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Intra-host variation structure of classical swine fever virus NS5B in relation to antiviral therapy

Andy Haegeman [a,*], Robert Vrancken [b], Johan Neyts [c], Frank Koenen [a]

[a] Veterinary and Agrochemical Research Centre (VAR), Groeselenberg 99, 1180 Brussels, Belgium
[b] Okapi Sciences NV, Ambachtenlaan 1, 3001 Heverlee, Belgium
[c] Rega Institute for Medical Research, University of Leuven (KU Leuven), Minderbroedersstraat 10, 3000 Leuven, Belgium

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ABSTRACT

Classical swine fever (CSF) is one of most important diseases of the Suidea with severe social economic consequences in case of outbreaks. Antivirals have been demonstrated, in recent publications, to be an interesting alternative method of fighting the disease. However, classical swine fever virus is an RNA virus which presents a challenge as intra-host variation and the error prone RNA dependent RNA polymerase (RdRp) could lead to the emergence/selection of resistant variants hampering further treatment. Therefore, it was the purpose of this study to investigate the intra-host variation of the RdRp gene, targeted by antivirals, in respect to antiviral treatment. Using the non-unique nucleotide changes, a limited intra-host variation was found in the wild type virus with 2 silent and 2 non-synonymous sites. This number shifted significantly when an antiviral resistant variant was analyzed. In total 22 nt changes were found resulting in 14 amino acid changes whereby each genome copy contained at least 2 amino-acid changes in the RdRp. Interestingly, the frequency of the mutations situated in close proximity to a region involved in antiviral resistance in CSFV and bovine viral diarrhea virus (BVDV) was elevated compared to the other mutations. None of the identified mutations in the resistant variant and which could potentially result in antiviral resistance was present in the wild type virus as a non-unique mutation. In view of the spectrum of mutations identified in the resistance associated region and that none of the resistance associated mutations reported for another strain of classical swine fever for the same antiviral were observed in the study, it can be suggested that multiple mutations confer resistance to some degree. Although the followed classical approach allowed the analysis the RdRp as a whole, the contribution of unique mutations to the intra-host variation could not be completely resolved. There was a significant difference in de number of unique mutations found between: 1/wild type virus and the antiviral resistant variant and 2/ between both and the number to be expected from the error rate of the RT-PCR process. This indicates that the some of the unique mutations contributed to the intra-host variation and that the antiviral pressure also shifted this pattern. This is important as one of the non-synonymous mutations found in the resistant variant and which was located in the antiviral resistance associated region, was present in the wild type virus as a unique mutation. The findings presented in this study not only show the importance of intra-host variation analysis but also warrants further research certainly in view of the potential inclusion of antivirals in a control/eradication strategy.

1. Introduction

Classical swine fever virus (CSFV) is a member of the *Flaviviridae* family, genus *Pestivirus* (Van Regenmortel et al., 2000). This single stranded RNA virus with a positive polarity is the causative agent of a highly contagious disease of the domestic pig as well as wild boar and has led to large epidemics in the European Union in the past decades (Greiser-Wilke et al., 2000) with severe socio-economic consequences (Meuwissen et al., 1999). The current European control strategy is based upon a "stamping out" policy (2001/89/EC) resulting in the slaughter and pre-emptive culling in the affected area. Notwithstanding the effectiveness of this approach, the public opinion is growing less tolerant regarding the slaughter of a large number of animals which are often uninfected and healthy (Le Potier et al., 2006; van Oirschot, 2003). The rising ethical concerns are even more pronounced in countries with a less favorable economic situation. Therefore it is not surprising that there is a demand for alternative control methods. Although vaccination can be viewed as an interesting alternative, the large scale application of the currently available vaccines is hampered by a number of reasons, such as the inability to serologically

* Corresponding author. Tel.: +32 02 379 06 44; fax: +32 02 379 06 66.
E-mail address: andy.haegeman@coda-cerva.be (A. Haegeman).

