Arterivirus molecular biology and pathogenesis

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Arteriviruses are positive-stranded RNA viruses that infect mammals. They can cause persistent or asymptomatic infections, but also acute disease associated with a respiratory syndrome, abortion or lethal haemorrhagic fever. During the past two decades, porcine reproductive and respiratory syndrome virus (PRRSV) and, to a lesser extent, equine arteritis virus (EAV) have attracted attention as veterinary pathogens with significant economic impact. Particularly noteworthy were the ‘porcine high fever disease’ outbreaks in South-East Asia and the emergence of new virulent PRRSV strains in the USA. Recently, the family was expanded with several previously unknown arteriviruses isolated from different African monkey species. At the molecular level, arteriviruses share an intriguing but distant evolutionary relationship with coronaviruses and other members of the order Nidovirales. Nevertheless, several of their characteristics are unique, including virion composition and structure, and the conservation of only a subset of the replicase domains encountered in nidoviruses with larger genomes. During the past 15 years, the advent of reverse genetics systems for EAV and PRRSV has changed and accelerated the structure–function analysis of arterivirus RNA and protein sequences. These systems now also facilitate studies into host immune responses and arterivirus immune evasion and pathogenesis. In this review, we have summarized recent advances in the areas of arterivirus genome expression, RNA and protein functions, virion architecture, virus–host interactions, immunity, and pathogenesis. We have also briefly reviewed the impact of these advances on disease management, the engineering of novel candidate live vaccines and the diagnosis of arterivirus infection.

Introduction

About two decades ago, genome sequencing established that arteriviruses qualified as a separate family of positive-stranded RNA (+ RNA) viruses (reviewed by Cavanagh, 1997; Snijder & Meulenberg, 1998). A family with, on the one hand, an intriguing but distant evolutionary relationship to coronaviruses and other members of the order Nidovirales but, on the other hand, characteristics unique among currently known +RNA viruses. These include virion composition and structure, and the conservation of only a subset of the replicate domains encountered in nidoviruses with larger genomes. Shortly after the family Arteriviridae was created, equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV) were the first nidoviruses for which reverse genetics systems became available (Meulenberg et al., 1998; van Dinten et al., 1997). This breakthrough not only revolutionized the structure–function analysis of RNA and protein sequences, but also facilitated studies into pathogenesis and immune responses, and the engineering of novel candidate live vaccines.

The consequences of arterivirus infection can range from persistent asymptomatic infection to acute disease, associated with abortion, fatal age-dependent poliomyelitis or lethal haemorrhagic fever. Three of the currently recognized arterivirus species [EAV, lactate dehydrogenase-elevating virus (LDV) of mice and simian haemorrhagic fever virus (SHFV)] were first isolated about 50 years ago (reviewed by Snijder & Kikkert, 2013; Snijder & Meulenberg, 1998). However, during the past two decades, it was particularly the fourth arterivirus, PRRSV, that moved into the spotlight by causing tremendous economic losses to the swine industry worldwide. In the late 1980s, highly diverged variants of this virus emerged almost simultaneously in Western Europe (genotype 1) and North America (genotype 2), causing numerous acute respiratory disease outbreaks and abortion storms. Subsequently, the ‘porcine high fever disease’ outbreaks in South-East Asia (Tian et al., 2007) and the emergence of the highly virulent MN184 strain in the USA (Han et al., 2006) have attracted special attention.

Disease prevention and control will clearly benefit from the dissection of the molecular and cellular biology of
arterivirus infection. In this review, we will summarize what has been learned, in particular since the advent of reverse genetics, with respect to arterivirus genome expression, RNA and protein functions, virion architecture, host responses, and pathogenesis. We will also briefly review the impact of these advances on disease management, vaccine development and the diagnosis of arterivirus infection.

The expanding order Nidovirales

About 15 years ago, the family Arteriviridae was united with the family Coronaviridae in a new order Nidovirales (Cavanagh, 1997; Snijder & Meulenberg, 1998). In spite of striking differences in genome size and virion structure, the genome organization and expression of arteri- and coronavirus are strikingly similar, and key replicase domains were postulated to share a common ancestry (den Boon et al., 1991). A prominent feature of nidovirus genome expression is the nested set of subgenomic (sg) mRNAs that was the basis for the order name Nidovirales (Latin nidus = nest). The complex evolutionary relationship between arteriviruses and other nidoviruses has been reviewed extensively elsewhere (Gorbalenya et al., 2000; Nga et al., 2011; Snijder & Meulenberg, 1998). Essentially, related replicase genes have become associated with seemingly unrelated sets of structural protein genes, with RNA recombination likely playing an important role in nidovirus evolution.

As nidovirus discovery continued, in a remarkably wide variety of hosts, the order was further expanded with nidoviruses infecting shrimp (family Roniviridae; Cowley et al., 2000), fish (genus Bafinivirus; subfamily Torovirinae in the family Coronaviridae; Schütze et al., 2006) and most recently also insects (proposed family Mesoniviridae; Nga et al., 2011; Zirkel et al., 2011). The recently identified wobbly possum disease virus (WPDV) appears most closely related to arteriviruses (Dunowska et al., 2012). Furthermore, several additional monkey arteriviruses, only distantly related to SHFV, were discovered recently (Lauck et al., 2013). Together with the large evolutionary distances between current arterivirus species (Fig. 1), this may well prompt a future revision of the internal taxonomic structure of the family.

Genome structure and expression

Genome properties and organization

The arterivirus genome is 12–16 kb long, 3’-polyadenylated and presumably 5’-capped. Full-length genome sequences (Table 1) have been obtained for European and North American EAV isolates, a large number of genotype 1 and 2 PRRSVs, two LDV strains, and five distantly related monkey viruses, tentatively grouped under the name SHFV.

The arterivirus genome is a polycistronic + RNA (Figs 2 and 3), with 5’ and 3’ NTRs of 156–224 and 59–117 nt, respectively, that flank an array of 10–15 known ORFs. The large replicate ORFs 1a and 1b occupy the 5’-proximal three-quarters of the genome, with the size of ORF1a being much more variable than that of ORF1b. ORF1a translation yields replicase polyprotein (pp) 1a (1727–2502 aa), whereas ORF1b is expressed by −1 programmed ribosomal frameshifting (PRF) (den Boon et al., 1991), which C-terminally extends pp1a into pp1ab (3175–3959 aa). Recently, a short transframe (TF) ORF was found to overlap the nsp2-coding region of ORF1a in the +1 frame and to be expressed by −2 PRF (Fang et al., 2012). Remarkably, the TF ORF is conserved in the genomes of PRRSV, LDV and SHFV, but this part of the EAV genome is considerably truncated.

The 3’-proximal genome part has a compact organization and contains eight to 12 relatively small genes, most of which overlap with neighbouring genes (Fig. 2). These ORFs encode structural proteins and are expressed from a 3’-co-terminal nested set of sg mRNAs (Fig. 3). The organization of these ORFs is conserved, but downstream of ORF1b, SHFV and all recently identified SHFV-like viruses contain three or four additional ORFs (~1.6 kb) that may be derived from an ancient duplication of ORFs 2–4 (Godeny et al., 1998; Lauck et al., 2013). Together with the size variation in ORF1a, this presumed duplication explains the genome size differences among arteriviruses.
RNA structures in replication, transcription and translation

Several RNA signals involved in arterivirus replication have been identified. In EAV, a minimum of ~300 nt from both genome termini is required for efficient replication, meaning that replication signals extend into the coding sequences (Molenkamp et al., 2000a; Tijms et al., 2001). Detailed RNA secondary structure models were developed for the EAV 5' and 3' NTRs (Fig. 4). The 5' NTR is involved in translation, replication and transcription (van den Born et al., 2004), and contains the so-called

### Table 1. Arterivirus genomes and database accession numbers

<table>
<thead>
<tr>
<th>Virus (isolate)</th>
<th>Host</th>
<th>Genome size (kb)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAV (Bucyrus)</td>
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</tr>
<tr>
<td>LDV (P)</td>
<td>Mouse</td>
<td>14.1</td>
<td>NC_001639</td>
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<tr>
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<td>PRRSV genotype 2 (16244B)</td>
<td>Swine</td>
<td>15.4</td>
<td>NC_001961</td>
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<tr>
<td>SHFV (LVR 42-0)</td>
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<td>NC_003092</td>
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<tr>
<td>SHFV-like (krtg2)</td>
<td>Red-tailed guenon</td>
<td>15.3</td>
<td>JX473849</td>
</tr>
</tbody>
</table>

