known as equine arteritis. It is manifested in oedema of the head and extremities. This manifestation of the disease has also led to the synonyms “pink eye” and “equine influenza”. An economically important form of the disease is abortion in pregnant mares, which can occur epidemically in stud farms.

Only horses seem to be susceptible to the infection. The virus is transmitted by direct contact and through aerosols. The infection occurs more frequently at tournaments, fairs or exhibitions, where many horses from different regions come together. The virus can cause a persistent infection; some stallions excrete viruses in the ejaculate for many years. This is epidemiologically very important and constitutes an animal health problem because the virus can be spread in this way among the population even when artificial insemination is used.

Clinical Features

After an incubation period of about 3 days to 2 weeks, the animals develop fever and oedemas in the head, limbs and abdomen (preputial and scrotal oedema in stallions), as well as conjunctivitis. The disease is normally transient, and deaths are rarely observed. In pregnant mares, abortion can occur 10–30 days after infection. In a seronegative, and therefore susceptible livestock population, up to 80 % of gestating mares can abort ("abortion storms"). Temporary infertility can also be observed in stallions as a complication of equine arteritis virus infections. However, the infection usually develops subclinically and diseases are rarely observed.

Pathogenesis

The main target cells of the virus are macrophages and endothelial cells. This tropism explains both the symptoms and the pathological aspects of the disease. With the exception of the central nervous system, the virus can very quickly reach virtually every organ system, where it induces vascular perturbations and pathological alterations. Local infarctions and bruises (oedema) are combined with systemic hypovolaemic symptoms. Intrauterine transmission leads to an infection of the fetus, which also dies, and is aborted owing to generalized oedemas. Abortion is habitually associated with the complete detachment of the placenta.

Immune Response and Diagnosis

The diagnosis can be made by neutralization tests. The examination of serum pairs allows a retrospective diagnosis of acute infections. Detection of the virus is achieved by cultivation of the pathogen in equine cell lines or by using RT-PCR.

Control and Prophylaxis

In several countries, vaccines based on both inactivated and attenuated viruses have been licensed. Both vaccines have proven to be basically effective.

14.7.5.2 Porcine Reproductive and Respiratory Syndrome Virus

Epidemiology and Transmission

PRRSV has only been known since around 1985, when it emerged almost simultaneously in Europe and North America. Sequence analyses of the first viruses isolated on both continents revealed a sequence identity of only about 60 %. The isolates are referred to as genotypes owing to that divergence. Genotype I includes the isolates of the European type, whereas genotype II includes those of the North American type. The origin of these genotypes is uncertain. One hypothesis assumes a host switch from mice to swine which occurred independently in Europe and North America. Alternatively, it is also discussed that wild boars might have been infected by mice with an LDV-like virus in Europe, and were then transported from Europe to North America. Inasmuch as PRRSV is considered as the virus with the

highest known mutation rate, it is quite conceivable that it might have developed in separated swine populations into the known genotypes.

The virus seems to infect only swine and is transmitted by direct contact between animals. Epidemiologically important are clinically healthy swine with persistent PRRSV infection which spread the virus in susceptible herds. The complete viral contamination occurs within a few weeks.

Clinical Features

PRRSV infections cause a disease in pigs which is very similar to equine arteritis. Typical symptoms include fertility problems, which are principally manifested as late abortions after a gestation period of more than 110 days (the normal gestation period in pigs is115 days). In addition, dead and mummified fetuses can also be aborted. In contrast to infection with porcine parvoviruses (▶ Sect. 20.1.6), pathological changes can be observed in aborting sows. These include endometritis and myometritis, frequently accompanied by haemorrhagic placenta. In non-pregnant animals, the infection is present as a febrile systemic disease that is often accompanied by respiratory symptoms. A classic symptom is an abnormal blue discoloration of the ears, the snout and the vulva owing to reduced blood flow.

Pathogenesis

The target cells of the virus are macrophages and endothelial cells. The virus persists in macrophages despite the presence of neutralizing antibodies. These antibodies may contribute to the pathogenesis of the disease by an antibody-dependent cytotoxicity (▶ Chap. 7). There are indications that PRRSV prevents the induction of IFN-β: it inactivates IFN-β promoter stimulator 1 (IPS-1), an adapter molecule for RIG-I helicase, and thus inhibits the RIG-I-mediated signalling cascade.

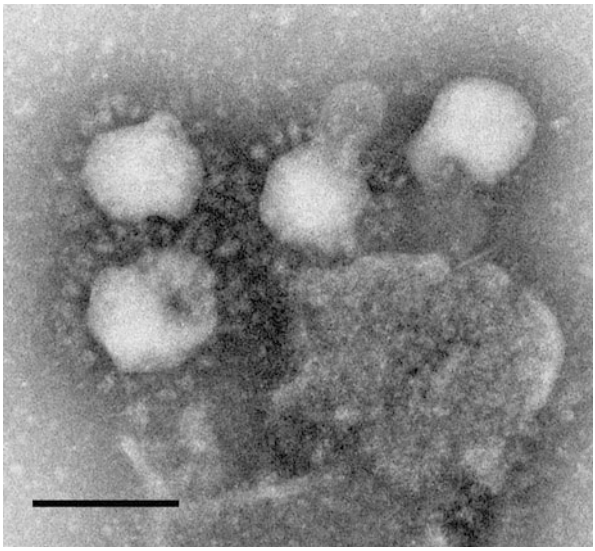
Immune Response and Diagnosis

The virus can be detected by immunofluorescence in stillborn piglets. Cultivation of the virus in cell culture is possible in porcine macrophages or in the monkey kidney cell line MA-104. Antibodies are detectable using commercial ELISAs. However, serology is of only limited diagnostic significance owing to the widespread vaccination of pigs.

Control and Prevention

There are live vaccines for protection against infections with the two genotypes as well as inactivated vaccines, the effectiveness of which is controversially discussed. The currently available inactivated vaccines seem to be considerably less efficient than the attenuated vaccines. A very lively debate is being conducted about the effectiveness of the vaccines beyond the limits of the genotypes and the possible back mutation of attenuated vaccine viruses of both genotypes into virulent pathogens.

14.8 Coronaviruses



The family *Coronaviridae* is classified, together with the families *Arteriviridae* (Sect. 14.7) and Roniviridae, into the order *Nidovirales*. Human coronaviruses were discovered by David A.J. Tyrrell and colleagues during cold epidemics in 1965. They were originally defined as their own family on the basis of morphological differences from other viruses in 1968. Electron micrographs revealed virus particles that are surrounded by an envelope containing embedded proteins, which confer on them the appearance of a "halo" (Latin, *corona*). When the molecular details of the genome structure and replication mechanisms became known later, they confirmed the original classification based strictly on morphological analyses.

Coronavirus infections cause predominantly harmless colds and infections of the upper respiratory tract in humans. Infections with coronaviruses are also known in some domestic mammals. Essentially, they are associated with acute gastroenteritis in cattle, swine, cats and dogs. However, there are also other disease patterns, such as encephalitis in pigs and a fatal systemic general infection in cats, known as feline infectious peritonitis, which is caused by the feline coronavirus. The mouse hepatitis virus, which provokes liver inflammation and bronchitis in rodents, is an important model system for unravelling pathogenetic mechanisms. Besides the different coronaviruses that infect mammals, there are some types that cause severe infections in poultry. Most notable is avian infectious bronchitis virus.

