Virology 438 (2013) 51–55

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Minireview

Approaches to define the viral genetic basis of classical swine fever virus virulence

Immanuel Leifer [a,*], Nicolas Ruggli [a], Sandra Blome [b]

[a] Institute of Virology and Immunoprophylaxis (IVI), Sensemattstrasse 293, CH-3147 Mittelhäusern, Switzerland
[b] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald Insel Riems, Germany

ARTICLE INFO

Article history:
Received 15 October 2012
Returned to author for revisions
20 November 2012
Accepted 18 January 2013
Available online 13 February 2013

Keywords:
Classical swine fever virus
Pestivirus
Virulence

ABSTRACT

Classical swine fever (CSF), a highly contagious disease of pigs caused by the classical swine fever virus (CSFV), can lead to important economic losses in the pig industry. Numerous CSFV isolates with various degrees of virulence have been isolated worldwide, ranging from low virulent strains that do not result in any apparent clinical signs to highly virulent strains that cause a severe peracute hemorrhagic fever with nearly 100% mortality. Knowledge of the molecular determinants of CSFV virulence is an important issue for effective disease control and development of safe and effective marker vaccines. In this review, the latest studies in the field of CSFV virulence are discussed. The topic of virulence is addressed from different angles; nonconventional approaches like codon pair usage and quasispecies are considered. Future research approaches in the field of CSFV virulence are proposed.

Introduction

Classical swine fever virus (CSFV), the infectious agent of classical swine fever (CSF), can cause important economic losses, representing a constant and serious threat for the pig industry (Edwards et al., 2000; Vandeputte and Chappuis, 1999). The disease can manifest as acute hemorrhagic fever with respiratory, gastrointestinal and neurological symptoms resulting in high mortality rates, as a chronic disease with atypical symptoms, or even remain mostly unapparent (Kaden et al., 2000). Besides host factors like the age and general health status of the animals, it is mainly the virulence of the CSFV isolate that determines disease severity (Moennig et al., 2003). Virulence of a virus can be defined as fitness advantage or as the ability of the virus to cause clinical and pathological symptoms in a susceptible host. In some reports virulence was related to cell killing *in vitro* (Herrera et al., 2007). In this review, virulence of CSFV is defined as the ability of the virus to cause clinical and pathological signs in pigs. The virulence of CSFV isolates ranges from avirulent to highly virulent with up to 100% mortality (Kaden et al., 2000). Avirulent vaccine strains were obtained from highly virulent strains by attenuation through multiple passaging in cell culture or in non-natural hosts such as guinea pigs and rabbits. One example is the live vaccine C-strain (Chinese strain) "Riems" (Kaden et al., 2000, 2004; Kaden and Lange, 2001).

CSFV possess a single positive-strand RNA genome of approximately 12,300 nucleotides flanked by 5' and 3' non-translated regions (NTR) (Meyers and Thiel, 1996). The large open reading frame encodes typically a polyprotein of 3898 amino acids that undergoes co- and post-translational processing by cellular and viral proteases (Meyers and Thiel, 1996). The polyprotein processing generates the four structural proteins C, E^{RNS}, E1, and E2 and the eight non-structural proteins N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Rümenapf et al., 1993; Tautz et al., 1997; Thiel et al., 1991).

Several groups studied the molecular biology of CSFV extensively with the aim of identifying molecular determinants of virulence (Fernandez-Sainz et al., 2009, 2010; Gladue et al., 2012; Leifer et al., 2011; Tamura et al., 2012; Risatti et al., 2005a, 2005b; Risatti et al., 2006, 2007; Sainz et al., 2008; Van Gennip et al., 2004). Different aspects are discussed regarding their influence on and correlation with CSFV virulence. This review shall help to consider different molecular viewpoints in the context of virulence.

Towards defining viral genetic determinants of CSFV virulence

Attenuation of CSFV through serial passages in non-natural hosts

Avirulent vaccine strains are derived from highly virulent strains by attenuation through multiple passages in cell culture or non-natural animal hosts. One example is the "GPE^{-}" vaccine [NCBI: D49533] that was derived from the strain "ALD" [NCBI:D49532]. With the aim of identifying the mutations responsible for the loss of

* Corresponding author. Fax: +41 31 848 9271.
E-mail address: Immanuelleifer@hotmail.com (I. Leifer).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.01.013

virulence, the genomes of the vaccine and parent viruses were completely sequenced (Ishikawa et al., 1995). In this study, 225 nucleotide differences spanning the whole genome were found. Six of these differences were located in the non-coding regions. As mutations in the NTR of hepatitis C virus are responsible for the loss of virulence (Friebe and Bartenschlager, 2002), it was postulated that the exchanges in the NTR of strain "ALD" may also be relevant concerning virulence. However, no conclusive statements on the involvement of specific nucleotide and amino acid exchanges in attenuation of the "GPE$^-$" strain could be drawn.