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differentiate vaccinated from infected pigs (live attenuated vaccines as C-strain), the reliability of the accompanying discriminatory ELISA test (subunit vaccines; Floegel-Niesmann, 2001) and the variable results in viral transmission studies (Ahrens et al., 2000; De Smit et al., 2001; Depner et al., 2001; Uttenthal et al., 2001). Even if these issues could be resolved by the next generation of vaccines (Beer et al., 2007), the time needed to establish sterile immunity following application (immunity gap) remains a potential problem. A second alternative is represented by the use of small selective and potent inhibitors of the viral replication cycle. One inherent property that makes them so attractive is their ability to act almost immediately following administration thereby closing the immunity gap (Collett, 2005). Although the use of antiviral agents is well established in human medicine, veterinary application is still in its early stages although more and more studies report their potential. Publications by Vrancken et al. (2008, 2009a,b), demonstrating the blocking of CSFV replication in vitro as well as the target species and the reduction in CSFV transmission by targeting the viral polymerase (NS5B) with the imidazo[4,5-c]pyridine derivative BPIP, showed the possibility of using antivirals in a control and eradication strategy for CSFV.

Notwithstanding these advantages, the emergence of antiviral resistance is an issue that needs to be addressed. This is even more important in view of the nature of CSFV, namely an RNA virus. It is well documented that viral RNA dependent RNA polymerases have a higher error rate than DNA polymerases due to the lack of proofreading activity (Ferrer-Orta et al., 2006; Friedberg et al., 2006), resulting in a higher mutation rate (Drake and Holland, 1999; Sanjuán et al., 2010). Although this provides the virus with an adaptive character, it also results in a higher genomic variability. The combination of a high mutation rate and an extremely large population resulted in the development of the quasispecies theory as originally proposed by Eigen (1971). Although the dynamics and true nature of quasispecies is still under debate (Holmes, 2010) and beyond the scope of this article, the presence of multiple viral variants in the same host is well documented. This diversity is important as it entails the potential presence of drug resistant variants (even if at a very low frequency) within a drug susceptible main population. In fact, the presence of such resistant variants against antivirals has already been demonstrated in human medicine (for example: Hedskog et al., 2010; Nájera et al., 1994), in particular also for Flaviviridae (Bartels et al., 2008; Kuntzen et al., 2008). The latter forms a significant challenge for designing antiviral therapies (Fanning, 2008) as the presence of natural occurring resistance mutations have been associated with non-responders to antiviral treatment in humans (Kurbanov et al., 2010; reviewed by Domingo and Gomez, 2007). Notwithstanding the fact that CSFV is reported as being relatively stable (Vanderhallen et al., 1999) some studies have reported the presence of intra-host variability (Leifer et al., 2010; Kiss et al., 1999).

With the development of next generation sequencing technology (NGS), the possibility to detect nucleotide differences at very low frequency has aided quasispecies/intra-host variation research (example; Rozera et al., 2009). A drawback of the current NGS technologies is the obtainable sequencing read length which is limited to around 500 bp. This means that for larger fragments (>500 bp) the linkage between amplicons is lost if no sequence markers, such as mutations, are present. Although new software packages are becoming available allowing contig assembly, it remains problematic in cases when the variability is not distributed relatively homogenously or when it is unknown (Prosperi et al., 2011). A heterogeneous genome population, such as intra-host variability, further complicates the problem and can result in an uncertainty of the true degree of gene variability. This information is valuable when investigating the emergence of resistance or the impact of antiviral treatment on the gene variant variability. The more classical approach of PCR combined with cloning has its own pitfalls such as the introduction of artificial variation due to polymerase errors and reduced detection sensitivity. One of the advantages of the classical approach is to analyze longer fragments and look at the gene variability as a whole. The latter is beneficial when looking at antivirals, resistance mutations and gene function as it allows the potential linking of different compensatory or synergistic mutations. Therefore, the classical approach was considered to be more suited for this study.

In view of the potential of the use of antivirals to control CSFV disease, the purpose of this study was to evaluate the intra-host variability of the NS5B gene of CSFV. Also, we wanted to determine whether previously reported resistance associated mutations in the viral polymerase (Vrancken et al., 2008; Paeshuyse et al., 2006, 2007, 2009) pre-exist in the wild-type virus population.