Fig. 2. Arterivirus genome organization. The family prototype EAV is shown at the top. The replicate ORFs 1a and 1b (the latter expressed by −1 programmed ribosomal frameshifting (PRF)) are followed by the genes encoding the minor and major envelope proteins and the N protein. GP, glycoprotein; M, membrane; E, envelope; N, nucleocapsid. The 3'-proximal region of the SHFV genome carries a large insertion (pink) containing four ORFs that may encode additional virion proteins. With the exception of EAV, arterivirus genomes contain an alternative transframe (TF) ORF in the non-structural protein 2 (nsp2)-coding region, which is expressed by −2 PRF (Fang et al., 2012). The positions corresponding to (known or predicted) polyprotein cleavage sites are depicted above the replicate ORFs; red arrowheads, sites cleaved by the nsp4 SP (S); blue arrowheads, sites cleaved by PLP domains (P) in the nsp1/nsp2 region. The processing scheme of the SHFV nsp1 region remains to be elucidated. Three ORF1a-encoded (putative) transmembrane domains (TM) and four highly conserved ORF1b-encoded domains are depicted: RNA-dependent RNA polymerase (R), (putative) multinuclear zinc-binding domain (Z), RNA helicase (H), and NendoU endoribonuclease domain (N). Modified from Snijder & Kikkert (2013).
leader TRS hairpin (LTH) that is crucial for sg mRNA production. The relevance of other EAV 5’ NTR structures remains to be investigated as few of these are conserved in other arteriviruses (Lu et al., 2011; van den Born et al., 2004).

A 3’-proximal EAV RNA hairpin is required for RNA synthesis and its loop was implicated in an essential pseudoknot interaction with an upstream hairpin within the N protein gene (Beerens & Snijder, 2007). This conformation appears conserved in all arteriviruses and may constitute a molecular switch that, for example, regulates a step in negative-strand RNA synthesis. In addition, a 3’-terminal CC motif upstream of the poly(A) tail was implicated in a critical step in replication, possibly the initiation of negative-strand RNA synthesis (Beerens et al., 2007). In PRRSV, a ‘kissing interaction’ between the loops of RNA hairpins in the 3’ NTR and N protein gene was found to be crucial for replication (Verheije et al., 2002a).

Arterivirus genome translation presumably initiates through ‘conventional’ ribosomal scanning of the 5’ NTR (van den Born et al., 2005), but also entails one or two ribosomal frameshifting events. As in all other nidoviruses, a −1 PRF in the short ORF1a/1b overlap region is used to express ORF1b. The estimated frameshift efficiency is 15–20% (in a reporter system), which derives from the concerted action of a ‘slippery’ sequence and a downstream RNA pseudoknot structure (den Boon et al., 1991; Firth & Brierley, 2012). In addition, all arteriviruses except EAV employ −2 PRF to express a conserved TF ORF that overlaps the nsp2-coding region (Fig. 2) (Fang et al., 2012). This frameshift site dictates both efficient −2 and −1 PRF (estimated efficiencies in PRRSV-infected cells of 16–20% and 7%, respectively) and consequently three N-terminally collinear products are produced: nsp2, nsp2TF and nsp2N. A downstream RNA sequence (CCCCANCUC) is a critical determinant of this first documented case of −2 PRF in eukaryotic cells, but is not predicted to be part of a particular frameshift-directing RNA structure. The two PRF mechanisms create a complex series of non-structural protein expression ratios. Of the ribosomes that translate the PRRSV nsp1 region, ∼20% and ∼7% synthesize nsp2TF and nsp2N, respectively, with ∼73% translating the remainder of ORF1a (nsp3–8) and probably only ∼15% subsequently translating ORF1b (nsp9–12).

**Proteinases and replicase polyprotein processing**

As in all nidoviruses (Ziebuhr et al., 2000), the post-translational processing of arterivirus replicase polyproteins involves a complex proteolytic cascade that is directed by three (EAV) or four (PRRSV/LDV) ORF1a-encoded proteinase domains (Figs 2 and 5). Cleavage of pp1a and pp1ab generates 13 (EAV) or 14 (PRRSV/LDV) processing end products, named nsp1–12, including nsp7/1β (PRRSV/LDV/EAV) and nsp1α/1β (PRRSV/LDV). In PRRSV, LDV and SHFV, the −2 PRF event described above yields the truncated nsp2 variants nsp2TF and nsp2N (Fang et al., 2012). The nsp3–8 region is subject to two alternative processing cascades, with cleaved nsp2 acting as a co-factor to promote the nsp4/5 cleavage in the ‘major’ processing.
pathway (Wassenaar et al., 1997). EAV cleavage-site mutagenesis underlined the critical role of replicase poly-protein processing in virus replication (van Aken et al., 2006b; van Dinten et al., 1999).

Two papain-like proteinases (PLPs) and one chymotrypsin-like serine proteinase (SP) are functional in all arteriviruses, and reside in nsp1/1b (PLP1b), nsp2 (PLP2) and nsp4 (SP) (for reviews, see Fang & Snijder, 2010; Snijder & Meulenberg, 1998; Ziebuhr et al., 2000). In PRRSV and LDV, a fourth non-structural proteinase (PLP1a) mediates internal nsp1 cleavage into nsp1a and nsp1b (den Boon et al., 1995). The presence of two PLP domains may reflect an ancient duplication event, but PLP1a has become inactivated in EAV. The sequence analysis of the SHFV nsp1 region revealed a potentially even more complex organization, with an array of three (potential) PLP domains in the 480 aa region upstream of the (predicted) nsp1/2 junction.

During the past decade, crystal structures have been reported for all four arterivirus proteinase domains (Fig. 5). Consistent with earlier bioinformatics, expression and mutagenesis studies (den Boon et al., 1995), PLP1x (Sun et al., 2009) and PLP1β (Xue et al., 2010) are very compact PLP domains employing a Cys–His tandem as active-site residues. They appear to exclusively cleave their own C-terminus, which is retained in the substrate-binding pocket, thus precluding further proteolytic activity.

In contrast, the PLP2 domain in nsp2 exhibits both cis and trans cleavage activities (Han et al., 2009; Snijder et al., 1995). In addition to performing the critical nsp2/3 cleavage, PLP2 is capable of removing ubiquitin (Ub) and Ub-like modifiers like ISG15 (an IFN-stimulated gene) from host cell substrates (Frias-Staheli et al., 2007; Sun et al., 2010, 2012b; van Kasteren et al., 2012). Structurally, PLP2 also differs from PLP1a/b and was proposed to be distantly related to so-called ovarian tumour domain-containing (OTU) deubiquitinases (DUBs) (Makarova et al., 2000). However, the compact EAV PLP2 structure (Fig. 5d; van Kasteren et al., 2013) is distinct from other OTU superfamily members and incorporates a structurally important zinc finger (ZF) domain. Given these features, PLP2 represents a unique subclass of zinc-dependent OTU DUBs.

Crystal structures were solved for both EAV and PRRSV nsp4 (Fig. 5e), which includes the SP that is the arteriviral main proteinase and cleaves all sites downstream of nsp3 (Barrette-Ng et al., 2002; Tian et al., 2009). The protein contains two domains that form the typical chymotrypsin-like two-β-barrel fold and a C-terminal domain that is dispensable for proteolytic activity, but may be involved in fine-tuning of replicase polyprotein processing (van Aken et al., 2006a).

Non-structural protein functions
During or following pp1a and pp1ab cleavage, arterivirus non-structural proteins assemble into a membrane-associated
enzyme complex that directs viral replication and transcription. In addition to encoding the proteinases discussed above, ORF1a encodes three (putative) transmembrane proteins (nsp2, nsp3 and nsp5) that are thought to anchor the viral RNA-synthesizing machinery to modified intracellular membranes. These membrane structures as well as the role of nsp1 proteins in transcriptional control and innate immune evasion will be discussed separately below.