Infections with the severe acute respiratory syndrome (SARS)-related coronavirus emerged in humans for the first time in the winter of 2002–2003, mainly in Southeast Asian countries (China, Hong Kong, Taiwan), but also in Canada. Worldwide, this unique outbreak caused more than 8,000 manifest infections and 700 deaths, corresponding to a mortality rate of about 10 %. The origin of this novel virus was

unclear for a long time until an almost identical SARS-related coronavirus was found in China in bats (*Rhinolophus* spp., greater horseshoe bat). They transmit the pathogen to civet cats, which in turn can pass the pathogen on to humans in animal markets.

14.8.1 Classification and Characteristic Prototypes

Coronaviruses are subdivided into two subfamilies, *Coronavirinae* and *Torovirinae*. In the subfamily *Torovirinae*, white bream virus is a member of the genus *Bafinivirus* and is pathogenic for fish, whereas bovine torovirus equine torovirus, human torovirus and porcine torovirus belong to the genus *Torovirus* and cause gastrointestinal infection in the respective animals. The biology of toroviruses and the diseases that they cause have barely been explored, however. The subfamily *Coronavirinae* is classified into four genera according to differences in genome organization and sequence (Table 14.18). Coronaviruses that infect humans as well as many mammals such as ungulates, carnivores and bats have recently been assigned into the genera *Alphacoronavirus* and *Betacoronavirus*. SARS-related coronavirus has been classified into the latter genus along with various related coronaviruses isolated from civet cats and bats. The genera *Gammacoronavirus* and *Deltacoronavirus* contain species that cause disease in various birds.

14.8.2 Structure

14.8.2.1 Virus Particle

The enveloped virions of coronaviruses have a diameter of 80–180 nm. The single-stranded, positive-sense RNA genome is associated with the N protein as a nucleocapsid in the interior of the particle (Fig. 14.22). The nucleocapsid has a helical shape. The helix has a diameter of 10–20 nm. Particular amino acids of the N protein interact with the carboxy-terminal domain of the M protein that is inserted in the envelope. In this way, the nucleocapsid is associated through protein interactions with the inner side of the envelope. Apart from the M protein, an amino-terminally glycosylated protein of 20–30 kDa, two other viral proteins are embedded in the envelope: the glycosylated S protein (180–200 kDa) is present in club-shaped trimers, which protrude about 20 nm from the envelope surface and are responsible for the appearance of the corona, and the E protein (9–12 kDa), which is present in only small amounts. Another membrane-associated protein, haemagglutinin esterase (HE), is present only in members of the genus *Betacoronavirus*. It has a molecular mass of 65 kDa, is present as a dimer and exhibits haemagglutination activity.

14.8.2.2 Genome Organization and Structure

Coronaviruses possess the largest genome of all known RNA viruses: it has a length of 27,000–32,000 nucleotides (human coronavirus 229E, 27,317 nucleotides; avian infectious bronchitis virus, 27,608 nucleotides; porcine

Table 14.18 Characteristic prototypes of coronaviruses

Subfamily	Genus	Human virus	Animal virus
Coronavirinae	**Alphacoronavirus**	**Human coronavirus 299E**	**Alphacoronavirus 1**
			Porcine transmissible gastroenteritis virus
			Feline coronavirus (feline enteric coronavirus, feline infectious peritonitis virus)
		Human coronavirus NL63	**Miniopterus bat coronavirus 1**
			Miniopterus bat coronavirus HKU8
			Rhinolophus bat coronavirus HKU2
	Betacoronavirus	**Betacoronavirus 1**	**Betacoronavirus 1**
		Human coronavirus OC43	Bovine coronavirus
			Equine coronavirus
			Porcine hemagglutinating encephalomyelitis virus
		Human coronavirus HKU1	**Murine coronavirus**
			Mouse hepatitis virus
			Rat Coronavirus
		SARS-related coronavirus	**SARS-related coronavirus**
			SARS coronavirus (Bat coronavirus HKU3, SARS coroanvirus civet)
			Pipistrellus bat coronavirus HKU5
			Rousettus bat coronavirus HKU9
	Gammacoronavirinae		**Avian coronavirus**
			Avian infectious bronchitis virus
			Turkey coronavirus
			Beluga Whale coronavirus SW1
	Deltacoronavirus		**Bulbul coronavirus HKU11**
			Thrush coronavirus HKU12
Torovirinae	**Bafinivirus**		**White bream virus**
	Torovirus	**Human torovirus**	**Bovine torovirus**
			Equine torovirus
			Porcine torovirus

SARS-Severe acute respiratory syndrome. The names for virus species are given in bold, in combination with selected virus types

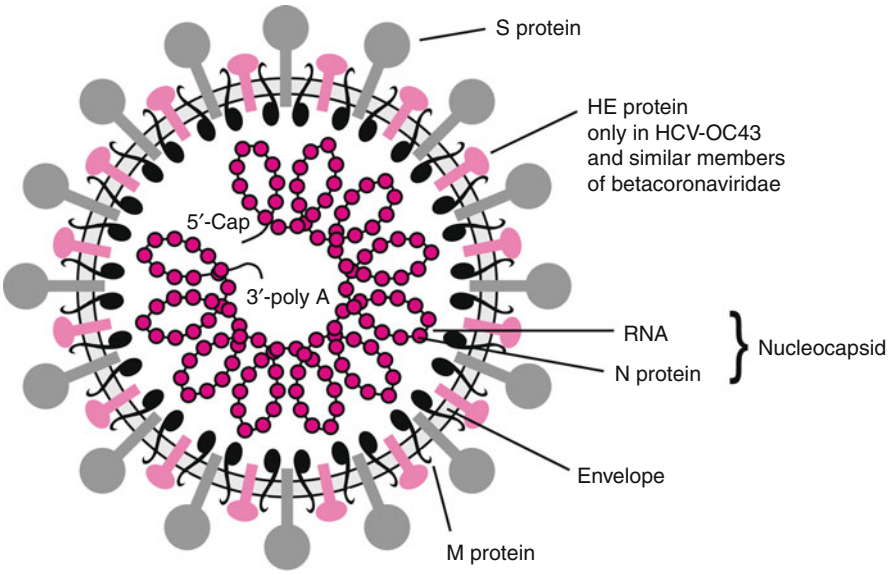


Fig. 14.22 Structure of a coronavirus particle. The RNA genome is complexed with N proteins into a helical nucleocapsid on the inner side of the particle. It is surrounded by an envelope, in which the S and HE glycoproteins and the non-glycosylated M protein are integrated. *HCV* human cornonavirus

transmissible gastroenteritis virus, 28,580 nucleotides; SARS-related coronavirus, 29,727 nucleotides; mouse hepatitis virus, 31,357 nucleotides), is single-stranded and has a positive-sense polarity, and is modified at the 5′ end with a cap-structure and at the 3′ terminus with a poly(A) tail (Fig. 14.23). The RNA is infectious. The genome contains multiple coding regions: two relatively large open reading frames, 1a and 1b, overlap at their ends by 40–60 nucleotides (1a starts immediately adjacent to the 5′ terminus). They span approximately 20,000 nucleotides and code together for a polyprotein, 1ab (pp1ab), of theoretically 700–800 kDa. A ribosomal frameshifting during translation leads to skipping the stop codon at the end of open reading frame 1a, facilitating the continuous synthesis until the end of open reading frame 1b. This process occurs in 20–30 % of all translation events and renders possible the synthesis of pp1ab, the precursor of the non-structural proteins. If this ribosomal frameshifting does not occur, then the translation will end at the stop codon at the end of open reading frame 1a, resulting in polyprotein 1a (pp1a; 450–500 kDa). These polyproteins contain the genetic information for two (in SARS-related coronavirus and avian infectious bronchitis virus) or three (in other coronaviruses) proteases, which autocatalytically cleave the precursor proteins pp1a and pp1ab, as well as a functionally active RNA-dependent RNA polymerase and an RNA helicase. The reading frames for the structural proteins are situated within the last third of the genome. In the 5′ to 3′ direction, they encode the envelope proteins S, HE (only in most betacoronaviruses, not in SARS-related coronavirus), E, M and, just