Gain of pathogenicity by passaging the "GPE$^-$" vaccine strain in pigs

Under field conditions, the "GPE$^-$" vaccine virus is not transmitted. Artificial serial passages of the attenuated "GPE$^-$" vaccine strain in pigs by injection of infected tonsil homogenates resulted in the recovery of a pathogenic virus after 11 passages (Tamura et al., 2012). Sequence analyses revealed one exchange in the E2 protein and two exchanges in the NS4B protein. By introducing these exchanges into the "GPE$^-$" genome using reverse genetics, it was demonstrated that these three exchanges were responsible for the regaining of pathogenicity. While the exchange in the E2 protein may influence viral entry and release, the exchanges in the NS4B protein enhanced the replication efficiency in porcine cells. Evidences from other studies on the potential involvement of E2 and NS4B in virulence are discussed below. The study of Tamura and colleagues is the first that identified specific amino acid sequences of CSFV related to virulence based on gain of pathogenicity. It is postulated that the recovery of virulence is due to re-adaptation of the "GPE$^-$" virus to its original host, the pig. The process of virus adaptation involves probably both genome mutation and selection of sequences from the quasispecies population. This is underlined by the findings that exchanges in the viral NS4B protein found after several "GPE$^-$" passages in pigs were already present as a minor quasispecies in the original "GPE$^-$" vaccine. From these experiments it could not be concluded whether these genome positions are universal CSFV virulence determinants.

Codon and codon pair usage are not related to virulence.

It is well established that differences in codon usage influence protein expression (Gouy and Gautier, 1982). In addition, the codon pair usage was found to influence virulence of poliovirus and influenza virus, for which attenuation was generated by artificially modifying the codon pair bias. This was referred to as de-optimization of the codon pair usage (Coleman et al., 2008; Mueller et al., 2010). In case of CSFV, no remarkable differences in codon usage related to virulence were observed (Tao et al., 2009). This is supported by the observation that codon usage of RNA viruses is related to the host (Su et al., 2009). Comparative analyses of the codon pair usage of highly, moderately, and avirulent CSFV strains did not reveal any direct correlation between codon pair usage and virulence either (Leifer et al., 2011). Instead, it was observed that differences in codon pair usage of CSFV isolates cluster the strains into genotypes, similar to the way primary nucleotide sequence analysis and codon usage do.

A possible role of quasispecies distribution in virulence

Another aspect that has never been investigated in the context of CSFV virulence so far is the role of the quasispecies distribution of the viral RNA. Deep sequencing facilities and accompanying analysis software have made in-depth quasispecies analyses possible. A potential role of the quasispecies composition of CSFV isolates in influencing their virulence should be considered, as it had already been determined to affect the virulence of other viruses like foot-and-mouth disease virus (Ojosnegros et al., 2010a, 2010b). Several reports show the occurrence of different quasispecies in CSFV isolates (Kiss et al., 1999; Leifer et al., 2010a, 2010b). In order to further investigate this, the quasispecies composition of the E2 und NS5B encoding genes of highly and low to moderately virulent strains was analyzed. Here, differences in the nucleotide variability and quasispecies entropy were observed (Leifer et al., this issue). The highest genome diversity was found in the highly virulent strains "Brescia" and "Koslov" compared to the low and moderately virulent isolates "Paderborn", "Hennef", and "Uelzen". From these limited preliminary data it is not possible to state whether the higher genome variability observed in highly virulent isolates does contribute to the virulence phenotype. As severe clinical signs were observed after infections with CSFV "Eystrup" and "Brescia" derived from cDNA which have a rather homogeneous genome composition (Mayer et al., 2003; Risatti et al., 2005), a high quasispecies diversity is unlikely to be a virulence determinant *per se*, but its potential involvement in influencing virulence cannot be excluded yet. For conclusive argumentation, further in depth analyses are required.

In vitro cytopathogenic CSFV are attenuated

In some studies, virulence is related to the ability to kill cells *in vitro* (Herrera et al., 2007). Cytopathogenicity of pestiviruses in cell culture is linked to high amounts of free NS3 protein resulting from more effective cleavage of the NS2 and NS3 proteins from the NS2-3 precursor protein. For CSFV, cytopathogenicity is a phenotype that has only rarely been observed in cell culture with natural occurring isolates (Aoki et al., 2001; Kosmidou et al., 1998). Nevertheless, several cytopathogenic CSFV were generated artificially. In particular, insertion of the NS2 cofactor Jiv-90 for enhanced NS2-3 cleavage resulted in a virus that was attenuated in pigs (Gallei et al., 2008).