The functions of nsp6–8 and a conserved cysteine-rich domain in the C-terminal part of nsp2 have remained enigmatic thus far. Interestingly, the latter domain is lacking from the truncated, 2PRF-derived nsp2TF and nsp2N proteins. nsp2N lacks a hydrophobic domain and is predicted to be cytosolic, whereas nsp2TF is fitted with an alternative transmembrane region that appears to target the protein to the exocytic pathway (Sun et al., 2009), including the N-terminal zinc finger (ZF) domain (blue, zinc-binding residues highlighted in yellow) and C-terminal PLP1α domain (red/pink). After cleavage, the ‘C-terminal extension’ (CTE) domain (green) remains in the substrate-binding pocket. (b) PRRSV nsp1α (Sun et al., 2009), including the N-terminal zinc finger (ZF) domain (blue, zinc-binding residues highlighted in yellow) and C-terminal PLP1α domain (red/pink). After cleavage, the ‘C-terminal extension’ (CTE) domain (green) remains in the substrate-binding pocket. (c) PRRSV nsp1β (Xue et al., 2010), including N-terminal domain (NTD; blue), linker sequence (grey), PLP1β domain (red/pink) and CTE (green). (d) EAV PLP2 proteinase domain (van Kasteren et al., 2013), including its ZF with zinc-binding residues highlighted (yellow). (e) PRRSV nsp4 (Tian et al., 2009), including the typical chymotrypsin-like two-β-barrel fold of the SP domain (red/pink) that is the arterivirus main proteinase. The pink bidirectional arrow represents a loop devoid of visible electron density in the crystal structure. The nsp4 C-terminal domain (CTD; blue) is dispensable for proteolytic activity in EAV.

(Fang et al., 2012; Han et al., 2010), but their biological significance needs to be studied in more detail. It is interesting to note that nsp2N and nsp2TF both contain the N-terminal PLP2 domain, and may thus target this viral DUB to alternative locations in the cell.

In addition to PLP2 and the −2 PRF mechanism, arterivirus nsp2 stands out for its ‘hypervariable region’ in which deletions and insertions have occurred in both EAV and PRRSV (Balasuriya et al., 2004b; reviewed by Fang & Snijder, 2010). Although its interactions with the immune system remain to be elucidated, a cluster of immunodominant B cell epitopes and potential T-cell epitopes is associated with the hypervariable region. Variation in this nsp2 region was initially linked to viral virulence, in particular for the highly pathogenic Asian PRRSVs, but this claim continues to be debated (reviewed by Fang & Snijder, 2010). Nevertheless, certain nsp2 regions that are non-essential for replication may play an important role in PRRSV pathogenesis in vivo.

Arterivirus ORF1b encodes the two core enzymes for viral RNA synthesis: the RNA-dependent RNA polymerase and PRF. In addition to the PRF mechanism, arterivirus nsp2 stands out for its ‘hypervariable region’ in which deletions and insertions have occurred in both EAV and PRRSV (Balasuriya et al., 2004b; reviewed by Fang & Snijder, 2010). Although its interactions with the immune system remain to be elucidated, a cluster of immunodominant B cell epitopes and potential T-cell epitopes is associated with the hypervariable region. Variation in this nsp2 region was initially linked to viral virulence, in particular for the highly pathogenic Asian PRRSVs, but this claim continues to be debated (reviewed by Fang & Snijder, 2010). Nevertheless, certain nsp2 regions that are non-essential for replication may play an important role in PRRSV pathogenesis in vivo.
Arterivirus sg mRNA contains a common 5’-proximal nested set of sg mRNAs, from which the structural proteins are translated (Fig. 6). Each arterivirus sg mRNA contains a common 5’-proximal nested set of sg mRNAs, from which the structural proteins are translated (Fig. 6). Each arterivirus sg mRNA contains a common 5’-proximal nested set of sg mRNAs, from which the structural proteins are translated (Fig. 6). Each arterivirus sg mRNA contains a common 5’-proximal nested set of sg mRNAs, from which the structural proteins are translated (Fig. 6). Each arterivirus sg mRNA contains a common 5’-proximal nested set of sg mRNAs, from which the structural proteins are translated (Fig. 6).

A fourth conserved arterivirus ORF1b domain encodes a puzzling endoribonuclease function (originally named NendoU, for nidovirus endonuclease specific for uridylate; Ivanov et al., 2004; Snijder et al., 2003a). Its importance for arterivirus replication was established by site-directed mutagenesis (Posthuma et al., 2006), but the exact function of NendoU and, in particular, the identity of its natural substrate(s) in infected cells remains enigmatic. Recombinant EAV and PRRSV nsp11 display broad substrate specificity in vitro, cleaving ssRNA and dsRNA substrates 3’ of pyrimidines (Nedialkova et al., 2009). The individual expression of nsp11 is extremely toxic and a similar broad activity towards viral RNA substrates in infected cells would clearly make NendoU expression potentially ‘suicidal’. This suggests that its access to RNA substrates is strictly controlled in the context of infection, possibly by compartmentalization of nsp11 in replication structures. Two ORF1b-encoded replicate domains that remain to be characterized are the conserved N-terminal third of nsp9 and the small C-terminal nsp12 cleavage product. It is also of note that the enzymes and signals ensuring the 5’ capping and 3’ polyadenylation of arterivirus mRNAs remain to be identified.

Synthesis and translation of sg mRNAs

A hallmark of arterviruses and other nidoviruses is the synthesis of a 3’-co-terminal nested set of sg mRNAs, from which the structural proteins are translated (Fig. 6). Each arterivirus sg mRNA contains a common 5’ end ‘leader sequence’ (156–211 nt) that is identical to the 5’-proximal part of the genome (de Vries et al., 1990). This property is shared with coronaviruses, but not with several other groups of nidoviruses. The fusion of the 5’ ‘leader’ to the coding part (or ‘body’) of the sg mRNA is thought to rely on discontinuous RNA synthesis – a mechanism that resembles copy-choice RNA recombination and that has been reviewed extensively elsewhere (Pasternak et al., 2006; Sawicki et al., 2007; Sola et al., 2011). Briefly, discontinuous negative-strand RNA synthesis is thought to generate a nested set of subgenome-length, negative-stranded RNAs, which subsequently serve as templates for sg mRNA synthesis. Base pairing of short conserved transcription regulatory sequences (TRSs) plays a key role, as was first verified for EAV using reverse genetics (Pasternak et al., 2001; van Marle et al., 1999).

At the 3’ end of the nascent subgenome-length negative strand, the body TRS complement can base pair with the TRS that is present at the 3’ end of the leader sequence (‘leader TRS’) in the genomic template.

Arterivirus sg mRNAs are produced in non-equimolar, but relatively constant amounts, thus providing a mechanism to regulate structural protein expression. Transcription depends on TRS duplex formation and, in general, the relative amounts of sg mRNA correlate with the calculated stability of this duplex (Pasternak et al., 2003). Structural studies (van den Born et al., 2004) placed the leader TRS in a single-stranded loop referred to as the LTH (Fig. 4a) – a critical signal in EAV discontinuous RNA synthesis (van den Born et al., 2005).

Several replicase subunits have been implicated in transcriptional regulation, with specific mutations in both EAV nsp1 and nsp10 causing the (near-) complete inactivation of sg mRNA synthesis (Tijms et al., 2001; van Dinten et al., 1997). EAV nsp1 is thought to control the accumulation of genome and sg mRNAs by determining the levels at which their negative-stranded templates are produced (Nedialkova et al., 2010). An N-terminal ZF domain was implicated in this function, but other nsp1 domains are also important (Tijms et al., 2001). In PRRSV, where nsp1 is internally cleaved into nsp1z and nsp1β, the ZF-containing nsp1z subunit appears to fulfil a similar role in transcriptional regulation (Fig. 5b; Kroese et al., 2008; Sun et al., 2009).

With the exception of the smallest transcript, arterivirus sg mRNAs are structurally polycistronic but are presumed to be functionally monocistronic. Notable exceptions are the functionally bicistronic mRNAs from which the partially overlapping gene sets E/GP2 and ORF5a/GP5 are expressed (Firth et al., 2011; Godeny et al., 1998; Johnson et al., 2011; Snijder et al., 1999).