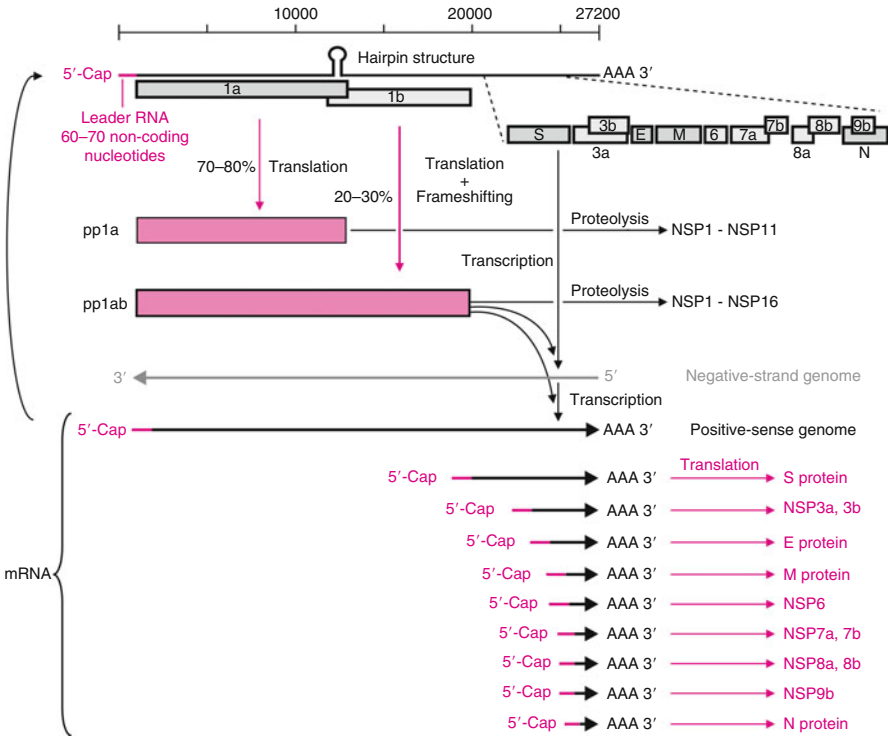


Fig. 14.23 Genome organization and replication in coronaviruses (here SARS-related coronavirus). The viral RNA genome functions as a mRNA, and is translated in the cytoplasm. The two overlapping reading frames encode non-structural polyproteins 1a and 1ab (*pp1a* and *pp1ab*). A hairpin structure induces a frameshifting during translation that leads in about 15–20 % of translation processes to the synthesis of non-structural polyprotein 1ab, which harbours in its carboxy-terminal region the RNA-dependent RNA polymerase activity. It is cleaved by the autocatalytic activity of a cysteine protease in the centre of the precursor protein. Papain – like protease sequences reside within the N-terminal domains of proteins 1a and 1ab, they are autocatalytic active and perform their own cleavage from the precursor proteins. The RNA-dependent RNA polymerase transcribes the positive-sense RNA genome into a complementary negative strand. It serves as a template for the synthesis of new positive-sense RNA genomes and for the transcription of various subgenomic mRNA species, which are modified at the 5′ end with a cap structure, and include identical sequences of the leader region in all mRNA molecules. The structural proteins are translated from the different subgenomic mRNAs, whose reading frames are localized in the 3′-oriented part of the positive-sense RNA genome. They are partially overlapping. Besides the genes specified here, there are some additional small reading frames in this region of the genome in the different coronavirus types which commonly encode non-structural proteins of unknown function. They are not shown here

before the 3′ end, N. In addition, there are various small open reading frames (2a, 3a, 3b, 6, 7a, 7b, 8a, 8b and 9b) in the part of the genome encoding the structural proteins. The coronaviruses of different genera differ significantly in the occurrence of these small open reading frames that predominantly encode very small accessory proteins; most of them are not essential for viral replication.

14.8.3 Viral Proteins

14.8.3.1 Non-Structural Proteins

The viral genome serves as mRNA for the synthesis of the precursor proteins pp1a and pp1ab (486 and 790 kDa, respectively, in SARS-related coronavirus). To synthesize pp1ab, a ribosomal frameshift is necessary to skip the stop codon at the end of open reading frame1a. This occurs because of a defined secondary RNA structure, which forms a stem loop at the end of the first reading frame. The large, experimentally not isolable precursor protein pp1ab is autocatalytically cleaved by the activity of the proteases into 13–16 cleavage products, depending on the various virus types. The function of the resulting non-structural proteins has not been ascertained in all cases. The best investigated activities are those of the non-structural proteins NSP1–NSP16 in SARS-related coronavirus; here, there is an RNA-dependent RNA polymerase (NSP12) that is required for both replication of the viral RNA genome and synthesis of subgenomic mRNAs. It constitutes part of the large precursor protein pp1ab along with the RNA helicase (NSP13) that probably binds Zn^{2+} ions, an exoribonuclease (NSP14), an endoribonuclease (NSP15) and a 2′-*O*-ribose methyltransferase (NSP16). SARS-related coronavirus includes in precursor protein pp1a (486 kDa), which is cleaved in this virus into 11 proteins (NSP1–NSP11), the sequence of papain-like protease 2 ($PL2^{pro}$; NSP3) and an enzyme that resembles the 3C protease of picornaviruses ($3CL^{pro}$, NSP5). The enzyme $3CL^{pro}$ constitutes the most active protease and is responsible for 11 of the cleavage reactions in the precursor polyprotein. These two proteases are present in all coronaviruses. Most of them contain an additional papain-like cysteine protease ($PL1^{pro}$) at the amino terminus of pp1a and pp1ab, which autocatalytically cleaves an amino-terminal domain from these polyproteins. Its function is not completely understood (Table 14.19). In SARS-related coronavirus, instead of this proteolytic activity of non-structural protein NSP1, there is a function that interferes with the metabolism of the cells and causes the degradation of cellular mRNAs, including the transcripts that are necessary for the synthesis of class I interferons. In addition, the different coronaviruses encode some other non-structural proteins which are produced during the infectious cycle. The genetic information resides in the gene region encoding the structural proteins, but not regularly in all virus types. The function of these small ancillary proteins is largely unknown.

14.8.3.2 Structural Proteins

Three types of envelope proteins are found in all coronaviruses: the M protein (formerly also known as E1 protein), the S protein (also called E2 protein) and the E protein (also called sM protein). An additional glycoprotein (HE) is only present in most betacoronaviruses (Table 14.19).