2. Materials and methods

2.1. Cells and viruses

A porcine kidney cell line (PK_{15}, CCL-33) was maintained in Minimal Eagle Medium (MEM) supplemented with 10% BVDV-free heat inactivated fetal calf serum (FCS), 0.25 μg/ml amphotericin, 50 μg/ml gentamicin, 1000 U/ml sodium benzyl penicillin and 2 mM Glutamine. The cells were kept at 37 °C in a CO_2-incubator (5% CO_2).

An isolate obtained originally during the 1993–94 CSFV outbreak in Belgium (isolate Wingene), later characterized to be similar to an isolate known as "souche Lorraine" (Koenen and Lefebvre, 1995) and belonging to genotype 2.3, was used for generating the antiviral resistant variant and to evaluate the intra-host variation of the isolate. The original isolate has been amplified twice on PK_{15}.

2.2. Antiviral compound

The synthesis of 5-[(4-Bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-c]pyridine (BPIP) is described by Puerstinger et al. (2006).

2.3. Reference sequence

RNA was purified from isolate Wingene (which was also used for generating $BPIP^r$ variants; see Section 2.5) using the RNeasy kit (Invitrogen, Merelbeke, Belgium) according to the manufacturer's recommendations. The RNA extracts were directly stored at −80 °C until further use. The cDNA synthesis mixture consisted of 10 mM DTT, 0.5 mM of each dNTP, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 80 pmol random hexamer, 10 U RNase OUT Inhibitor (Invitrogen, Merelbeke, Belgium), 100 U M-MLV (Invitrogen, Merelbeke, Belgium) to 4 μl extracted RNA in a total volume of 20 μl. The following thermal profile was used: one cycle of 37 °C for 1 h; one cycle of 65 °C for 10 min.

Primers and corresponding PCRs were designed in conserved regions of CSFV based upon a global alignment of publicly available CSFV sequences using ClustalW (Thompson et al., 1994). PCRs were designed to have at least 100 bp overlap in order to allow contig assembly. The amplified PCR fragments were purified using micro concentrators (Amicon Inc., Beverly MA, USA) according to the supplier's instructions. Sequencing was performed as described below. Primers and PCR conditions amplifying the rest of the isolate Wingene can be provided upon request.

2.4. Ns5b RT-PCR

The PCR was carried out in a total volume of 50 μl and consisted of 4 μl of RT-product, 5 μl High Fidelity buffer (10×), 2.5 mM

$MgSO_4$, 0.2 mM of each dNTP (Roche Applied Science, Vilvoorde, Belgium), 1 U Platinum High Fidelity Polymerase (Invitrogen, Merelbeke, Belgium). The amplification of the complete NS5B region was carried out by adding 33.75 pmol of a forward and reverse primer (5′-GAAATGATGAAAAGGGGGAAA-3′ and 5′-CAATGGGGTTCTACTGATAGGTC-3′; respectively) and by using the following thermal program: one cycle of 94 °C for 2 min; 35 cycles of 94 °C 30 s, 52 °C 30 s, 68 °C 2.5 min; one cycle of 68 °C for 10 min.

2.5. Cloning/plasmid preparation

Four μl of each purified PCR fragment were ligated into the pCR2.1-Topo vector using TOPO TA Cloning (Invitrogen, Merelbeke, Belgium). Following blue/white screening on X-gal containing kanamycin (50 μg/ml) LB plates, plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions. Insert verification was carried out by Eco RI digestion and gel electrophoresis.

2.6. Sequencing

The purified plasmids were sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City CA, USA). The sequence reactions were purified by precipitation with 80% ethanol and centrifugation at 12000*g* for 15 min at 4 °C. After washing with 70% ethanol the pellet was air-dried and dissolved subsequently in 25 μl template suppression reagent (Applied Biosystems Foster City CA, USA). Next, the purified product was denatured by incubation of 2 min at 94 °C and was subsequently analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City CA, USA). The obtained sequences were identified and compared with publicly available sequences and with the obtained reference sequence using the "blast" engine at "http://www.ncbi.nlm.nih.gov/BLAST/" (Tatusova and Madden, 1999). The complete fragment was reconstituted from the individual sequence file using a CAP contig assembly program (Huang, 1992).