Virion, structural proteins and assembly

Virion and nucleocapsid properties

The arterivirus genome is encapsulated by a single N protein of 110–128 aa, forming a core that is wrapped in a lipid envelope containing various surface GPs and other membrane proteins (Fig. 7). The roughly spherical or oval-shaped virions have a diameter of 50–60 nm and reported buoyant densities of 1.13–1.17 g cm\(^{-3}\) in sucrose (Snijder & Meulenberg, 1998; Spilman et al., 2009). They possess a relatively smooth envelope (Fig. 7), which is likely explained by the small ectodomains of the two major envelope proteins, GP5 and M protein.

The arterivirus nucleocapsid has long been assumed to be isometric, but recent cryo-electron tomography studies of PRRSV particles rather suggest a pleomorphic core structure (mean diameter 39 nm), possibly resembling that proposed for coronaviruses in terms of being a helical coil or an even more loosely organized filamentous structure (Dokland, 2010; Spilman et al., 2009). The N protein is phosphorylated, which may modulate nucleic acid binding.
or protein–protein interactions (de Vries et al., 1992; Wootton et al., 2002). Crystal structures were obtained for the capsid-forming C-terminal domain of the PRRSV and EAV N proteins (Fig. 7f). The protein’s N-terminal domain contains many positively charged residues and presumably binds RNA (reviewed by Dokland, 2010). Based on cryo-EM studies of frozen hydrated PRRSV particles, the core was proposed to consist of two intertwined layers of N protein dimers that ‘sandwich’ the genome (Fig. 7g–i; Spilman et al., 2009). Specific RNA encapsidation signals that interact with the N protein have not been identified thus far.

The arterivirus envelope is now presumed to contain two major and five minor envelope proteins (Fig. 7e). With the exception of the recently discovered ORF5a protein (Firth et al., 2011), they are all critical requirements to produce infectious progeny (Molenkamp et al., 2000b; Wissink et al., 2005). In reverse genetics studies, EAV mutants lacking one of the minor GP genes (see below) produced non-infectious subviral particles that consisted of RNA, and the N, M and GP5 proteins, suggesting that these form the basic virion scaffold. The defect could be complemented in trans by expression of the deleted gene, thus generating a system to produce ‘disabled infectious single-cycle viruses’ with potential vaccine applications (Welch et al., 2004; Wieringa et al., 2004; Zevenhoven-Dobbe et al., 2004).

**Major envelope proteins**

The two major envelope proteins are the ‘major’ GP (GP5 in EAV, PRRSV and LDV) and the non-glycosylated M protein, which form a disulphide-linked heterodimer (de Vries et al., 1995; Faaberg & Plagemann, 1995; Snijder et al., 2003b). The EAV GP5/M heterodimer is essential for virus assembly, possibly by inducing the membrane curvature required for virus budding. The N-terminal half of the conserved M protein presumably traverses the membrane three times, exposing only a short ectodomain (10–18 aa) on the virion surface (de Vries et al., 1992; Faaberg & Plagemann, 1995). The major GP is the most variable structural protein, although important structural features are conserved between arteriviruses. The proteins consist of
an ectodomain, a central hydrophobic region predicted to span the membrane three times and a sizeable cytoplasmic C-terminal domain of 50–75 aa. An N-terminal signal sequence is predicted to be cleaved from the ectodomain (Dokland, 2010), which is post-translationally modified with one to three N-linked glycans (de Vries et al., 1992; Faaberg & Plagemann, 1995; Zhang et al., 2010) that can become very heterogeneous due to the attachment of poly-N-acetyllactosamine structures of different size during GP5 maturation in the Golgi complex. The GP5 ectodomain is an important target for neutralizing antibodies and for LDV (Li et al., 1998), for example, its variable N-glycosylation was linked to important differences in neutralization and pathogenesis (see below).
Minor envelope proteins

The structural properties of arterivirus minor envelope proteins have been reviewed recently by Dokland (2010). The minor GPs (GP2, GP3 and GP4) are present in virions as disulphide-linked GP2–GP3–GP4 heterotrimers and GP2–GP4 heterodimers (Das et al., 2010; de Lima et al., 2009; Wieringa et al., 2003a, b; Wissink et al., 2005). GP2 is a conventional class I integral membrane protein containing an N-terminal signal peptide, an ectodomain with one to four potential N-glycosylation sites, and a C-terminal transmembrane segment and short cytoplasmic tail. Cysteine residues in the GP2 ectodomain are critical for the formation of intramolecular and intermolecular disulphide bridges, and the interaction with the GP4 ectodomain (Wieringa et al., 2003a). Arterivirus GP3 is a heavily glycosylated integral membrane protein containing a non-cleaved N-terminal signal sequence and a hydrophobic C-terminal domain (de Lima et al., 2009; Wieringa et al., 2002). The protein may be anchored in the membrane with both termini (Wieringa et al., 2002). Like GP2, GP4 is a class I membrane protein with an N-glycosylated ectodomain from which a signal peptide is removed (Meulenberg et al., 1997; Wieringa et al., 2002). A recent PRRSV study suggests that, in addition to being part of the GP2–GP3–GP4 heterotrimer, PRRSV GP4 interacts with the major GP, GP5 (Das et al., 2010).

The two remaining arterivirus envelope proteins are small non-glycosylated polypeptides that are predicted to be largely embedded in the membrane. In EAV (Snijder et al., 1999) and PRRSV (Wu et al., 2001), the small E protein was identified as a minor structural protein, which associates with the minor GP heterotrimer (Wieringa et al., 2004). The E protein is essential for virus infectivity and, although its membrane topology is unknown, was proposed to oligomerize and form an ion channel that could play a role during viral entry and/or fusion (Lee & Yoo, 2006). The E protein contains a conserved myristoylation signal (Thaa et al., 2009) that is important but not absolutely required for virion infectivity.

Recently, a second small hydrophobic protein, encoded by the previously undiscovered ORF5a, was identified for EAV and PRRSV (Firth et al., 2011; Johnson et al., 2011). The ORF5a protein was detected in purified PRRSV. Although EAV reverse genetics demonstrated that it is not essential for infectivity, knockout mutants produce significantly reduced progeny titres.

Assembly and release

Since the N protein is found in association with viral replication structures, arterivirus genome encapsidation may be initiated at the site of viral RNA synthesis (Tijms et al., 2002). Recent electron tomography studies of EAV-infected cells revealed a network of N protein-containing sheets and tubules of unknown function, which appear intertwined with the membranous replication structures (see below). Arterivirus budding involves the wrapping of a preformed nucleocapsid by membranes of the smooth endoplasmic reticulum (ER) or Golgi complex (Magnusson et al., 1970; Wood et al., 1970), in which the viral envelope proteins appear to be retained. The formation of the GP5/M heterodimer is a primary determinant of virus assembly and triggers the transport of both proteins to the Golgi complex – a step correlated with the production of infectious progeny (Snijder et al., 2003a; Verheije et al., 2002b; Wieringa et al., 2004; Wissink et al., 2005). Once released into the lumen of secretory pathway compartments, virions are transported to the plasma membrane and released, while undergoing extensive glycan processing in the Golgi complex.

Arterivirus–host interactions

Tropism, entry and receptors

With the exception of EAV, arterivirus tropism is very restricted (Plagemann & Moennig, 1992; Snijder & Meulenberg, 1998). EAV replicates efficiently in primary horse macrophages and kidney cells, and in a remarkable variety of cell lines. LDV grows in primary mouse macrophages, but not in macrophage or other cell lines. In addition to replicating in primary macrophages from their respective hosts, SHFV and PRRSV replicate in certain African green monkey kidney cell lines (MA-104) and derivatives thereof, such as MARC-145 (Duan et al., 1998).

Arterivirus entry occurs through standard clathrin-mediated endocytosis, and the viral nucleocapsid is released into the cytosol following endosome acidification and membrane fusion (Kreutz & Ackermann, 1996; Nauwynck et al., 1999). However, fusion activity has not been convincingly assigned to any of the viral envelope proteins. Likewise, the debate on which protein(s) determine tropism and receptor binding has not been settled. In cell culture, a chimeric PRRSV equipped with the EAV minor GPs and E protein gained the broad tropism that is typical of EAV (Tian et al., 2012), whereas EAV/PRRSV chimeras with swapped GP5 and M ectodomains did not display such a changed tropism (Dobbe et al., 2001; Verheije et al., 2002b). Thus, the GP5/M heterodimer may be the key player in envelope formation and budding, while the minor GPs are the prime determinants of host cell binding and possibly also fusion and entry.