The S protein (“S” for “surface” or “spike”) has a molecular mass of 180–200 kDa. It is glycosylated and anchored in the envelope of the virus, but also in the cytoplasmic membrane by a transmembrane domain at the carboxy terminus, which is modified by an aliphatic acid. The S protein exists as a dimer or trimer and forms

Table 14.19 Functions and properties of coronavirus proteins

Reading frame	Protein	Size	Properties	Function
ORF1a	NSP1	180 aa		Virulence factor; activity described only in SARS-CoV, causes degradation of cellular RNA and allows the virus to replicate unimpaired, blocks the synthesis of IFN-α and IFN-β
	NSP2	638 aa	?	Function unclear; interaction with the cellular proteins prohibitin 1 and prohibitin 2; deletion has no effect on replication
	$PL1^{pro}$	?		Papain-like cysteine protease; autocatalytic cleavage of the N-terminal domain from pp1a and pp1ab (not in SARS-CoV)
	$NSP3/PL2^{pro}$	1,922 aa	Zinc finger motif, 180–200 kDa	Papain-like cysteine protease; cleaves pp1a and pp1ab between NSP2 and NSP3; causes protein deubiquitylation; ADP phosphatase activity
	NSP4	500 aa	Membrane-associated	Influences formation of intracellular membrane vesicles, active in virus morphogenesis?
	$NSP5/3CL^{pro}$	306 aa	30 kDa, dimer	Serine protease with homology to 3C protease of picornaviruses; principal protease
	NSP6	290 aa	Hydrophobic, transmembrane domain	?
	NSP7	83 aa	Forms supercomplexes with NSP8	?
	NSP8	198 aa	Forms supercomplexes with NSP7, nucleic acid binding properties	Alternative RNA-dependent RNA polymerase to NSP12; primase activity for primer synthesis in genome replication and translation?
	NSP9	113 aa	Forms homodimers, interaction with NSP8	?
	NSP10	139 aa	Zinc finger motif, nucleic acid binding properties	?
	NSP11	13 aa	?	?

ORF1b	NSP12/RNA-dependent RNA polymerase	932 aa	106 kDa	Synthesis of genomic and subgenomic RNA species
	NSP13/RNA helicase	601 aa	Zn^{2+} ion-binding, 67 kDa	ssRNA/dsRNA helicase; NTPase, dNTPase; necessary for genome replication
	NSP14/3–5′ exoribonuclease (ExoN)	527 aa		Exoribonuclease, active during RNA synthesis, possibly involved in recombination and repair processes
	NSP15/endo-ribonuclease (NendoU)	346 aa		Uridylate-specific endoribonuclease; active during RNA synthesis
	NSP16	298 aa		Active during 5′ capping of mRNAs and viral genomes
ORF2a	2a (mouse hepatitis virus)	30 kDa	Non-structural protein	Cyclic phosphodiesterase, RNA processing
ORF2	S	180–200 kDa	Glycosylated membrane protein; trimer; located in virus and plasma membrane of infected cells	Viral attachment/receptor binding Induces membrane fusion Induces production of neutralizing antibodies ADCC response
ORF2.1	HE	65–70 kDa	Glycosylated membrane protein, present in most betacoronaviruses (not in SARS-CoV)	Haemagglutinin and esterase responsible for secondary adherence to acetylated neuramic acid residues
ORF3	3a	274 aa	Membrane protein, O-glycosylated; tetramer; component of virus particles	Ion channel protein; induces production of proinflammatory cytokines in SARS-CoV
	3b	154 aa	Transport into nucleus	Function unclear; inhibits production of IFN-α and IFN-β?
ORF4	E	9–12 kDa	Membrane protein	Necessary for particle assembling, ion channel protein (viroporin), in some coronaviruses proapoptotic
ORF5	M	20–30 kDa	Glycosylated membrane protein; localized in ER and viral membranes of infected cells	Interaction with N protein Initiates viral morphogenesis by budding within the ER lumen

(*continued*)

Table 14.19 (continued)

Reading frame	Protein	Size	Properties	Function
ORF6	ORF6	63 aa	Betacoronaviruses (SARS-CoV): associated with ER membrane and Golgi vesicles, component of the virus particle Alphacoronaviruses and gammacoronaviruses: non-structural protein, function unclear	Function unclear; interaction with karyopherin-α_2, whereby there is inhibition of nuclear transport of Stat1?
ORF7	7a	122 aa	Localized in ER/ERGIC/Golgi, also component of the virus particle, interaction with M/E proteins, and with other ancillary or cellular proteins	Apoptosis induction, activation of cellular kinases, interaction with BiP and proteasome
	7b	44 aa	Membrane protein (SARS-CoV), detectable in virus particles, in infected cells in Golgi vesicles	Function unclear
ORF8	8ab 8a 8b		SARS-CoV isolated from animals (civet cats, bats) contain a continuous ORF8 producing a single 8ab protein; in human isolates ORF8 contains a deletion of 29 nucleotides, thus generating ORFa and ORFb	Function unknown
ORF9	N	60–70 kDa	Phosphorylated, dimeric, strongly basic, localization cell type-specific, also in the nucleus	Binding to the RNA genome forming helical nucleocapsids Interaction with the cytoplasmic domain of M protein Interaction with smad3, interferes with cellular transcription and cell cycle regulation
	9b			Function unclear; morphogenesis?

Data and indications refer to SARS-CoV, unless otherwise noted. The protein succession order in the table corresponds to the real order of their reading frames in the viral genome

ADCC antibody-dependent cellular cytotoxicity, *BiP* binding immunoglobulin protein, *ERGIC* ER–Golgi intermediate compartment, *pp1a* polyprotein 1a, *pp1ab* polyprotein 1b, *ssRNA* single-stranded RNA

club-like protuberances on the surface of the virus. Neutralizing antibodies are produced against the S protein in the course of an infection. Three important epitopes have been identified within the amino acid sequence. The virus attaches to cell surface molecules by specific domains of the S protein. The fact that the S protein is also present in the cytoplasmic membrane of infected cells makes them targets for the antibody-mediated cytotoxic cell lysis by killer cells. Furthermore, the viral fusogenic activity resides in the S protein. This refers to the ability of the viral envelope to merge with the cytoplasmic membrane and to induce the fusion of the membranes of infected and uninfected cells to form polykaryocytes. To induce the fusogenic activity, the S protein of some coronaviruses must be cleaved by a cellular trypsin-like protease at a highly basic amino acid sequence in the middle of the protein (mouse hepatitis virus, infectious bronchitis virus of birds and similar viruses). This process probably occurs in the Golgi apparatus during late stages of viral maturation. It results in an amino-terminal moiety S_1 that is non-covalently linked to the carboxy-terminal half S_2, and can be detached from the surface of the virus. The fusion effect is not mediated as in paramyxoviruses, in which a new hydrophobic amino terminus in the S_2 moiety is generated by cleavage (▶ Sect. 15.3). The molecular mechanism of membrane fusion in coronaviruses has not been conclusively unravelled. Moreover, virus types whose S protein is not proteolytically cleaved can also induce cell fusions in spite of all that. Two hydrophobic segments of the S_2 protein seem to be involved in this process, as shown in the mouse hepatitis virus. If they are altered by mutation, the fusion activity is lost.

The HE protein is present only in betacoronaviruses; SARS-related coronavirus does not possess such a gene. HE protein is glycosylated, has a molecular mass of about 65 kDa and forms dimers by disulphide bonds. The viruses that encode and express the HE protein have the capability of haemagglutinating and binding to erythrocytes. In this process, the HE protein interacts with the 9-O-acetylated neuraminic acid (sialic acid), which is a modification of lipid and protein components on cell surfaces. The HE protein has an esterase activity, which enables the virus to remove acetyl groups from sialic acid molecules. The HE protein of coronaviruses has a strong sequence homology to the HEF protein of influenza C viruses (▶ Sect. 16.3).