2.7. Selection of BPIP-resistant Wingene (BPIP^r Wingene)

Three cultures of semi-confluent PK_{15} cells were infected for 1 h at 37 °C with 100 $TCID_{50}$ of the isolate Wingene, from a stock culture. Following trypsinization, each of the cell cultures were seeded in 25 cm^2 flasks containing growth medium supplemented with 1 μM BPIP and were subsequently incubated for 3 days at 37 °C. This sequence was repeated with a stepwise increase in BPIP concentration (up to 20 μM) and until BPIP-resistant virus was obtained. In total 17 passages (~50 days) were needed.

2.8. Antiviral assays

PK_{15} cells were seeded at a density of 5×10^3 per well in 96-well cell culture plates in MEM-supplemented with 10% heat-inactivated FCS and antibiotics (0.25 μg/ml amphotericin, 50 μg/ml gentamicin, 1000 U/ml sodium benzylpenicillin). Following 24 h incubation at 37 °C (5% CO_2) medium was removed and cultures in each well were infected with 100 $TCID_{50}$ of CSFV. After incubating for 1 h at 37 °C, cells were washed three times with PBS and fivefold serial dilutions of the antiviral compound were added in a total volume of 100 μl. Uninfected cells, cells receiving virus without compound and uninfected cells receiving the antiviral compound were included in each assay plate. After 3 days, medium was removed and cells were washed three times with PBS and left to dry at 37 °C. After fixation in a Pasteur oven for 1.5 h at 80 °C, cells were incubated with an in-house produced biotine conjugated polyclonal anti-CSFV serum for 1 h at room temperature, plates were then washed three times with PBS + 0.05% Tween20 after which they were incubated with streptavidine coupled peroxidase antibody (Amersham Biosciences Europe, Freiburg, Germany) for 1 h at rt. Following three wash steps, plates were incubated with detection buffer [4 mg 3-amino-9-ethylcarbazole dissolved in 1 ml N,N-dimethylformamide and 19 ml of 0.05 M acetate buffer (pH 5.2) containing 10 μl of 30% H_2O_2] for 15 min, until a dark red color appeared. The 50% effective concentration (EC_{50}) was defined as the concentration offering 50% protection of cultured cells against viral infection. The cytotoxicity was determined by the evaluation of the cell morphology by microscopic evaluation and CC_{50} values (=cytotoxic effects affecting 50% of uninfected cultured cells) were determined. Both the EC_{50} and CC_{50} values were calculated using the method of Reed and Muench (1938).

2.9. Statistical analysis

2.9.1. Cluster analysis

A pure temporal or spatial Bernoulli model was assumed to analyze the single mutations in the genome sequence. The analysis was performed in SATSCAN v9.1.1 (Kulldorff, 2006). The cases (single mutations) and non-cases were represented by a 1/0 variable. Fictive dates were assigned to each nucleotide of the whole sequence (01/01/2011 until 04/12/2017). The whole sequence (bp = 2530), including the single mutations (=1), was scanned for high rate clusters.

2.9.2. Error rates

The calculated error rates were compared to the data found in the literature and to each other. To this end a software package, WINPEPI, was used to compare the unbiased estimates (ODDS) for two proportions.

3. Results

3.1. Reference sequence

The complete genome of wild type Wingene, with the exception of the first and last 22 nt, was obtained following PCR amplifications, sequence analysis and contig assembly. Each nucleotide was at least sequenced twice with two different primers in order to reduce sequencing errors. The obtained sequence was deposited at GenBank (Accession Number: JQ595295) and was further used for developing sequencing primers. The PCR amplifying the complete NS5B region was further optimized for use in the intra-host variability study. The nucleotide and amino acid (aa) numbers listed in this study correspond to their position in this reference sequence.

3.2. Intra-host analysis of wild type Wingene

In order to reduce the impact of errors incorporated into PCR fragments by the RT and PCR amplification, each step was performed in multiple formats (three for RT and 10 for PCR) followed by pooling of the cDNA and PCR amplicons respectively. Using primers, flanking the NS5B genomic region, a 2530 bp fragment was amplified and subsequently cloned. In total 54 clones, containing the desired fragment, were selected and completely sequenced. Sequencing primers (Table 1) were chosen in order to provide significant sequence overlap and thereby providing additional verification.