The host factors that mediate entry have only been studied in detail for PRRSV (for recent reviews, see Van Breedam et al., 2010a; Welch & Calvert, 2010). Heparin-like molecules on the cell surface (Deputte et al., 2002) and sialic acids on the virion surface (Deputte & Nauwynck, 2004) were implicated in PRRSV binding to porcine alveolar macrophages. Subsequently, internalization would be mediated by sialoadhesin (CD169) – a macrophage-restricted member of the siglec family of immunoglobulin-like lectins that carries sialic acids with which the ectodomains of the GP5/M heterodimer can interact (Van Breedam et al., 2010b). Expression of porcine CD169 in non-susceptible cell lines can mediate PRRSV internalization, but not disassembly and productive infection. However, a CD169 homologue is not
expressed by the MARC-145 cell line that fully supports PRRSV infection (Duan et al., 1998), suggesting the existence of alternative receptors. In particular, CD163, a member of the scavenger receptor cysteine-rich family, was identified as its expression rendered a variety of non-permissive cell lines susceptible to PRRSV infection (Welch & Calvert, 2010). It was postulated that CD169 and CD163 work together in porcine macrophages, with the former serving as a receptor for internalization and the latter playing a key role in uncoating and genome release (Van Gorp et al., 2008). Clearly, several questions regarding arterivirus entry remain to be addressed, despite the considerable recent progress made for PRRSV.

Replication structures

As in all +RNA viruses, arterivirus replicative proteins assemble into an enzyme complex for viral RNA synthesis that is associated with virus-induced membrane structures. In the case of arteriviruses and other nidoviruses, these are not ‘simple’ invaginations, but more complex networks of modified intracellular membranes in the perinuclear region of the infected cell (Fig. 8a–c; Li et al., 2012; van der Meer et al., 1998). All arterivirus replicase subunits studied to date co-localize in this area, except for the nsp1 and nsp2TF proteins (Chen et al., 2010; Fang et al., 2012; Tijms et al., 2002). Infection triggers the formation of large numbers of ‘double membrane vesicles’ (DMVs) (Fig. 8d), with which the viral replication and transcription complex (RTC) is thought to be associated (Pedersen et al., 1999; van Hemert et al., 2008). The formation of this membranous ‘scaffold’ for the RTC has been attributed to the ORF1a-encoded (putative) membrane-spanning proteins nsp2, nsp3, and nsp5, and paired membranes and DMVs can be induced by the expression of nsp2 and nsp3 alone (Snijder et al., 2001). A recent electron tomography study revealed that, as in coronaviruses, DMVs can be interconnected by their outer membranes (Fig. 8e–h), thus forming a network of modified ER in EAV-infected cells (Knoops et al., 2012). A striking additional similarity with coronaviruses is the presence of dsRNAs inside the DMVs. Although these may represent double-stranded intermediates of replication and transcription, their enclosure by a double membrane is puzzling and their functionality in RNA synthesis remains to be demonstrated.

Little is known about host proteins that may interact with arterivirus RNA or transmembrane non-structural proteins, or that may be recruited to replication structures. Common sets of (as-yet unidentified) host proteins bind in vitro to the 3’ end of the genome or anti-genome of SHFV, EAV and PRRSV, and were implicated in the initiation of arterivirus RNA synthesis (Hwang & Brinton, 1998; Maines et al., 2005). The RNA-synthesizing activity of EAV RTCs is associated with crude membrane fractions from infected cells does depend on membrane integrity. Moreover, an as-yet unidentified host protein factor or complex from the cytosolic fraction of the cells is required for in vitro RTC function (van Hemert et al., 2008). Recent EAV and PRRSV inhibitor studies with the drug cyclosporin A suggest the involvement of members of the cyclophilin family, most likely cyclophilin A, in arterivirus RNA synthesis (de Wilde et al., 2013). Furthermore, it has been suggested that the autophagy marker microtubule-associated protein 1 light chain 3 (LC3) and the ER degradation-enhancing z-mannosidase-like1 (EDEM1) associate with EAV DMVs (Monastyrska et al., 2013), but the functional relevance of this observation for viral RNA synthesis remains unclear since the analysis was only performed late in infection. In any case, in this study EAV replication was unchanged in autophagy-deficient cells, although it was affected by the depletion of LC3, specifically in its non-lipidated form. In contrast, several recent PRRSV studies (for example Sun et al., 2012; Liu et al., 2012) suggested that autophagy promotes arterivirus replication, as judged from the effect of inhibiting the autophagy pathway using pharmacological and siRNA approaches. Clearly, additional research is needed to establish the precise role of autophagy or individual factors from this pathway in the arterivirus replicative cycle.

Arterivirus proteins in the nucleus

Despite the cytoplasmic replication cycle, some arterivirus proteins are directed (in part) to the nucleus of infected cells, specifically the nsp1 proteins and the N protein. Their possible involvement in modulating host cell responses is discussed in the next section, together with other viral proteins implicated in similar functions.

Whereas the signals or interactions that target arterivirus nsp1 proteins to the nucleus remain to be defined, residues 41–47 of the PRRSV N protein were identified as a nuclear localization signal that interacts with the nuclear transporters importin a and b (Rowland et al., 2003). Like its EAV counterpart (Tijms et al., 2002), the PRRSV N protein accumulates in the nucleoli of infected cells. Remarkably, a leptomycin B-induced block of CRM1-mediated nuclear export resulted in the nuclear accumulation of the EAV N protein, indicating that the protein must shuttle between the cytoplasm and nucleus before playing its role in cytoplasmic virus assembly. Various nuclear host proteins interact with the PRRSV N protein (reviewed by Sun et al., 2012a), including fibrillarin, nucleolin and poly(A)-binding protein, but the functional implications of these findings remain to be studied. Using reverse genetics, a knockout mutant for the nuclear localization signal in the PRRSV N protein was engineered, which was viable, but seriously attenuated. Pigs infected with this mutant developed reduced viraemia and significantly higher neutralizing antibody titres (Lee et al., 2006).

Immune responses

Innate immune responses

Arterivirus infections generally elicit poor innate immune responses, probably explaining the weak adaptive immune
responses and viral persistence. For a detailed overview of the immune response to arterivirus infection, the reader is referred to other reviews (Darwich et al., 2010; Kimman et al., 2009; Sun et al., 2012a) and references therein. Viral protein functions that may help arteriviruses to modulate or evade host immune responses will be discussed in the next section.

It has not been firmly established which pattern recognition receptors of the innate immune system are involved in the sensing of arterivirus infection. The involvement of Toll-like receptor 3 (TLR3) during PRRSV infection in macrophages has been suggested (Darwich et al., 2010 and references therein), but also other TLRs were implicated in

Fig. 8. Arterivirus replication structures. (a–c) Subcellular localization of EAV (a, b) and PRRSV (c) non-structural proteins in infected Vero-E6 (a, b) or MARC-145 (c) cells as observed by immunofluorescence microscopy. (a) Double labelling for EAV nsp3 (red) and dsRNA (green), presumably highlighting structures involved in viral RNA synthesis. (b) Staining for EAV nsp9, which contains the RdRp domain. (c) Double labelling showing that PRRSV nsp2TF (red) is not targeted to replication structures (labelled for nsp2; green). (d–h) Electron micrographs and tomograms of EAV-infected cells. (d) Membrane changes in infected BHK-21 cells with the rectangle indicating a region containing many DMVs. Arrows, virions budding from smooth intracellular membranes. Bar, 250 nm. (e, f). Tomogram slices showing close-ups of DMVs in EAV-infected Vero-E6 cells preserved by high-pressure freezing. Arrows indicate connections of DMV outer membranes with ER (e) or other DMVs (f). Bars, 50 nm. (g, h) Three-dimensional surface-rendered model of DMVs from a tomogram of EAV-infected Vero-E6 cells (7 h post-infection). DMV membranes, brown; DMV cores, blue; ER, beige; mitochondria, red; N protein tubules, green. Bar, 250 nm. (h) Close-up showing neck-like connections of DMVs to ER (arrows). (a, b) Courtesy of Dr Yvonne van der Meer (Leiden University Medical Center); adapted from Snijder & Kikkert (2013). (c, d, e–h) Adapted from Fang et al. (2012), Fang & Snijder (2010) and Knoops et al. (2012), respectively, with permission.
triggering innate immune response. Experimental LDV infection of knockout mice established the importance of TLR7 in the induction of the type I IFN response (Ammann et al., 2009). An EAV study in knockout mouse embryonic fibroblasts suggested that the retinoic acid inducible I (RIG-I)-like receptors, including melanoma differentiation-associated protein 5 (MDA5) and RIG-I, both have a role in countering arterivirus infection (van Kasteren et al., 2012).