The E protein (9–12 kDa) is found within infectious virus particles in different concentrations; it is necessary for particle assembly and morphogenesis. In some coronaviruses (mouse hepatitis virus and SARS-related coronavirus), the E protein has a proapoptotic function. Furthermore, it seems that E protein acts as viroporin, forming ion channels and altering the membrane permeability.

M protein (“M” for “matrix”) is a surface protein with a molecular mass of 20–30 kDa and is glycosylated at the amino-terminal domain. The sugar groups are mainly linked to serine or threonine residues. In contrast to the normal N-glycosylation at asparagine residues, the glycosylation is an O-glycosylation. Only a few amino-terminal regions of this protein are exposed on the surface, and it has three transmembrane domains. The carboxy terminus resides inside the virus particle and interacts with the N protein of the nucleocapsid.

The M protein is not transported to the plasma membrane via the Golgi apparatus, but it remains in the ER membrane throughout the infection cycle. By the interaction of theM protein with the nucleocapsid, the first steps of viral assembly occur at those sites, which initiate the budding process into the ER lumen. The E protein is also involved in viral morphogenesis. If M and E proteins are produced by genetic engineering methods in eukaryotic cells, they self-assemble into virus-like particles.

The N protein ("N" for "nucleic acid binding") interacts with the viral genome; it is rich in basic amino acids and phosphorylated. It can also interact specifically with the carboxy-terminal regions of the M protein. Phylogenetic trees based on the nucleic acid sequences of the N genes correlate well with the classification of coronaviruses into different genera. N proteins are detectable in both the cytoplasm and the nucleolus of infected cells. Because of the cell-type-specific localization in the nucleus/nucleolus, it is assumed that N proteins interfere with various cellular processes.

14.8.3.3 Accessory Proteins

Besides the classical structural proteins, there are many products that are also detectable in the virus particles. They are encoded by the small reading frames within the structural gene region at the 3′ half of the genome. These include proteins 3a, 7a, and 7b, and the ORF6 protein. These are predominantly gene products with accessory functions: deletions of the corresponding genes do not fundamentally affect the infectiveness or the replicative capacity in vitro. Presumably, these proteins have important roles as virulence factors for infecting humans or animals. Little is known with regard to the function of these proteins (Table 14.19).

14.8.4 Replication

Human coronaviruses 229E attach to target cells by interaction with some still uncharacterized domains of the S protein with the zinc metalloprotease CD13 (aminopeptidase N) on the cell surface. There is no evidence that the protease CD13 cleaves the S protein during binding. Feline coronavirus uses also an aminopeptidase as a receptor. On the other hand, SARS-related coronavirus and human coronavirus NL63 bind by a domain of the S_1 protein to another metalloprotease, angiotensin-converting enzyme 2 (ACE-2). This protein is present on the surface of pneumocytes, but also on enterocytes and cells of other tissues and organs (heart, kidney, endothelium). In addition, binding to some lectins such as DC-SIGN, L-SIGN and LSECtin, facilitates entry of SARS-related coronavirus into the cell. Mouse hepatitis virus uses different isoforms of the carcinogenic embryonic antigen as cellular receptors; these isoforms belong to the immunoglobulin superfamily. Coronaviruses, which in addition to the S protein contain the HE protein in the membrane, can also interact with 9-O-acetylated neuraminic acid residues on the cell surface. However, this initial interaction of HE protein with

sugar groups is not enough to infect a cell. It must be strengthened by specific binding of the S protein with unidentified cellular proteins.

Penetration of the particle seems to occur by receptor-mediated endocytosis, and subsequent fusion of the endosomal membranes with the viral envelope (as in flaviviruses and togaviruses; Sects. 14.5 and 14.6). Binding between S_1 and ACE-2 proteins induces conformational changes in the S proteins of SARS-related coronavirus, whereby a fusogenic domain in the S_2 moiety develops its activity at low pH, promoting fusion between the viral envelope and the cell membrane. A protease, cathepsin L, which is associated with endosomal membranes, promotes the infectivity of the virus by processing still uncleaved S polypeptides into S_1 and S_2 proteins.

All replication steps are executed in the cytoplasm. The RNA genome of coronaviruses contains a cap structure at the 5′ terminus and this mediates binding of ribosomes. As already mentioned, the non-structural polyproteins pp1a and pp1ab are primarily translated from the genomic RNA; pp1ab arises by induction of a translational frameshifting mechanism, and contains the RNA-dependent RNA polymerase. The precursor polyprotein pp1ab has a molecular mass of 700–800 kDa; however, it has not yet been directly detected in the cell during viral replication. Nevertheless, in vitro translation experiments in which the genomic RNA was used as a template revealed that this translation product is actually synthesized. The proteases that are encoded in its sequence ($PL1^{pro}$, $PL2^{pro}$ and $3CL^{pro}$) cleave pp1ab into 16 non-structural proteins, one of which is the RNA-dependent RNA polymerase (NSP12).

In the next step, the complementary strand is synthesized by the concerted activities of RNA-dependent RNA polymerase (NSP12), RNA helicase (NSP13), exoribonuclease (NSP14) and endoribonuclease (NSP15) using the genomic RNA as a template. The newly synthesized complementary strand comprises the entire genome and has a negative polarity. It has two functions in the replication cycle: it serves as a template for the synthesis of new viral genomes and several subgenomic mRNA species. Eight subgenomic mRNAs have been found in SARS-related coronavirus infections, from which the various accessory and structural proteins are translated. They are characterized by their discontinuous synthesis: all have the same 3′ terminus, but their initiation sites are different and are located in the genomic region between the end of the reading frame of pp1ab and the 3′ end of the genome. Despite their different initiation sites, all subgenomic mRNA molecules possess a standard sequence of approximately 60–90 nucleotides at the 5′ terminus: the leader RNA. It has a capped 5′ end and is complementary to the 3′ terminus of the negative strand. It is believed that the leader sequence serves as a primer for the synthesis of subgenomic mRNA species; whether the activity of NSP8 is required for the synthesis of these short RNA molecules is unclear. The leader RNA has a conserved sequence (UCUAAAC) at the 3′ terminus. Sequences complementary to this motif are found in the negative-sense RNA at different regions. They are located upstream of the different initiation sites for the synthesis of the subgenomic mRNA species: the leader RNA can hybridize with them, thus providing a small double-stranded region with a free 3′-OH end to resume

polymerization. Probably, the RNA polymerase is not capable of initiating synthesis of the subgenomic mRNA species at the different start sites. Therefore, it was probably necessary for the virus to develop the transfer mechanism of a leader RNA. The finding that multiple elements of the conserved heptamer sequence are present upstream of some of the transcription start sites, e.g. upstream of the start of the coding sequences for the N protein, suggests that RNA synthesis is preferably initiated at these sites, thus facilitating regulation of the quantity of the various proteins.