In total 47% of the fragment was sequenced by two different primers. Following contig assembly of the individual sequences, the complete fragments of 54 clones were aligned and analyzed for nucleotide variation. Overall, 123 unique and 5 non-unique

Table 1
NS5B sequencing primers; ∗: In addition to the M13F and M13R primers provided in the Topo TA cloning kit.

Sequencing primers*	Type	Position
5′-GAGGTTGGTGCAAAATGT-3′	Forward	9863
5′-GGAGAGAGGAATAAACAGAAGG-3′	Forward	10532
5′-GACCTATCAGTAGAACCCCATTG-3′	Reverse	12075

Table 2
Summary of the non-unique mutations in the wild type Wingene population. (a): position in the NS5B protein (position in the polyprotein).

Nucleotide change	AA change	Genomic position	Protein position (a)	Freq (%)
T > C	Silent	10047	50 (3230)	14.6
A > G	N.D	10428	178 (3358)	3.6
G > A	S.N	10612	239 (3419)	23.6
C > T	Silent	10997	367 (3547)	3.6
A > G	3′UTR	12056		3.6

Table 3
Error frequencies.

Region	Length (bp)	Nr. Subst.
Single read regions		
1–375	375	21
689–1042	353	13
1165–1487	322	12
2233–2530	297	16
Total	1347 53.2%	62 50%
Two or more read regions		
376–688	312	25
1043–1164	120	9
1488–2233	751	28
Total	1186 46.8	62 50%

Table 4
Summary of previously published mutations linked to antiviral resistance to BPIP in pestiviruses.

Compound	Virus/isolate	Mutation	Reference
BPIP	CSFV Alfort 187	T259S	Vrancken et al. (2008)
	BVDV	F224S	Paeshuyse et al. (2006)
AG110	BVDV	E291G	Paeshuyse et al. (2007)
LZ37	BVDV	F224Y	Paeshuyse et al. (2009)

Fig. 1. Part of the BVDV NS5B crystal structure (Choi et al., 2006). The residues: (1) in blue: previously published mutations linked to antiviral resistance in pestiviruses (Table 4); (2) in green: residues which interatct with BPIP in addition to F224 (Vrancken et al., 2008); (3) in white: N178D and S239N mutations found in the wild type Wingene population; (4) in yellow: the Q12R, F225Y, R227C and R227H mutations found in the BPIP-resistant Wingene population.

substitutions were found. Two of five non-unique mutations resulted in an amino acid substitution (see Table 2). Subsequently, the impact on the data of the introduction of artificial errors due to the followed methodology was assessed. The introduction of errors by sequencing and sequencing analysis was first examined. No differences were observed in error frequency between regions which were sequenced with one (53%) or two (47%) primer sets (Table 3).

Next, the contribution of errors introduced by misincorporation during the RT-PCR process was assessed by looking at potential clustering of the 123 unique mutations. As misincorporation is a random process and the fact that there are no special features in the NS5B region which could result in an increase in polymerase errors, the distribution of the unique mutations should be random (Bracho et al., 1998). No evidence was found of clustering using the Statcan program suggesting their random nature. When, on the other hand, all 123 unique mutations are assumed polymerase errors, a polymerase error rate of 2.5×10^{-5}/nt/cycle is obtained. This obtained error rate is significantly different from the values reported in literature for the combined RT and PCR process ($2–8 \times 10^{-6}$; Malet et al., 2003) suggesting that not all unique mutations are caused by RT-PCR errors. The data are summarized in Table 6.

When the non-unique mutations were considered, the two mutations resulting in an amino acid change are the most likely to have an influence on the NS5B activity and where therefore localized on the published crystal structure of the BVDV NS5B (Choi et al., 2006). The BVDV crystal structure was used as the crystal structure for the CSFV polymerase is to our knowledge not available. Their position was subsequently compared on the protein to the position of known resistance mutations in CSFV strain Alfort and BVDV (Table 4, Fig 1). Neither mutations were situated in the vicinity of any up to date identified resistance mutations (Fig. 1).

