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Development of an improved live attenuated antigenic marker CSF vaccine strain candidate with an increased genetic stability

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ABSTRACT

Controlling classical swine fever (CSF) involves vaccination in endemic regions and preemptive slaughter of infected swine herds during epidemics. Live attenuated marker vaccines that confer effective protection against the disease and allow differentiation between infected and vaccinated animals (DIVA) could impact CSF control policies. Previously, we reported the development of FlagT4 virus (FlagT4v), a rationally designed live attenuated marker vaccine. During its vaccine assessment, FlagT4v reverted to a virulent virus during successive passages in piglets. Sequence analysis revealed deletions and substitutions almost exclusively in the areas of E1 and E2. To improve genetic stability of FlagT4v, we introduced changes in the codon usage in those areas. The newly developed virus, FlagT4Gv, was shown to retain the attenuated phenotype after successive passages in piglets. As observed with FlagT4v, the newly developed FlagT4Gv conferred effective protection against challenge with virulent CSFV at early (7 days) and at late (28 days) times post-vaccination.

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Introduction

Classical swine fever (CSF) is a highly contagious disease of swine. The etiological agent, CSF virus (CSFV), is an enveloped virus with a positive-sense, single-stranded RNA genome, classified as a member of the genus *Pestivirus* within the family *Flaviviridae* (Becher et al., 2003). The 12.5 kb CSFV genome contains a single open reading frame that encodes a 3898-amino-acid polyprotein and ultimately yields 11–12 final cleavage products (NH_2–Npro–C–E^{rns}–E1–E2-p7–NS2–NS3–NS4A–NS4B–NS5A–NS5B–COOH) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Rice, 1996). Structural components of the CSF virion include the Core (C) protein and glycoproteins E^{rns}, E1 and E2. E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor; E^{rns} loosely associates with the viral envelope (Thiel et al., 1991; Weiland et al., 1990; 1999). E2 is the most immunogenic of the CSFV glycoproteins (Konig et al., 1995; van Gennip et al., 2000; Weiland et al., 1990), inducing neutralizing antibodies that provide protection against lethal CSFV challenge.

The two main policies used for CSFV control are prophylactic vaccination or non-vaccination with "stamping out" of exposed animals during an outbreak. Countries considered free of CSF do not recommend the use of currently available live attenuated viruses (LAVs) as tools to control outbreaks of the disease, despite the proven efficacy of these vaccines in eliciting a rapid and solid protection against the virus (van Oirschot, 2003). The humoral immune response induced by these vaccines does not differ from that elicited by infections caused by wild-type viruses; hence, the use of CSFV LAVs has been hampered by their inability to induce a response that differentiates infected and vaccinated animals (i.e., DIVA capability). The use of a CSFV LAV with DIVA capabilities could significantly impact policies of disease control.

We have previously reported (Holinka et al., 2009) the development of a CSFV double antigenic marker LAV strain, FlagT4v. This LAV contains an inserted synthetic epitope, Flag® (Sigma, St. Louis, MO), that serves as a positive antigenic marker, as well as a negative antigenic marker characterized by the abolition of a highly conserved CSFV-specific epitope recognized by monoclonal

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antibody WH303 (Edwards et al., 1991) where a series of 6 amino acid substitutions were introduced into glycoprotein E2 (mutation T4) causing loss of WH303 reactivity. Immunization with FlagT4v induced complete protection against challenge with virulent CSFV Brescia (as Brescia virus, we use BICv, a virus derived from and infectious clone containing the full length genome of Brescia strain), even within the first week of vaccination, and serological responses against both the positive (Flag) and negative (mAb WH303) antigenic markers allowed the discrimination of vaccinated from infected animals (Holinka et al., 2009).

During the assessment of FlagT4v as a candidate vaccine strain, reversion to a virulent phenotype was observed. The genomic sequence of the revertant virus contained deletions and mutations primarily in the areas encoding for the Flag insertion in glycoprotein E1 (Holinka et al., 2009) and in the T4 mutation in E2 (T4) (Risatti et al., 2006) when compared with FlagT4v. A new virus, FlagT4Gv, was constructed with improved genetic stability relative to FlagT4v. This virus contains changes in the codon usage within the Flag and T4 genomic regions, with RNA sequences diverging as much as possible from the original FlagT4v sequence. FlagT4Gv was shown to be stably attenuated when assessed in reversion to virulence experiments using highly susceptible 5–6 week old piglets. In addition, FlagT4Gv was as efficient as FlagT4v in protecting swine against challenge with CSFV Brescia at early (7 days) and at late (28 days) times post-vaccination.