The nested transcripts are modified by the ribose methyltransferase (NSP16) with a cap structure at the 5′ terminus; this mediates binding of ribosomal subunits and translation of the respective reading frames into proteins. However, in the different coronavirus types, many of the different subgenomic RNAs are bicistronic or tricistronic, and from these two or three proteins are translated. Among the subgenomic RNAs, a total of five bicistronic RNAs have been found in SARS-related coronavirus, and in avian infectious bronchitis virus there is one tricistronic mRNA. The translation of the reading frames, which are not localized adjacent to the 5′ end of the capped subgenomic transcripts, is performed by an alternative mechanism, frequently by translational frameshift. However, in the case of subgenomic RNAs 3 and 5 of avian infectious bronchitis virus and mouse hepatitis virus, an IRES-like secondary RNA structure facilitates binding of the ribosomes before the start of the downstream reading frames. In addition, it seems that there are other ways which allow translation initiation of alternatively used reading frames. However, they are usually not very effective and result in a low expression of the corresponding proteins. Which mechanisms also play a role in the regulation of protein synthesis has not been clarified. In infected cells, the most abundant polypeptide is the N protein, which is translated from the shortest transcript.

During virus morphogenesis, N proteins interact with genomic RNA molecules to shape the helical nucleocapsids and bind to the carboxy-terminal domains of the M and E proteins, which are integrated in the ER membrane. This triggers the budding process, during which the nucleocapsid is surrounded by the envelope containing M proteins and glycoproteins. The particles produced are released into the ER lumen and are transported by Golgi vesicles to the cell surface, where they are released into the environment.

14.8.5 Human Pathogenic Coronaviruses

14.8.5.1 Human Coronaviruses 229E and OC43

Epidemiology and Transmission

Human coronaviruses 229E and OC43 have been known since 1966 and 1967, whereas the species NL63 and HKU1 together with some additional virus types were identified only a few years ago during an intensive search for human coronaviruses as a result of the SARS epidemic. All these coronavirus types have a worldwide distribution: between 75 % and 65 % of children of 3.5 years of age

and up to 90 % of adults have antibodies against coronaviruses – an indication of their wide dissemination. It is estimated that globally 10 % of all infections of the upper and lower respiratory tract are caused by them. Coronaviruses are transmitted by infected people through droplet infection. Like other droplet infections, these viruses can also be transmitted by smear infections due to poor hygiene. Infections occur more frequently during the winter months. Reinfections – even with the same strain of the virus – are common and usually have an asymptomatic course.

Clinical Features

Coronaviruses cause cold diseases of the upper respiratory tract, rarely infecting the lower airways. Infections are often asymptomatic, or have only mild symptoms. The incubation period lasts 2–5 days; the duration of the illness with coryza, cough, sore throat and headache associated with low-grade fever lasts for about 1 week. In infants and young children, the infection can take a much severer course and can be associated with croup-like symptoms. It can lead to asthma attacks and in some individual cases to bronchitis and pneumonia. In cases of pre-existing respiratory diseases such as asthma and chronic bronchitis, these symptoms can be strengthened in both children and adults. Human coronaviruses have also been associated with disorders of the gastrointestinal system. This appears to occur only in immunologically compromised individuals, e.g. in AIDS patients, who can also have prolonged diarrhoea.

Pathogenesis

The human coronaviruses multiply in the ciliated epithelial cells of the respiratory tract, which express the corresponding receptor molecules ACE-2 or CD13. Electron microscopy studies suggest that they can also proliferate in the intestinal epithelium. The infection is usually restricted to the epithelial cells of these organs.

Diseases in which the virus infects macrophages and lymphocytes, proliferates in these cells and then spreads via the bloodstream into liver, endothelial, glial and kidney epithelial cells and infects these organs and tissues are only known from animal coronaviruses and SARS-related coronavirus. Whether the HE protein plays a role in the infection of these cell types and in the pathogenesis of the disease is not known. Mutations in the S protein alter both virulence and tropism, i.e. the specificity for different cell types. In addition to virus-specific factors, genetic factors appear to be important for the establishment of coronavirus infections: e.g. the susceptibility to human coronavirus 229E infections is apparently determined by a factor encoded on chromosome 15.

Immune Response and Diagnosis

During a coronavirus infection, IgM, IgG and IgA antibodies are produced. Immunoglobulins against the S protein are neutralizing. IgA antibodies and interferons, which are secreted in the nasal secretion, seem to be important for protecting against infections. Very little is known about the importance of the cellular immune response in human infections. From mouse hepatitis virus is known that cytotoxic T cells are involved in the clearance of the virus from the organism.

Coronaviruses can be cultivated only to a limited extent in cell culture. Human coronavirus 227E can be propagated in tissue cultures of embryonic trachea, human rhabdomyosarcoma cells or in the cell line MA-177, a diploid cell line from the intestinal epithelium. The cultivation of human coronavirus OC43 is much more difficult.

The diagnosis of coronavirus infections is usually done retrospectively by detecting virus-specific IgM and IgG antibodies in ELISA. Alternatively, the occurrence of viral nucleic acids can now be demonstrated by PCR from appropriate clinical materials.

Therapy and Prophylaxis

Since coronavirus infections are largely harmless, no attempts have ever been undertaken to develop a vaccine. There is no antiviral therapy.

14.8.5.2 SARS-Related Coronavirus

Epidemiology and Transmission

The first infections with SARS-related coronavirus emerged in Foshan and Heyuan in the Chinese province of Guangdong in November 2002. Most patients had direct or indirect contact with animal markets. Until January 2003, the infections spread through human-to-human transmissions to Guangzhou, the capital of Guangdong province, where they affected mainly people who were working in public health services. There was talk of an "infectious atypical pneumonia", and a number of infectious agents were suspected as possible causes, among others, chlamydia. When the number of patients increased to over 300, including 100 nurses and physicians, and five of them had died, the WHO was informed of this new infection on 11 February 2003. In March 2003, an infected nephrologist from Guangdong province stayed in a hotel in Hong Kong, and verifiably infected at least ten other hotel guests, who in turn carried the infection to various countries, including Singapore, Vietnam, Ireland, the USA and Canada. During a second infection wave in Hong Kong and all the afore-mentioned countries, particularly physicians and nurses were infected, and these in turn passed on the infection in the hospitals and to their families, thus exporting it to other countries. The pathogen arrived in central Europe (Germany) via Singapore. On 10 March 2003, this new disease received the name severe acute respiratory syndrome (SARS). In the following months until 5 July 2003, the SARS disease emerged worldwide in 8,400 patients in 29 countries, and about 800 of them died. As a result of massive countermeasures by the WHO, it was possible to control the infection relatively rapidly. These measures included isolation/quarantine, entry bans/travel restrictions and checks at airports, and an open and timely information policy. In March 2003, Christian Drosten of the Bernhard Nocht Institute in Hamburg and other international research groups independently identified a coronavirus as the aetiologic agent of the disease, and determine its entire genome sequence. This facilitated the development of specific diagnostic test systems. At that time, a peculiarity was that all information was made immediately accessible and divulged, e.g. via the Internet.

Inasmuch as it was assumed that this new human virus had been transmitted to humans by zoonotic means, various animal species which are traded on

animal markets in Guangdong were examined for the presence of analogous infections. Coronaviruses were found in nasal swabs and faecal samples from civet cats (*Paguma larvata*) and raccoons (*Nyctereutes procyonoides*). Their genomes were almost identical to that of human SARS-related coronavirus. Animal traders frequently exhibited antibodies that reacted with the proteins of SARS-related coronavirus. It was found that the virus can infect a number of other animals, such as cats, mice, ferrets and macaques; however, wild civet cats and raccoons were not infected. This suggested that these animals do not represent the natural hosts. The natural hosts are probably bats of the genus *Rhinolophus* (greater horseshoe bat), in which the SARS virus was detected in high concentrations. They excrete the pathogens in their faeces. The bats transmitted the virus to civet cats under the limited space conditions at the animal markets, and from these the virus was further transmitted to animal dealers, buyers and exploiters (cooks, furriers). The occurrence of further mutations facilitated the adaptation to human hosts, leading to the rapid transmission from infected patients to other people. The transmission among humans occurs preponderantly by airborne infection, whereby it was primarily assumed that every infected person develops symptoms within 10 days. However, there are clear indications that the virus is also excreted in the stool, and faecal–oral smear infections may contribute to its dissemination. With the exception of a few infections in Guangdong in January 2004 which were associated with animal contacts again, and some cases that could be attributed to laboratory contacts with SARS-related coronavirus, no more SARS infections and illnesses have occurred since the SARS outbreak of 2003.