3.3. Generation of BPIP^r-Wingene variants and variation analysis

In order to evaluate the spectrum of possible antiviral resistance mutations, BPIP-resistant variants were generated. In total, three independently selected cultures of BPIP^r viruses were obtained after 17 consecutive passages of the isolate Wingene in increasing concentrations of BPIP. All three obtained drug-resistant viruses were at least 15-fold less sensitive than the wild type virus to BPIP (EC_{50} of 24 ± 4.0 μM) as determined in an the antiviral assay.

As all three BPIP-resistant cultures proved equally resistant to BPIP, one culture was selected at random for variation analysis. In an identical approach to the wild type analysis, 47 transformants were selected and completely sequenced and compared.

In total 75 unique and 22 non unique (Table 5) nucleotide changes were identified. Similarly to the wild type population, the impact of induced errors by RT-PCR was analyzed. The obtained error rate was 1.76×10^{-5}/nt/cycle and again no clustering was found for the unique mutations (Table 6). The calculated error rate was significantly different from what is mentioned in the literature for RT-PCR induced errors suggesting that some unique mutations are not PCR errors. When the number of unique mutations found in the wild type Wingene ($n = 123$) is compared to that found in the BPIP-resistant Wingene population ($n = 75$), a significant difference was found. From the 22 non-unique mutations, 14

Table 5
Summary of the non-unique mutations in the BPIP-resistant Wingene (a): position in the NS5B protein (position in the polyprotein).

Nucleotide change	AA change	Genomic position	Protein position	Freq.
G > A	SILENT	9893	1 (3179)	4.2
A > G	SILENT	10151	85 (3265)	4.2
G > A	SILENT	10259	121 (3301)	4.2
A > G	Q > R	10267	124 (3304)	97.9
A > T	H > L	10273	126 (3306)	25
A > G	SILENT	10328	144 (3324)	4.2
A > G	SILENT	10343	149 (3329)	4.2
A > G	SILENT	10376	160 (3340)	4.2
C > T	T > I	10381	162 (3342)	20.8
T > A	F > Y	10570	225 (3405)	47.9
C > T	R > C	10575	227 (3407)	6.3
G > A	R > H	10576	227 (3407)	33.3
G > A	S > H	10612	239 (3419)	100
A > T	I > L	10755	287 (3467)	22.9
C > T	SILENT	10778	294 (3474)	100
A > G	K > E	11040	382 (3562)	4.2
C > A	A > D	11071	392 (3572)	4.2
G > C	V > L	11082	396 (3576)	6.3
A > G	D > G	11344	483 (3663)	6.3
A > G	E > G	11821	642 (3822)	4.2
A > G	SILENT	11903	669 (3849)	12.5
C > T	T > I	11929	678 (3858)	4.2

Table 6
Summary table. (a) based upon an error rate of $2–8 \times 10^{-6}$/nt/cycle and (b) an additional mutation was found in the 3′UTR.

	WT	BPIPr
Clones analyzed	54	47
Unique mutations	123	75
Expected number (a)	10–38	9–34
Non-unique mutations	4 (b)	22
Amino acid changes		
Total	2	14
Near BPIP binding pocket	0	5

of them resulted in an amino acid change with frequencies varying from 4% to 100%. Interestingly, the S239N mutation which was present in the wild type Wingene population with a frequency of 24%, was present in all the clones of the BPIP-resistant Wingene population. The F224S resistance mutation described in literature for BVDV is present in the BPIP-resistant Wingene population at a homologous position, i.e. F225Y, but with a frequency of only 47%. In contrast, none of the mutations described for the Alfort resistant variant (Table 4) were observed in the BPIP-resistant Wingene population. None of the observed non-unique mutations are present in the wild type Wingene population with the exception of an R to H mutation at position 227. The latter has a frequency of 33.3% in the BPIP-resistant population while it was a unique mutation in the wild type Wingene population. Interestingly, this mutation is in close proximity to the F225Y resistance associated mutation.

All of the 47 clones contained non-synonymous mutations (ranging from 2 to 5 mutations; with the majority having 4), indicating that each genome had undergone some nucleotide change with an effect on protein level. Following the mapping of all the non-synonymous mutations on the BVDV NS5B model, it was observed that a number of them were allocated to the BPIP binding pocket (Fig. 1; Q124R, H126L, F225Y, R227C, R227H, A392D, V396L), the region previously associated with BPIP resistance (Paeshuyse et al., 2006; Vrancken et al., 2008). Interestingly, all the clones analyzed contained a least 1 mutation (with a maximum of 3) in that particular region.