The extent to which arterivirus infection suppresses the innate immune response varies among genetically diversified isolates. It was demonstrated that different PRRSV field isolates varied in their ability to induce IFN-α expression in porcine alveolar macrophages—an observation that may account for differences in virulence and pathogenicity (Lee et al., 2004). Multiple studies reported that PRRSV infection induces IL-8 expression, while the induction of IL-6 and IL-10 is still debated (Darwich et al., 2012, but their effect on virus replication and pathogenesis remains to be elucidated (Coutelier et al., 1995; Markine-Goriaynoff et al., 2001). LDV-induced IFN-α expression was also reported, but the virus is insensitive to a systemic IFN-α response in mice (Ammann et al., 2009). LDV infection activates NK cells and consequently a large increase in IFN-γ production was observed, but these responses were unable to control LDV replication in vivo (Markine-Goriaynoff et al., 2002).

**Innate immune evasion**

Arteriviruses likely employ a combination of functions to modulate the host’s innate immune response and several proteins have been implicated: PRRSV nsp1α, nsp1β, nsp2, nsp11 and N protein, and EAV nsp2 and N protein. However, many of these studies employed expression of individual viral proteins rather than infected cells, which may influence parameters like expression kinetics and subcellular localization of viral proteins. Furthermore, thus far only a few studies addressed the (postulated) immune-modulating activities of PRRSV non-structural proteins during replication and pathogenesis in animals. Thus, corroborating these (proposed) innate immune evasion functions in infected cells and animal models remains a critical step. An interesting example is the proposed modulation of IFN-β production by the PRRSV nsp11 NendoU endoribonuclease (Sun et al., 2012a), which is known to be highly cytotoxic upon its individual expression in a variety of systems. The cytosolic version of the enzyme may target the overall RNA population of the cell, thus inducing a translational shut-off (see above; Nedialkova et al., 2009), whereas NendoU activity in infected cells is likely to be restricted by, for example, association with membranous replication structures.

PRRSV nsp1α and nsp1β were proposed to be major modulators of the type I IFN response (reviewed by Fang & Snider, 2010; Sun et al., 2012a). Their expression suppresses IFN-β activation following the induction of innate immune responses by Sendai virus infection or dsRNA transfection (Beura et al., 2010; Chen et al., 2010; Song et al., 2010). The nsp1α is able to suppress the activation of NFXB, while nsp1β has the ability to inhibit IFN regulatory factor 3 (IRF3)-mediated type I IFN production. Both proteins localize to the nucleus of infected cells and a recent study suggests that nsp1α interferes with IFN-β production through degradation of the transcription factor CREB (cAMP response element-binding protein)-binding protein (CBP) in the nucleus (Han et al., 2013). In addition, nsp1β is able to inhibit downstream IFN-induced signalling pathways by interrupting STAT1 phosphorylation and nuclear translocation of IFN-stimulated gene factor 3 (ISGF3) in the JAK (Janus kinase)–STAT (signal transducer and activator of transcription) pathway (Chen et al., 2010; Patel et al., 2010). The nsp1α and nsp1β domains critical for their function in IFN suppression have been identified (e.g. by alanine-scanning mutagenesis) (Beura et al., 2012). Most recently, a highly conserved nsp1β motif was implicated in inhibiting the IFN response (Li et al., 2013), as its mutagenesis yielded viable recombinant viruses inducing increased expression of IFN-α, IFN-β and ISG15.

The arterivirus PLP2 (Fig. 5) is a dual-specificity proteinase that not only cleaves the nsp2/3 junction in the replicase polyproteins (see above), but is also able to disrupt innate immune signalling by removing Ub and Ub-like modifiers from host cell substrates. EAV and PRRSV PLP2 were found to exhibit general DUB activity towards cellular Ub conjugates and to also remove the IFN-induced Ub homologue ISG15, which is thought to have antiviral activity (Frias-Staheli et al., 2007; Sun et al., 2012b). The biological significance of this activity was supported by the fact that PLP2-DUB could inhibit type I IFN production by interfering with NFXB activation, which is regulated by polyubiquitination (Sun et al., 2010). PLP2 overexpression in cell culture can suppress IFN-β induction by RIG-I-like receptors. The proteinase is able to remove K63-linked polyubiquitin from RIG-I in order to inhibit downstream signalling (van Kasteren et al., 2012). Recently, based on the structure of the EAV PLP2–Ub complex, targeted mutagenesis was used to inhibit DUB activity without affecting nsp2/3 cleavage (van Kasteren et al., 2013). The resulting virus mutants displayed WT replication properties and their strongly reduced PLP2-DUB activity translated into strikingly enhanced innate immune signalling in infected equine cells. The mRNA levels for IFN-β increased by nearly an order of magnitude, suggesting that this approach could be applied to next-generation vaccine development (see below).
As described above, arterivirus N proteins are transported to the nucleus and interactions with various host cell proteins have been described (reviewed by Sun et al., 2012a). However, no firm conclusions on the functional significance of these interactions have been drawn. The PRRSV N protein may be responsible for the early upregulation of IL-10 during infection, suggesting that it modulates the production of regulatory T cells to suppress the innate immune response (Wongyanin et al., 2012), but this hypothesis requires further investigation.

**Humoral immune responses**

In general, arterivirus infection induces the early production of high antibody levels. Antibodies in EAV-infected horses mainly recognize the structural proteins of GP2, GP5, M and N (Chirnside et al., 1995; MacLachlan et al., 1998), and non-structural proteins of nsp2, nsp4, nsp5 and nsp12 (Go et al., 2011b). In PRRSV-infected animals, antibody responses against all GPs, M and N proteins were described, with the anti-N response being the first and strongest (Darwich et al., 2010; Lopez & Osorio, 2004). PRRSV nsp1α, nsp1β, nsp2 and nsp7 also induced a humoral immune response (Brown et al., 2009; Johnson et al., 2007), with antibodies specific for these proteins being detected as early as 14 days post-infection (d.p.i.) and lasting to at least 202 d.p.i.

Neutralizing antibodies in EAV-infected horses can develop as early as 7–14 d.p.i. In contrast, neutralizing antibodies in PRRSV- or LDV-infected animals are generated late and their titres remain low (reviewed by Balasuriya et al., 2004a; Lopez & Osorio, 2004). In arterivirus-infected animals, neutralizing antibodies are predominantly directed to the major GP, GP5. Various neutralizing epitopes were mapped to the GP5 ectodomain in EAV, PRRSV and LDV (Balsuriya et al., 1997; Plagemann et al., 2002; Plagemann, 2004), and also to the PRRSV GP4 ectodomain (Meullenberg et al., 1997). Synthetic peptides representing neutralizing epitopes from LDV and PRRSV GP5 did not induce virus-neutralizing antibodies, suggesting that additional residues or structural features are critical for antigenicity (Lopez & Osorio, 2004; Plagemann, 2001). The four major neutralizing epitopes in EAV GP5 are conformation dependent and GP5/M heterodimerization is thought to be critical for the induction of neutralizing responses in both PRRSV and EAV (Balsuriya et al., 2004a; Lopez & Osorio, 2004).

The delayed or weak induction of neutralizing antibodies upon arterivirus infection has frequently been linked to GP5 glycosylation, particularly for PRRSV and LDV. It was postulated that non-neuropathogenic LDV strains can establish persistent infections due to the fact that neutralizing antibodies bind less efficiently to their highly glycosylated GP5 ectodomain (Chen et al., 1997; Li et al., 1998). In PRRSV, expression of a hypoglycosylated GP5 variant induced significantly higher neutralizing antibody levels in infected piglets (Vu et al., 2011). An immuno-dominant decoy epitope was identified in PRRSV GP5, which is thought to subvert the immune system’s ability to focus on an adjacent neutralizing epitope (Ostrowski et al., 2002). It has been proposed that LDV- and PRRSV-specific antibodies may contribute to antibody-dependent enhancement of infection (Cafruny & Plagemann, 1982; Yoon et al., 1996), in particular through virus opsonization by antibodies recognizing certain non-neutralizing N and GP5 epitopes, leading to enhanced virus internalization by macrophages (Cancel-Tirado et al., 2004). Finally, PRRSV infection was proposed to manipulate and delay neutralizing antibody production by preventing the development of a normal B cell repertoire in piglets (Butler et al., 2008).