Results

Genetic modifications observed in FlagT4v during the reversion to virulence study

To assess the genetic stability and attenuated phenotype of FlagT4v, it was tested in a conventional five passage reversion to virulence experiment utilizing IM inoculation of piglets with pooled virus-positive tonsils obtained from the previous passage (see Materials and methods). During the first three passages inoculated piglets did not demonstrate clinical signs of CSF while at passage number 4, all animals presented a transient rise in body temperatures, although none of these piglets had additional CSF-related clinical signs. At passage number 5, two of the five piglets presented severe CSF-related clinical signs and were euthanized, while the other three piglets presented with a transient increase in body temperature, appeared depressed exhibiting limited CSF-related signs (data not shown). Thus, attenuated FlagT4v reverted to a virulent phenotype after successive back passages in susceptible piglets.

To identify the genetic changes in the FlagT4v genome that led to reversion to virulence, full genome sequencing of the virus isolated from the spleen of one of the euthanized piglets (FlagT4SPv) was performed. The obtained sequence was then compared to parental (attenuated) FlagT4v (Table 1). Compared to FlagT4v, the revertant FlagT4SPv had three amino acid substitutions, a deletion of a stretch of 18 amino acids, and a nucleotide deletion (Table 1). FlagT4SPv harbors mutations that resulted in a A109V substitution in the Core structural protein and N850S/A993E substitutions in glycoprotein E2. Interestingly, N850 in FlagT4v is located in the center of the T4 area, a series of 6 amino acid substitutions introduced into BICv (specifically S832 in Brescia strain) that causes the loss of reactivity to monoclonal antibody WH303 (constituting the negative antigenic marker of FlagT4v) along with attenuation of T4v in swine (Risatti et al., 2006). All amino acid substitutions observed in FlagT4SPv were caused by single nucleotide mutations: nucleotide C687T resulted in the A109V substitution, nucleotide A2910G resulted in the N850S substitution, and nucleotide C3339A resulted in the A993E substitution (Table 1).

FlagT4SPv also harbors a deletion that comprises the entire insert situated at the carboxyl-end of glycoprotein E1 of FlagT4v, between residues 688–706 of the BICv polypeptide encoding for the Flag epitope. This 19 residue insert was introduced into the backbone of BICv by transposon insertion mutagenesis (Risatti et al., 2005b) and further modified to harbor the Flag epitope in a 18mer insert (Holinka et al., 2009). Finally, a deletion of a cytosine was observed at position 12342 within the 3′UTR of FlagT4SPv (Table 1).

Table 1
Amino acid and nucleotide changes observed in revertant FlagT4Sv.

Position changed	CSFV genomic area
Substitution residue 109 (A–V), nucleotide C687T	N^{Pro}
Deletion residues 688–706, nucleotides 2424–2477	E1
Substitution residue 850 (N–S), nucleotide A2910G	E2
Silent mutation residue 861, nucleotide A2944G	E2
Silent mutation residue 866, nucleotide A2960C	E2
Substitution residue 993 (A–E), nucleotide C3339A	E2
Silent mutation residue 1360, nucleotide T4507C	NS23
Silent mutation residue, nucleotide A8170G	NS4B
Silent mutation residue, nucleotide A10177G	NS5B
Deletion nucleotide C-12342	3′UTR

Identification of FlagT4v mutations leading to virulence in FlagT4Sv

To assess the individual contribution of each mutation to the virulence of revertant virus FlagT4Sv, a set of recombinant viruses were constructed that harbor each of the observed genetic modifications (Table 2). Thus, six different mutant viruses were developed: FlagT4v harboring the A109V substitution (FlagT4A109Vv), FlagT4v harboring the N850S substitution (FlagT4N850Sv), FlagT4v harboring the A993E substitution (FlagT4A993Ev), T4v (a FlagT4v lacking the Flag insertion) harboring the A109V substitution (T4A109Vv), T4v harboring the N850S substitution (T4N850Sv), and T4v harboring the A993E substitution (T4A993Ev). Each of these recombinant viruses was assessed for their virulence in swine. Groups of four 40–60 lbs pigs were inoculated IM with 10^5 $TCID_{50}$ of each virus and the presence of CSF-related clinical signs was recorded throughout the observation period (21 days). Animals inoculated with these recombinant viruses remained clinically normal with the exception of the group of pigs inoculated with recombinant T4N850Sv which presented a clinical disease that was indistinguishable from that observed in animals infected with virulent BICv (Table 2). These results indicate that deletion of the synthetic Flag insertion within the E1 glycoprotein, along with the amino acid substitution N850S in the E2 glycoprotein,

Table 2
Swine survival and fever response following infection with FlagT4 and T4 recombinant viruses containing individual revertant mutations observed in FlagT4S virus.

Virus	No. of survivors/ total no.	Mean time to death (days ± SD[b])	Fever	
			No. of days to onset (days ± SD)	Duration (days ± SD)
BICv	0/4	9.76 (0.95)	4 (0.0)	0.75 (0.5)
FlagT4A109V	4/4	–	–	–
FlagT4N850S	4/4	–	–	–
FlagT4A993E	4/4	–	–	–
T4A109V	4/4	–	–	–
T4N850S	0/4	11.25 (0.96)	3.5 (0.5)	7.75 (2.1)
T4A993E	4/4	–	–	–