4. Discussion

Antiviral therapy for controlling CSFV is an attractive alternative to the current approach of stamping-out. An inherent risk factor, however, of such a therapy is the emergence of drug resistant variants. Most RNA viruses are known to have a high mutation rate, hence drug-resistant variants may develop rapidly. The evolutionary dynamics of RNA viruses are complex and differ from that of traditional population genetics. Although the quasispecies model/evolution is still under debate (Comas et al., 2005; Holmes, 2003, 2010; Holmes and Moya, 2002; Jenkins et al., 2001; Más et al., 2010; Wilke, 2005) and is not the focus of this article, intra-host variation, certainly in RNA viruses has been well established. Alternatively, the high mutation rate associated with RNA viruses frequently interferes with therapeutic intervention due to acquisition of new mutations and leads to resistance (Gerrish and García-Lerma, 2003). The aim of this study was therefore to investigate for the first time the presence of antiviral resistance associated mutations in a wild type CSFV population.

The classical approach followed to demonstrate intra-host variation has some inherent drawbacks, which we addressed in this study. Firstly, the overestimation of the true degree of variability due to the introduction of artificial mutations linked to the reverse transcription and polymerase amplification process (Malet et al., 2003; Mullan et al., 2004) was limited by the use of a high fidelity polymerase and the PCR set-up (multiple format and pooling). In addition, no differences were seen between single and multiple read regions indicating that the impact of "artificial" mutations due to the sequencing and analysis is minimal. A possible overestimation was further reduced by considering only the non-unique nucleotide (nt) changes as mutations. However, this resulted probably in an underestimation as the calculated error rates of the RT-PCR process, using the unique nt changes, in both groups is significantly higher than reported in the literature (Malet et al., 2003). This suggests that not all unique nt changes are RT-PCR errors. This is further substantiated by the fact that the calculated error rate of the drug resistant population differs significantly from that of the wild type population. Antiviral pressure with BPIP clearly resulted in the elimination of a number of the unique nt changes found in the wild type population. Secondly, the limited sequence depth of the classical approach whereby variants with very low frequency could be missed cannot be completely resolved. However, Gretch and Polyak (1997) showed that the analysis of 20 clones gives a confidence of 95% to find all major variants (frequency of at least 10%). Similarly, Gao et al. (2005) showed that 40 clones gave the best cost/information content outcome regarding quasispecies using the classical approach. Thus, although the number of clones analyzed in this study does not give the depth of NGS, it provides nevertheless a very good picture of the intra-host variability of CSFV. Although the impact of mutations linked to in vitro-passaging cannot be 100% excluded, it is probably very limited as: (i) /previous work carried out in our institute showed that extensive passaging (in vitro and in vivo) or the application of an artificial bottleneck did not result in any change in the NS5B genomic sequence (Vanderhallen et al., 1999); (ii) /the specific design of generating the drug resistant variant (increasing antiviral concentration at each cell culture passage) assured that the main selection pressure is caused by the antiviral and not by the cell culture passage.

The presence of intra-host variability in the NS5B region identified during this in vitro study in wild type Wingene population confirms the data of Leifer et al. (2010). The variability remained limited to 2 silent and 2 amino acid (aa) changes. In contrast, the intra-host variation identified in the BPIP resistant population is significantly more complex with 22 nt changes (of which 14 were