Attenuation of CSFV by knocking out glycosylation sites of the envelope glycoproteins

Glycosylation of viral structural proteins is important for the virus life cycle (Doms et al., 1993; Hanna et al., 2005; Meyers et al., 2007; Risatti et al., 2005; Shi and Elliott, 2004; Shi et al., 2005; Van Gennip et al., 2004). Five to six putative N-glycosylation sites and one O-glycosylation site have been predicted for the E2 protein of CSFV (Moormann et al., 1990; Risatti et al., 2007). Five of these N-glycosylation sites in E2 are highly conserved among CSFV isolates. Only the isolates "CSF39," "LPC," and "Penevezys" show differences at the first and fourth N-glycosylation sites. Glycosylation is obviously necessary for the expression of virulence, but since the N-glycosylation sites are conserved among highly virulent and avirulent vaccine strains they are very unlikely represent virulence determinants for CSFV on their own. The sixth N-glycosylation site of the E2 protein is slightly more variable.

Seven N-glycosylation sites are described for the E^{RNS} protein (Sainz et al., 2008) and three for the E1 protein (Fernandez-Sainz et al., 2009). All these glycosylation sites are highly conserved among CSFV isolates, independent of their virulence. Only the N_{362} (asparagine) in the E^{RNS} is not found in the avirulent vaccine strain "GPE$^-$," where the asparagine is replaced by a serine (Leifer, unpublished data).

As shown by Risatti et al. (2007), mutation of the E2 N-glycosylation sites can result in less virulent phenotypes. The same results were obtained when mutating N-glycosylation sites in the E^{RNS} and E1 protein of CSFV (Fernandez-Sainz et al., 2009;

Sainz et al., 2008). This is not surprising since glycosylation of viral proteins is crucial for viral replication (Ansari et al., 2006; Deshpande et al., 1987; Gupta and Brunak, 2002; Horimoto and Kawaoka, 1994; Hulse et al., 2004). A lack of N-glycan chains can lead to protein misfolding, causing aggregation in the ER or degradation (Indyk et al., 2007; Parodi, 2000; Trombetta, 2003). Furthermore, these exchanges introduced experimentally might alter the protein structure independent of its glycosylation status, and may thereby influence virus growth and virulence.

A selective pressure analysis in the E2 protein turns up interesting results. At amino acid positions 72 and 75 surrounding the O-glycosylation site, a positive selection was observed (Tang and Zhang, 2007). These findings were confirmed by Wu et al. (2010) who tried to determine the correlation between virulence and evolution of the E2 protein in CSFV isolates. The polyprotein sequences of the O-glycosylation site in the E2 protein differ slightly among CSFV isolates. Changing the O-glycosylation site of the highly virulent strain "Brescia" (Risatti et al., 2007) did not alter its virulence. But investigations in this study were limited to replacing the amino acid proline with alanine. Proline is mainly found in avirulent and moderately virulent strains at the described position, while in highly virulent strains the proline is replaced by lysine. This region differs in up to three amino acids when comparing highly virulent strains to low virulent strains. Furthermore, O-glycosylation takes place at serine or threonine, whereas a nearby proline can alter the efficiency of glycosylation (Veluraja et al., 2001). The protein structure has been described as being important for efficient O-glycosylation (Aubert and Loucheux-Lefebvre, 1976; Dahms and Hart, 1986; Fiat and Jolles et al., 1980). In order to find out more about the O-glycosylation of the E2 protein and its role in virulence, replacing the region surrounding the O-glycosylation site of a low virulent strain by the sequence found in a highly virulent strain might be insightful.

The potential role of selected individual viral proteins in CSFV virulence

Different viral proteins of CSFV were associated with virulence. The N^{pro} protein possesses two different functions: an autoproteolytic function as protease (Rümenapf et al., 1998; Stark et al., 1993), and an inhibitory effect on the interferon (IFN) induction pathway (Bauhofer et al., 2007; Gil et al., 2006; Hilton et al., 2006; Horscroft et al., 2005; La Rocca et al., 2005; Ruggli et al., 2003, 2005, 2009) by mediating the degradation of the interferon regulatory factor 3 (IRF3). Animal experiments showed that CSFV mutants missing the N^{pro} protein were attenuated (Mayer et al., 2004; Tratschin et al., 1998), whereas mutations in the N^{pro} protein abolishing IRF3 degradation did only slightly alter virulence (Ruggli et al., 2009). This indicates that N^{pro} is not responsible for the different degrees of virulence among CSFV isolates. More likely, inhibiting the IFN response via the IRF3 pathway may help the virus to overcome the initial innate immune defense of the host. It may also influence the persistence of the virus in the host as suggested for BVDV (Meyers et al., 2007). Furthermore, an interaction of N^{pro} with IRF7 was reported (Fiebach et al., 2011). This manipulation of the IRF7 function is another way for CSFV to circumvent the host's innate immune defense. But again, this is probably similarly regulated in the different CSFV isolates, independent of virulence.