The Physician Who First Identified SARS Died of It

Carlo Urbani was one of the first people to recognize that SARS is a new, unusual pneumonia, from which a considerable risk to human health may emanate. He was an expert of infectious diseases who worked at the WHO. Urbani along with the WHO and the governments of the SARS-affected countries initiated the implementation of appropriate measures in Vietnam and Southeast Asia in February/March 2003 that were aimed at preventing and controlling the dissemination of the infection. Unfortunately, he infected himself as a result of his investigations, and died from SARS on 29 March 2003.

Clinical Features

The virus is transmitted from person to person by droplet or smear infections. The incubation period lasts 2–10 days, and the disease begins with flu-like symptoms, swollen lymph nodes and fever. Patients develop a dry cough associated with colds, limb pain, muscle pain, neck ache and headaches. At this stage, the disease is clinically virtually indistinguishable from an influenza infection. In addition to the severe respiratory symptoms, it is frequently also associated with disorders of the gastrointestinal system (diarrhoea, nausea) as well as with thrombocytopenia.

About 1 week after the appearance of the first symptoms, a subset of patients develop a severe pneumonia, which is often associated with pulmonary fibrosis, myocardial infarction, acute renal failure and ultimately multiple organ failure.

Pathogenesis

Probably, SARS-related coronavirus initially proliferates in the epithelial cells of the respiratory tract, like other pathogenic coronaviruses. The ACE-2 receptor proteins, to which the virus attaches by S proteins, are found on the surface of cells in many tissues; they are an integral part of the renin–angiotensin system and regulate it. The interaction of S proteins with ACE-2 decreases its concentration on the cell surface. This possibly induces a particular susceptibility to inflammations and lung failure in infected patients. In addition to ACE-2, the S proteins have the ability to attach to DC-SIGN. They are receptor proteins that are found on the surface of dendritic cells. However, they do not directly permit the entry of the virus to these cells, but they can transport the virus to the lymph nodes and other tissues containing ACE-2-positive cells, thus mediating infection in trans. The virus seems to spread via the bloodstream throughout the body, and subsequently infects – like animal coronaviruses – endothelial, lung, kidney and intestinal epithelial cells. Many patients have a myocardial infarction during the disease. Possibly, this is associated with binding of viral S proteins to ACE-2 proteins on the surface of myocytes.

In the early infection phase, a rapid activation of non-specific immune responses with increased production of various CC chemokines and chemokine receptors, proinflammatory interleukins and Toll-like receptor 9 are found in patients. This causes a rapid mobilization of monocytes and macrophages, which migrate into the infected organs, especially the lungs, and initiate the inflammatory process. Simultaneously, a rapid decrease in $CD4^+$ and $CD8^+$ T lymphocytes can be observed in patients during the acute phase of infection. The cause of this decline is unclear. SARS-related coronavirus is not able to infect lymphocytes; therefore, destruction of T cells cannot be a direct consequence of infection. Possibly, apoptotic processes induced by the infection may be involved. It has been found that several SARS-related coronavirus proteins, such as protein 7a, are able to induce a caspase-dependent apoptosis mechanism in cell lines from different tissues (lung, liver, kidney) in vitro.

Genetic factors in infected patients also seem to be important for the establishment of symptomatic SARS-related coronavirus infections. Individuals who can produce only small amounts of mannose-binding lectin appear to be predisposed to the establishment of a symptomatic infection. Mannose-binding lectin belongs, together with the pulmonary surfactant proteins A and D, to the collectins. They are proteins that attach to glycosylated regions of S proteins on the surface of the virus, facilitating penetration of the virus into granulocytes, and potentially preventing interactions with the target cells. In other studies, it has been found that SARS patients frequently exhibit haplotypes HLA-B*0703, HLA-B*4601 and HLA-DRB1*0301. However, these data are in part contradictory.

Immune Response and Diagnosis

During a SARS-related coronavirus infection, IgM, IgG and IgA antibodies are produced against N and S proteins. They are detectable at the earliest on the fourth day after the onset of symptoms; however, in many patients they are detectable considerably later. Mainly immunoglobulins against the S protein are neutralizing antibodies. They are directed predominantly against epitopes of the domain determined by amino acid residues 441–700. Little is known about the relevance of the cellular immune response in humans during infection.

ELISAs for detection of specific antibodies against the S and N proteins are available. Isolation and cultivation of the virus is time-consuming and not always successful, and must be performed only in biosafety 3 (or higher) laboratories. Therefore, acute SARS-related coronavirus infections are usually diagnosed by detecting viral genomes through RT-PCR in sputum, pharyngeal lavage, stool or serum. In such cases, a region of reading frame 1b, which encodes for the viral RNA polymerase, is usually amplified. A positive detection has to be verified in additional tests and reference laboratories. SARS-related coronavirus is well cultivable in a number of cell lines. The viruses exhibit strong lytic or rather persistent infections, depending on the cell type.

Control and Prophylaxis

There is no vaccine against SARS infections. During the SARS outbreak in 2003, infected patients were treated with ribavirin. In severe cases, corticosteroids were used for treatment because, at least in part, the symptoms are immunopathogenetically determined. It was also attempted to reduce the viral load in patients by use of high doses of immunoglobulins. Inhibitors of viral proteases are currently being developed.

14.8.6 Animal Pathogenic Coronaviruses

The family *Coronaviridae* encompasses many viruses that cause illnesses in animals. The most important are the pathogen of porcine transmissible gastroenteritis and feline coronavirus, which can cause peritonitis and polyserositis in cats. These viruses will be discussed in detail. Furthermore, there is also porcine haemagglutinating encephalomyelitis virus, which induces encephalitis in newborn piglets. Occasionally, it is also associated with diarrhoea. The infection of the central nervous system occurs via peripheral nerves, to which the viruses are transported from the initial reproduction site, the mucosa of the respiratory and gastrointestinal tract, without a viraemia developing. An immunoprophylaxis is not available. A globally important disease of poultry is caused by avian infectious bronchitis virus. Apart from an acute disease of the respiratory tract, substantial economic loss can arise from lesions of the fallopian tubes and the associated decline of egg production. Mouse hepatitis virus is widespread in mouse populations. It represents an important model system for studying the biology and pathogenesis of coronavirus infections. Mouse hepatitis virus causes a wide spectrum of clinical symptoms, which can range from gastrointestinal, hepatic and respiratory symptoms to central

nervous system disorders. Introduction into laboratory animal colonies occurs by inclusion of mice with persistent infections. The diagnosis is performed histopathologically or by cultivation of the virus. The elimination of the virus from infected colonies is almost impossible. Usually, it is necessary to annihilate the colony and to replace the infected livestock with new, virus-free animals. Therefore, regular examinations of mouse colonies are essential to detect infections with mouse hepatitis virus.

Coronavirus infections have been described in other species, particularly in dogs, but they do not have veterinary importance owing to their low clinical relevance.