The humoral immune response against SHFV infection varies between monkey species and virus isolates tested (Gravell et al., 1986). Virulent SHFV strains, which cause acute disease in patas monkeys, induced neutralizing antibodies by 7 d.p.i., leading to complete virus clearance by 21 d.p.i. In contrast, SHFV strains that cause persistent infections in these monkeys induce very low antibody titres.

**Cell-mediated immune responses**

The cell-mediated immune (CMI) response to arterivirus infection has not been characterized in great detail. Cell-mediated cytotoxic responses against EAV were studied in experimentally infected ponies, in which CD8+ T-cell-mediated cytotoxicity was virus strain-specific and genetically restricted (Castillo-Olivares et al., 2003). The EAV-specific CTL precursors persisted for at least 1 year after infection.

CMI responses, including CD4+/, CD8+ and CD4+/CD8+ double-positive T cells, have been detected in PRRSV-infected animals, and they appear transiently between 2 and 8 weeks post-infection (reviewed by Darwich et al., 2010; Murtaugh et al., 2002). The abundance of PRRSV-specific T cells and IFN-γ-producing cells in both acute and persistently infected swine appears to be highly variable, and there is no apparent correlation with the viral load in lymphoid tissues. It was reported that PRRSV has the ability to downregulate the expression of MHC class I and II molecules on the surface of antigen-presenting cells. Several PRRSV structural proteins (GP4, GP5, M and N) were found to be strong CMI inducers and a set of T-cell epitopes was identified (reviewed by Darwich et al., 2010). In addition, potential T-cell epitopes were identified in nsp9 and nsp10 (Parida et al., 2012).

LDV-specific CD4+ and CD8+ T-cell immune responses in infected mice did not suffice to achieve virus clearance (Even et al., 1995; van den Broek et al., 1997). Additional studies are required to determine whether there is a correlation between T-cell responses in vitro and protection in vivo, and overall it seems likely that arterviruses manipulate the CMI response, directly or indirectly.
Arterivirus disease, diagnostics and prevention

Epidemiology and disease

The natural host range of arteriviruses is restricted to equids (EAV), swine (PRRSV), mice (LDV), and several genera of African and Asian monkeys (SHFV). Macrophages appear to be the primary target cell for all arteriviruses (Plagemann & Moennig, 1992). Receptors for arterivirus entry have only been studied for PRRSV and have been discussed above.

Equine viral arteritis is a respiratory and reproductive disease that is encountered in equine populations worldwide. In the field, EAV infections are frequently subclinical or persistent, but the virus is also capable of inducing a variety of symptoms, including characteristic lesions of the small muscular arteries (arteritis), acute anorexia and fever, oedema, abortion in pregnant mares, and interstitial pneumonia in neonates (for reviews, see Balasuriya & MacLachlan, 2004; Holyoak et al., 2008; Timoney & McCollum, 1993). EAV persistence in the ampulla of the reproductive tract of infected stallions can result in a ‘carrier state’ and the risk of virus transmission through infected semen. The prevalence of infection varies between horse breeds and recent genome-wide association studies pinpointed genetic differences associated with susceptibility of CD3+ T lymphocytes to EAV infection in vitro (Go et al., 2011a), and possibly also with the risk of persistent infection in stallions (Go et al., 2012).

Following its emergence ~25 years ago, PRRSV has rapidly become one of the most important viral diseases of swine (Zimmerman et al., 2012). The virus continues to evolve (Stadejek et al., 2013), causing numerous acute disease outbreaks, resulting in swine mortality and abortion storms, or in atypical porcine reproductive and respiratory syndrome. The highly pathogenic PRRSV variants that emerged in China in 2006 caused mortality rates between 20% and 100%, depending on the age and health of the infected animals (Fig. 9). Reported primary target cells for PRRSV replication are fully differentiated porcine lung alveolar macrophages and other cells of the monocyte/macrophage lineage (Duan et al., 1997; Thanawongnuwech et al., 2000). Clinical manifestations include occasional discoloring of the skin, most often on the ears (‘blue ear disease’) and vulva. Further symptoms are fever, anorexia, breathing difficulties, lymphadenopathy, lung lesions and abortion. After spreading through the circulation, PRRSV can replicate persistently in the tonsils, lungs and lymphoid organs. The pathogenesis and epidemiology of PRRSV has been discussed in many excellent recent reviews (Shi et al., 2010; Zimmerman et al., 2012).

LDV infection causes increased serum levels of lactate dehydrogenase. The virus was initially isolated from laboratory mice, but subsequently also found in wild mice (Li et al., 2000). LDV infection is life-long but asymptomatic and is maintained by continuous rounds of cytocidal virus replication in a renewable subpopulation of macrophages. The virus has been used extensively as an in vivo research model due to its ability to escape immune surveillance (Cafruny, 1989; Plagemann & Moennig, 1992).

SHFV was originally discovered following an outbreak of fatal haemorrhagic fever in captive rhesus macaques (Tauraso et al., 1968), which likely contracted the virus from asymptotically infected African monkeys in the same animal facility. Clinical signs in macaques included early fever, oedema, dehydration and various haemorrhagic manifestations, usually culminating in death within 2 weeks with mortality rates approaching 100%. Although SHFV-like viruses have never been isolated from human specimens, the resemblance of the clinical symptoms in macaques with those caused by, for example, Ebola and Marburg viruses in humans has attracted considerable attention. SHFV and its recently identified distant relatives appear to be endemic among several species of African monkeys, including primates (Lauck et al., 2013; London, 1977). They apparently cause asymptomatic acute or persistent infections and the zoonotic potential of this arterivirus reservoir is a highly intriguing but as-yet unexplored matter.

Current practices and new diagnostic assays

For laboratory diagnosis of EAV, reverse transcriptase (RT)-PCR assays can be used on nasopharyngeal swabs or washings, conjunctival swabs, and blood samples. In addition, immunohistochemistry using specific mAbs to individual EAV proteins is a reliable method for EAV diagnosis in tissues. A virus neutralization assay remains the gold standard for detection of serum antibodies against EAV (Holyoak et al., 2008).

PRRSV can be detected using immunohistochemistry, virus isolation and (indirect) fluorescent antibody (IFA or FA) tests in serum or tissue samples. In serology, the IDEXX ELISA is the most widely used test to detect antibodies against PRRSV, but IFA and neutralization assays can also be used. Furthermore, (real-time) RT-PCR assays are available to specifically detect viral RNA in serum, semen and tissue samples. Sequence analysis of the ORF5 region has routinely been used for strain identification and differentiation, and a related sequence database has been established (Zimmerman et al., 2012). Although these traditional assays provide good sensitivity and specificity in controlled laboratory settings, experience from the field suggests that substantial improvement is needed in most of these areas.

Recently, assays using swine oral fluids have been evaluated as an alternative to serology (Langenhorst et al., 2012; Prickett & Zimmerman, 2010). Oral fluid is a complex mixture of saliva and gingival crevicular fluid, which more closely resembles serum than salivary gland secretions. Oral fluid sampling methods are an inexpensive, safe and non-invasive alternative to blood sampling to assess both acute infection and immune prevalence in a swine population. Oral fluid-based diagnostic assays, including ELISA and (real-time) RT-PCR, are being further developed and implemented in major animal disease diagnostic laboratories. In addition,
the fluorescent microsphere immunoassay (Luminex technology) has been adapted to the use of oral fluid and serum in order to improve the sensitivity of the assay. It allows the uniform, simultaneous detection of multiple antigens or antibodies within a small volume of a single sample (Langenhorst et al., 2012). In traditional antibody detection assays, PRRSV N protein was used as antigen, but recently also certain non-structural proteins (nsp1α, nsp1β, nsp2 and nsp7) have been explored as more accurate indicators of infection (Brown et al., 2009; Langenhorst et al., 2012).