The structural glycoproteins E^{RNS}, E1, and E2 are parts of the viral envelope membrane and are essential for replication, virus attachment, and entry (Rümenapf et al., 1993). In addition, the E^{RNS} protein possesses ribonuclease activity (Hulst et al., 1994; Langedijk et al., 2002; Schneider et al., 1993). Mutations that abrogate the ribonuclease activity led to virus attenuation (Meyers et al., 1999). For BVDV a role of E^{RNS} in the control of IFN-β activation is described (Iqbal et al., 2004). The E^{RNS} proteins are found as homodimers on the surface of the virion (Thiel et al., 1991). This dimerization is not essential for viral replication and infection (Van Gennip et al., 2005), but the loss of dimerization causes virus attenuation as well (Tews et al., 2009). Mutations in the E^{RNS} of strain "Brescia" C1.1.1 combined with adaptive mutations in the E2 protein led to reduced virulence (Van Gennip et al., 2004).

The E2 protein has also been proposed as a virulence determinant (Risatti et al., 2005, 2006; Van Gennip et al., 2004). E2 is the most immunogenic protein of CSFV and induces the formation of neutralizing antibodies, especially against the highly immunogenic TAV-epitope therein (amino acids TAVSPTTL) (Chen et al., 2010; Lin et al., 2000; Liu et al., 2006a, 2006b; Zhang et al., 2006). Replacing the TAV-epitope in CSFV with the corresponding epitope of BVDV strain NADL resulted in attenuated CSFV mutants (Risatti et al., 2006). Exchange of the complete E2 protein of the highly virulent strain "Brescia" with that of a CSFV vaccine strain resulted in a less virulent phenotype of the chimeric pestivirus (Risatti et al., 2005). By performing biostatistical analyses of evolutionary patterns within the E2 protein of CSFV, a correlation between adaptation and virulence was considered (Wu et al., 2010). These findings, and the findings made by Tamura and colleagues, point towards a potential role of the E2 protein in virulence (Tamura et al., 2012).

Besides the E2 protein, the NS4B protein appears to influence virulence too. As mentioned above, two amino acid exchanges in NS4B were involved in the partial recovery of virulence after artificial re-adaptation of the "GPE^{-}" vaccine virus to pigs (Tamura et al., 2012). This suggested that the mutations found in E2 and NS4B of CSFV during this re-adaptation process may act synergistically to influence pathogenicity in pigs. Importantly also, NS4B of CSFV harbors a putative Toll/interleukin-1 receptor-like domain (Fernandez-Sainz et al., 2010). This domain is located closely to the NS4B mutations identified above. Mutations in this domain in the highly virulent "Brescia" backbone resulted in an attenuated virus, probably by affecting the modulation of the host immune response. NS4B carries also a nucleoside triphosphatase motif. This activity is essential for CSFV replication, but there is no evidence of involvement in virulence yet (Gladue et al., 2011).

Conclusions

Viral entry into and release from the target cells, speed of viral replication, and the ability of the virus to circumvent the host's immune responses are critical factors for viral disease outcome. The E2 protein is involved in viral attachment to the host cells and the NS4B protein is part of the replication complex. Hence, both proteins can be related to virulence and pathogenicity. But so far no universal sequence patterns determining virulence of CSFV have been reported. Instead, virulence of CSFV is more likely to be a multigenic feature determined by a complex interplay of several viral genes or genome regions acting in concert. However, viral attenuation can easily be obtained *in vitro* by introduction of mutations in highly conserved sequence patterns that are necessary for viral replication, as shown in various studies. Furthermore, the quasispecies composition might likely have only a minor impact on CSFV virulence.

Outlook

In former studies, highly conserved molecular sequence patterns essential for viral replication were considered as potential virulence factors. By mutation of these elements, attenuated ("defect") viruses were created that lack specific properties, leading to less virulent phenotypes. Most notably, virulence and

sequence differences between isolates were not taken into account. As a prerequisite for determination of virulence factors, CSFV isolates should be grouped into different classes of virulence/pathogenicity (e.g., avirulent, low to moderately virulent, and highly virulent). Characteristic and conserved sequence patterns within these groups should be used as a basis for future investigations in the field of CSFV virulence. Virulence studies should also consider modifying low virulent isolates by reverse genetics with the aim of generating viruses of higher virulence to demonstrate the role of defined genome sequence patterns in determining virulence. It is also of interest to investigate whether certain genetic determinants of CSFV virulence may be related to other *Flaviviridae* like West Nile fever virus, dengue virus, or hepatitis C virus.

Acknowledgments

This study was supported by the Swiss Federal Veterinary Office (BVET), Grant # 1.10.13.

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