Bovine coronavirus infections are a major cause of calf diarrhoea in the first few days of life. The infection is local and viral replication is restricted to enterocytes. A vaccine is available in the form of a so-called maternal prophylaxis, in which the cow is vaccinated twice before parturition. The actual immunization consists in the intake of antibodies with the colostrum.

14.8.6.1 Porcine Transmissible Gastroenteritis Virus

Epidemiology and Transmission

Transmissible gastroenteritis is a disease of swine with high morbidity but low mortality. However, if animals become infected at the age of only a few days, the mortality rate can be very high. The virus is excreted over a period of about 14 days with the faeces. Persistent infections and chronic carriers are very rare. In the latter, the virus seems to persist in lung macrophages. The virus is usually transmitted by direct contact, but airborne transmissions have also been demonstrated.

A natural infection results in a durable immunity, which lasts approximately 1–2 years. It is based on a local mucosal immunity. Parenteral inoculation induces systemic antibodies which do not protect against an infection. Moreover, a systemic vaccination of sows does not stimulate the release of immunoglobulins with the milk.

Clinical Features

Infections with porcine transmissible gastroenteritis virus provoke severe diarrhoea and vomiting as its main symptoms. Other symptoms are rarely observed and other organ systems are normally not affected, except for the small intestine.

Pathogenesis

The virus is ingested orally, and infects enterocytes after gastric passage. They are lytically infected, destroyed and replaced by enterocytes from the Lieberkühn's crypts. This process explains the typical symptoms of transient, non-bloody diarrhoea. Histologically, the classic clinical picture is that of acute enteritis with atrophy of villi (in the posterior sections of the small intestine). The gastric passage of the acid-labile coronavirus is achieved by the pH-buffering effect of milk and is additionally supported by the slightly acidic pH in the stomach of young animals.

There is a naturally occurring mutant of transmissible gastroenteritis virus which carries a deletion within the gene encoding the S protein. This mutant virus has

completely lost its enterotropism, and replicates primarily in macrophages of the respiratory tract. It causes no or only mild symptoms, but interferes significantly with the serological surveillance during the control of transmissible gastroenteritis.

Immune Response and Diagnosis

The virus can easily be isolated. Alternatively, it can be detected by immunofluorescence in intestinal sections of dead animals. Examination of antibody levels in serum pairs allows indirect diagnosis. The infection status of a herd can be determined serologically by ELISA or neutralization tests in epidemiological studies.

Control and Prophylaxis

The successful control of infection is based on the elimination of seropositive animals and the establishment of and compliance with strict hygiene and husbandry requirements. A vaccine is available on the basis of inactivated viruses, which are propagated in cell culture; however, it is not very efficient. The local immune defence in the intestine of piglets is important for effective protection, which is most effectively established and transmitted to the piglets by the colostrum from naturally infected sows.

14.8.6.2 Feline Coronavirus (Feline Infectious Peritonitis Virus)

Epidemiology and Transmission

So-called feline infectious peritonitis is caused by infection of cats with feline coronavirus. Infections with this pathogen are widespread in cat populations. The virus is able to persist in cats, and is excreted primarily in the faeces. The cats are usually infected at a very early age by social contact with the mother or other persistently infected cats.

The exact host range of this virus is unknown. It infects domestic and large cats and probably also dogs, as some isolates of feline coronavirus have been identified as recombinants between feline and canine coronaviruses.

Clinical Features

The virus commonly causes a subclinical infection or mild and transient enteritis. The enteritis-causing virus can change by mutations, which create a new biotype with altered tissue tropism. This virus variant replicates no longer exclusively in enterocytes, but is also able to infect macrophages, thus inducing a systemic infection. The virus variant is scattered by macrophages in virtually all organs, where it can induce, starting from a vasculitis, pseudo-granulomatous inflammations. This clinical picture is referred to as feline infectious peritonitis. From a pathological point of view, this disease pattern is a generalized polyserositis and vasculitis/perivasculitis.

Pathogenesis

The virus binds to aminopeptidase N on cells of cats, dogs and pigs. The peritonitis-causing feline virus variant apparently emerges anew in each individual outbreak. In the course of this, the persisting virus mutates to a virulent variant. Transmission of the mutated virus from cat to cat is occasionally observed, but it

does not seem to be of significant epidemiological significance. Deletion of some nucleotides in gene 3c has been found in the virus mutant. This gene codes for a non-structural protein of unknown function during the viral replication cycle, which, however, probably influences the virulence of the isolates. Nevertheless, the deletions are not the same in all mutants that cause feline infectious peritonitis, but differ slightly in all viruses isolated from different outbreaks. The molecular-biological bases of these changes are largely unknown.

Immune Response and Diagnosis

The virological diagnosis is extremely difficult because a distinction between both the benign and the virulent virus variants is not possible with currently available techniques. Therefore, the unambiguous diagnosis of feline infectious peritonitis can only be performed by histopathologic examination.

Control and Prophylaxis

A vaccine is available on the basis of an attenuated vaccine containing a temperature-sensitive mutant of feline coronavirus; however, its efficacy is controversial. There are fundamental safety concerns regarding the use of live vaccines for the prevention of an infectious disease with unclarified (immuno) pathogenesis whose attenuated virus has scarcely been investigated and which possesses a broad host range. Furthermore, it has also been shown that coronaviruses from different species can recombine their genetic material to generate new virus variants. In the case of recombination with the vaccine virus, the emergence of virus variants with altered receptor binding is possible, and these in turn might infect other animal species.

14.8.6.3 Avian Infectious Bronchitis Virus

Epidemiology and Transmission

Infectious bronchitis of chickens is also caused by a coronavirus. It is distributed worldwide. Chickens are the only natural host for the virus. Avian infectious bronchitis virus causes a disease only in chickens. Similar coronaviruses have also been isolated from turkeys and pheasants, but they are considered to be their own species. Of particular importance is the huge antigenic diversity of this virus. On the basis of differences in the sequence of the S protein, a number of serotypes and genotypes are distinguished. The virus is very contagious, and it spreads very quickly within a flock through virus-containing faeces and nasal secretion. Between different flocks, the virus is predominantly transmitted indirectly by the farm staff.

Clinical Features

Avian Infectious bronchitis virus causes lesions in the kidney, in the oviduct and in the respiratory tract. The nature of the clinical manifestation depends on the virus strain and the host. In particular, the age and breed of the chicken influences the clinical picture. The disease is especially pronounced in few-day-old chicks. The morbidity is very high; thus, virtually all birds of a flock are infected. The mortality, however, is generally low (0–25 %).

Pathogenesis

The virus initially replicates in epithelial cells of the respiratory tract or the gastrointestinal tract and invades, after a subsequent viraemia, many organs, especially kidneys and fallopian tubes. Generally, the virus is eliminated by the immune response, but in rare cases the infection can persist for weeks.

Immune Response and Diagnosis

An infection produces a durable and protective immunity against the homologous virus, which is normally based on antibodies against the S protein. The degree of cross-immunity is different for individual strains. Diagnosis is performed by detection of the virus (isolation of the virus in cell culture, PCR) or immunofluorescence with identification of viral proteins in tissues of infected animals, or, at the livestock level, by detection of virus-specific antibodies by neutralization and haemagglutination-inhibition tests or ELISA.

Control and Prophylaxis

Infectious bronchitis of chickens is not subject to animal health regulations. Several live and inactivated vaccines are available and are generally applied according to the specific serotype.

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