**Arterivirus vaccines**

Both modified live virus (MLV) and inactivated vaccines are commercially available for the arteriviruses of veterinary importance. For EAV, Arvac (MLV; Ford Dodge Laboratories) and Artervac (inactivated; Ford Dodge Laboratories) have been used in the field. Since the first PRRSV MLV (RespPRRS/Ingelvac PRRS MLV; Boehringer Ingelheim Animal Health) became available in 1994, various other MLV and inactivated vaccines have been released in the USA and Europe (Mengeling, 2005). Together with management strategies, these vaccines suffice to control disease outbreaks. However, they are derived from single virus strains, and are not always efficacious in preventing reinfection and transmission. Furthermore, there are MLV safety concerns, in particular regarding reversion to virulence and the possibility of recombination with field isolates (Botner et al., 1997; Li et al., 2009; Storgaard et al., 1999). The efficacy of MLV vaccines in protecting against a broad spectrum of heterologous field isolates has also been questioned (Kimman et al., 2009). In general, killed vaccines are safer but less efficacious in inducing protection, which makes the development of a safe and broadly protective PRRSV vaccine a major challenge.

For EAV, several genetically engineered candidate vaccines have been tested in vivo. Vaccination of ponies with a subunit vaccine based on the GP5 ectodomain conferred certain levels of protection (Castillo-Olivares et al., 2001). More promising results were obtained with GP5/M-expressing Venezuelan equine encephalitis virus replicon particles. Horses vaccinated with this candidate vaccine produced neutralizing antibodies, shed little or no virus and developed only mild symptoms after a challenge (Balasuriya et al., 2002). Using reverse genetics, a candidate EAV live marker vaccine was constructed by deletion of the major neutralization domain of GP5 (Castillo-Olivares et al., 2003). This recombinant virus produced an asymptomatic infection in ponies, which were subsequently protected against a challenge with virulent EAV. A GP5 peptide-based ELISA was developed to distinguish vaccinated animals from those infected with WT virus.

Several experimental subunit/vectored vaccines based on plasmid DNA and viral vectors that carry PRRSV structural proteins (GP3, M and/or GP5) have been developed (reviewed in detail in the next section). Among these, a plasmid vaccine based on a combination of GP5 and M proteins was found to be protective in field trials (Cacho et al., 1999; Prater et al., 2000).

**Fig. 9.** Clinical symptoms typical of infection with highly pathogenic PRRSV. (a) Infected piglet shivering with high fever. (b) Characteristic purple/blue colouring of the ears. (c) Adult sow that has succumbed to porcine high-fever disease. In most cases, infection in pregnant sows leads to abortion, but occasionally also to death of the sow. (d) Aborted fetuses as a result of infection. (e) Kidney from an infected pig showing numerous blood spots (red arrows), found in 20–30% of the cases. (f) Severe lesions and haemorrhage (red arrows) of lungs unique to highly pathogenic PRRSV infection. (a, e, f) Courtesy of Dr K. Tian and Professor G. F. Gao, Chinese Academy of Sciences, Beijing, PR China; reprinted from Tian et al. (2007) under the Creative Commons license. (c, d) Courtesy of the Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agriculture Sciences, Fuzhou, PR China.
by Huang & Meng, 2010; Kimman et al., 2009). However, it remains to be determined whether such vaccines could generate better protection than the existing MLV and killed whole-viral vaccines. In terms of efficacy in the field, PRRSV MLV vaccines still are the most promising approach (Rock, 2007), and attempts to improve their safety and efficacy are ongoing. A recent development is the application of reverse genetics, recombinant chimeras of virulent field strains and attenuated vaccine strains were generated, which were attenuated to variable degrees in animal models (Kwon et al., 2008; Wang et al., 2008). Furthermore, recombinant viruses carrying specific mutations or deletions in viral immune evasion proteins have been engineered (Beura et al., 2012; Li et al., 2013; Sun et al., 2010, 2012b; van Kasteren et al., 2013).

Another drawback of current PRRSV vaccines is that vaccinated animals cannot be distinguished from pigs that have recovered from a natural infection. Several laboratories have explored the possibility of constructing genetically engineered marker vaccines (reviewed by Fang & Snijder, 2010). Using type 1 or 2 PRRSV infectious clones, an nsp2 B cell epitope was deleted to create a negative marker, whereas an antigenic protein or peptide tag was inserted into the nsp2-coding sequence of the same construct to add a positive marker. To monitor the vaccination status of animals, companion diagnostic assays were designed (Fang et al., 2008). These genetically modified live (marker) viruses may well provide the most rational approach to future PRRSV vaccine development, although the use of recombinant viruses in the field continues to be a matter of debate between vaccine developers, swine practitioners and animal health authorities.

**Outlook**

The arterivirus genome expression strategy is among the most complex of currently known +RNA viruses and includes an intriguing variety of regulatory mechanisms that operate at the co- and post-translational level. Consequently, EAV and increasingly also PRRSV are being used extensively as research models to study basic aspects of +RNA virus replication at large, and nidovirus molecular biology in particular. Thus far most of the ~25 arterivirus proteins have only been defined in basic terms and their functional characterization is one of the major challenges for future research. This will enhance our understanding of, for example, the intricacies of arterivirus RNA synthesis, replicase function, replication structures and virus–host interactions. The recently discovered non-canonical translation mechanism that produces nsp2TF/nsp2N adds another layer of complexity to arterivirus genome expression. This highly efficient −2 PRF event is unprecedented in eukaryotic systems and may have implications beyond the field of RNA virology. Furthermore, the in-depth characterization of nsp2TF/nsp2N function will likely reveal novel arterivirus–host interactions.

Following the discovery of the ORF5a protein as the eighth arterivirus structural protein, the dissection of the functional interactions between structural proteins during particle assembly and disassembly has become an even more complex issue. Recent studies revealed several unique features of the arterivirus nucleocapsid and envelope proteins, which in part link to unanswered questions regarding attachment and entry. Although progress has been made towards understanding host factors involved in PRRSV entry, issues like the specific functions of these host proteins and the role of carbohydrate moieties on the virion and cell surface remain to be addressed. The arterivirus–host molecular interplay may ultimately provide an explanation for the highly specific tropism of this group of viruses. This property, in turn, connects to the intriguing question whether arteriviruses are able to cross species barriers and emerge in other hosts. Considering the unapparent and persistent infections caused by currently known arteriviruses, it may be difficult to detect arterivirus-induced disease in other species. However, modern virus detection techniques may compensate for this and are increasingly likely to identify additional family members, as evidenced by the recent discovery of multiple additional SHFV-like viruses in African monkeys.

The development of improved vaccines and disease control strategies has been a major driving force behind studies into arterivirus molecular biology. EAV and PRRSV are significant veterinary pathogens, and the latter is one of the economically most important swine pathogens. The recent Asian PRRSV outbreaks continue to cause enormous economic losses. The complete control of PRRSV and EAV infection is hampered by natural characteristics common to all RNA viruses, in particular rapid evolution yielding genetically and antigenically diverse virus populations. Our poor understanding of arterivirus–host interactions and immunology further complicates the control of infection (e.g. due to major deficits in our knowledge of the events initiating the immune response upon infection, the key targets for B and T-cell-based protection, and the mechanisms regulating the maturation of the immune response). Persistent infection is another significant factor impeding arterivirus control, as current diagnostic methods are unable to reliably identify persistently infected animals. Recent advances in arterivirus non-structural protein research has resulted in the development of improved diagnostic assays and the identification of various potential innate immune antagonists, with implications for the rational design of novel vaccines. Reverse genetics has become a key tool to manipulate immune antagonist functions in the context of vaccine development. The initial steps toward genetically engineered MLV vaccines have been taken and illustrate how molecular virological knowledge of arterivirus infection, supported by structural biology and immunology, may provide a rational basis for future disease prevention and control.
Acknowledgements

We would like to acknowledge the contributions, over many years, of a large number of colleagues who worked with us in the arterivirus field. Our research was and is supported by the Netherlands Organization of Scientific Research (NWO grants 700.57.301, 700.10.352 and 700.59.008), the European Commission (grants 260444 and 264286), the US Department of Agriculture (grants 2005-35204, 2007-01745, 2008-55620 and 2011-02925) and the US National Pork Board (grants 05-155, 06-173, 09-234, 11-037, 11-143 and 12-127).

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