non-synonymous) on the genomic level. Not only the complexity is higher, there is a clear difference in the variant frequency in the BPIP resistant population between synonymous and non-synonymous changes. With the exception of C to T nt change at position 10778, all silent mutations in the BPIP-resistant population are present at a very low frequency. This can be expected as they do not provide an advantage for the virus. Therefore, it is not clear why the C10778T is present in 100% of the resistant clones. The latter could be the result of a linkage with a resistance mutation during antiviral selection. Although, the frequencies of the non-synonymous mutations in the BPIP-resistant population are variable, those in close proximity to the region identified in CSFV (Alfort187; Vrancken et al., 2008) and BVDV (Paeshuyse et al., 2006) as linked with BPIP resistance, are significantly higher, being 97% (Q124R), 25% (H126L), 47% (F225Y), 40% (R227H; R227C) compared to those situated elsewhere. The F225Y mutation is interesting as this is situated next to F244 which is the amino acid linked to antiviral resistance in BVDV, making it highly likely that F225Y is also involved in drug resistance. On the other hand, this mutation is only present in 47% of the population meaning that other mutations are involved. This is also confirmed by the fact that the T259S mutation seen in a BIBP-resistant population of Alfort187 (Vrancken et al., 2008) is not present in the BPIP-resistant Wingene population. The proposed resistance mechanism for BPIP as described by Vrancken et al. (2008) is based upon the decreased stacking interactions of the BPIP molecule with F224. However, hydrophobic interactions were also found between the BPIP molecule and residues in the vicinity of F224, namely A221, A222, G223, Y289 and A392 (Paeshuyse et al., 2006). Mutations in close proximity to these residues can be regarded as potential candidates as they can influence the stacking and/or hydrophobic interactions between BPIP and the NS5B protein by means of steric hindrance of the altered amino acid side chains. For this reason the Q124R, H126L, R227H and R227C mutations, directly located next to or in close proximity to the F224 (based on the 3D NS5B model of BVDV), need to be investigated. The main candidate would be the Q124R mutation as all but one clone contained this mutation (97.5%). This is even more remarkable as this residue is highly conserved within CSFV and even BVDV as are the residues at position 227 and 225. The fact that more than one mutation is responsible for the observed resistant phenotype in this study can be linked to the method by which the resistant population was generated. The gradual increase of the antiviral concentration could lead to the emergence (or selection) of a mutation resulting in resistance to the current concentration, thereby becoming the major variant, but not to the next concentrations. Consecutive passages with higher concentrations would then result in the emergence of new mutations whereby the previous major variant becomes a minor variant (Charpentier et al., 2004; Más et al., 2010). The passages in this study were stopped when based upon the antiviral assay the virus was found to be resistant to BPIP. No additional passages were performed with the maximal antiviral concentration. Therefore, the population was probably not yet in equilibrium. This could possibly have resulted in the retention of the early resistance mutations in the population.

One of the objectives of this study was to investigate the potential presence of antiviral associated mutations in a wild type population. Interestingly, one of the two mutations (S239N) identified in the wild type Wingene strain was also present in the BPIP-resistant Wingene population. A positive selection for this mutation was observed as its frequency increased to 100% in the BPIP-resistant population. Although this might suggest the involvement of the S239N in the resistant phenotype, this was found to be unlikely because of two reasons. First, in several other CSFV strains, such as strain Alfort 187, an N is already present in the wild type virus at this position and Alfort 187 is similarly inhibited by BPIP (Vrancken et al., 2008). Second, this mutation is not situated in a region of the polymerase that was shown to be involved with antiviral resistance to this class of compounds (Paeshuyse et al., 2006, 2007, 2009; Vrancken et al., 2008) following 3D mapping on the BVDV NS5B crystal structure. Another important finding was the R227H variant in the BPIP-resistant group. This was the only mutation in the BPIP resistant group which was already present in the wild type population. However, the mutation was present as a unique nt change in the wild type population which means that it could not be excluded as an RT-PCR error.

In conclusion, intra-host variability was observed in the wild type Wingene population, which shifted significantly when antiviral pressure was applied. The mutations in the BPIP-resistant Wingene variant suggested that more than one mutation was responsible for the observed resistant phenotype. Although no clear evidence was found for the presence of published resistance associated mutations in the wild type Wingene population, the finding of the R227H mutation in the wild type and BPIP-resistant Wingene population warrants further study as this mutation was mapped to a resistance associated region of NS5B. These findings clearly underline the importance of quasispecies/intra-host variation research in the framework of future antiviral therapy.

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Glossary

BBIP: 5-[(4-Bromophenyl)methyl]-2-phenyl-5H-imidazo[4,5-c]pyridine
BPIP^r: BPIP resistant virus (or variant)
CSFV: Classical swine fever virus
NS5B: Non-structural protein 5B, the viral polymerase
RT-PCR: Reverse transcription polymerase chain reaction
$TCID_{50}$: 50% Tissue culture infectious dose.