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Brief Communication

Classical Swine Fever Virus: Discrimination Between Vaccine Strains and European Field Viruses by Restriction Endonuclease Cleavage of PCR Amplicons

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Classical swine fever virus (CSFV; alias hog cholera virus; HCV) belongs to *Pestivirus* genus of the *Flaviviridae* family, together with bovine viral diarrhoea virus and border disease virus (*Wengler* 1991). Classical swine fever (CSF) is a highly contagious disease, which affects both domestic swine and wild boars. The disease is characterized by high fever and multiple hemorrhages, but it is frequently associated with very mild or unapparent clinical signs (*Van Oirschot* 1986). CSF is enzootic in most countries of Continental Europe, South America and the Far East. The control and eradication programmes are based on the restricted policy for movement of swine, stamping out of CSFV positive and suspicious pigs and testing of wild boars as potential reservoirs of the virus.

Although there are very potent vaccines against CSFV, many countries prohibit their application. The ban is due to the fact that by current techniques it is impossible to differentiate the antibodies raised against a vaccine from those produced against field viruses. Thus, serological screening, the common method of CSF eradication programmes, becomes useless if vaccination is practiced in the region. In addition, it has been observed that vaccine strains are able to spread from vaccinated to non-vaccinated animals (*Terpstra & Tielen* 1976, *Shimizu* 1980) and to circulate in the pig population, creating further problems for the control programmes. The use of novel vaccines, e.g., subunit vaccines expressing selected viral proteins, like E1 or E2, or the application of genetically modified live virus marker vaccines will hopefully circumwent these problems. However, the majority of the vaccines commercially available today are still the conventional live attenuated vaccines.

Considering the serious disadvantages and risk factors, the use of conventional CSFV vaccines is not allowed in the European Union. However, certain other European countries occasionally practice forced vaccinations and even the illegal use of vaccines is a possible hazard. Regarding these factors, there is a potential danger that vaccine strains can spread to the swine populations of the European Union, causing situations of serious epizootiological and economical importance.

Since the serological tests are not suitable to discriminate antibodies raised against wild viruses from those produced against vaccine

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Table 1. European field isolates and vaccine strains of CSFV analysed in this study.

Virus strain	Country of origin	Year of isolation	*Bbr* PI cleavage site in 5'NC region
A. European field isolates			
Zoelen	The Netherlands	1977	-
Bergen	The Netherlands	1977	-
Osterode	Germany	1982	-
D 4990	Italy	1983	-
D 5020	Italy	1983	-
D 5021	Italy	1983	-
Atzbull	Germany	1984	-
V694	Germany	1986	-
V744	Germany	1986	-
V750	Germany	1986	-
SF 10/86	United Kingdom	1986	-
SF 11/86	United Kingdom	1986	-
SF 32/86	United Kingdom	1986	-
Bas Rhine	France	1986	-
1185	Belgium	1986	-
1825	Belgium	1986	-
4H	Hungary	90ties	-
10H	Hungary	90ties	-
C3D	Italy	1990	-
S7D	Italy	1991	-
23326/94	Slovakia	1993	-
35577/94	Slovakia	1993	-
719/94	Poland	1994	-
B. Vaccine strains			
Thiverval	France		+
Egermann Riems	Germany		+
Suvac	Hungary		+
Cellpest	Poland		+
Vaccine USSR	Russia		+
TVM	Czech Republik		+
GPE	Japan		+
Norden	Mexico		+
Minnesota Anchor	Mexico		+
Colvasan Sanycon	Mexico		+
Porcivac	Mexico		+

strains, the rapid isolation and identification of the spreading virus, as a vaccine strain, would be an essential requirement. Nevertheless, no practical technique is available today for the rapid identification of the vaccine strains. The differentiation between CSFV vaccine strains and field viruses was attempted by biological methods (*Aynaud et al.* 1971, *Biront & Leunen* 1988), while the discrimination of vaccine strain C was achieved by using monoclonal antibodies (*Wensvoort et al.* 1986). These conventional techniques are laborious and time consuming.

The aim of the present study was to develop a

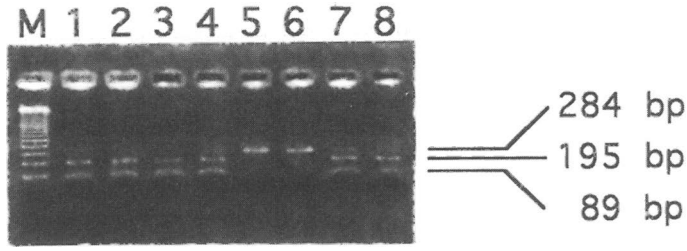

Figure 1. Cleavage of PCR products by restriction endonuclease *Bbr* PI. The 284 bp products were generated from the 5'-NC region of CSFV strains, by using 324/326 primers.
Lines: 1, Suvac vaccine: 2, Thiverval vaccine; 3, GPE-vaccine, 4, TVM vaccine; 5, D 4990 field strain (Italy, 1983); 6, Bergen, field strain (The Netherlands, 1977); 7, Egermann Riems vaccine; 8, Porcivac vaccine; M, 100 bp ladder (Gibco, BRL).

practical approach for discrimination between vaccine strains and recent field viruses in Europe. Our theory was based on previous phylogenetic studies, which revealed that vaccine and recent European field isolates formed different genogroups (*Hofmann et al.* 1994, *Lowings et al.* 1996, *Stadejek et al.* 1996, *Vilček et al.* 1996). Considering the genetic differences, we developed an assay, which is based on the amplification of a selected part of the 5'-non coding (5'-NC) region of the CSFV genome, followed by selective restriction endonuclease cleavage of the amplified DNA fragments.

Altogether 13 vaccine strains and 23 European field viruses were tested in this study, as listed in Table 1.

The procedures of RNA isolation by proteinase K digestion, followed by phenol/chloroform/ treatments, synthesis of cDNA with random primers and Moloney murine leukemia virus reverse transcriptase, as well as the PCR of a 284 bp fragment of the CSFV 5'-NC genome region with general pestivirus primers 324 and 326, were described in a previous publication (*Vilček et al.* 1994).

The PCR products (0,5-1,0 μg DNA) were cleaved with restriction endonuclease Bbr PI (5 U; Boehringer Mannheim, Germany) in a reaction volume 20 μl, according to manufacturers' instruction. Ten µl amounts of the cleaved DNA were run in 2% agarose gels and visualized by UV light.

The general pestivirus primers 324/326 consistently amplified a 284 bp DNA fragment from 11 of the 13 vaccine strains tested, as well as from all 23 European field strains of CSFV.

The restriction endonuclease cleavage with *Bbr* PI allowed the discrimination between vaccine strains and field isolates (Table 1). The PCR products, generated from 11 vaccine strains marketed in Europe, America and Asia, were all cleaved by *Bbr* PI, yielding 2 characteristic fragments of 195 bp and 89 bp in length, respectively (representatives are shown in Fig. 1, lines 1 to 4, 7, 8). In contrast, the PCR products generated from 23 European field strains of CSFV, collected from 9 countries over the period of 1977-1994, were not cleaved by *Bbr* PI (representatives illustrated in Fig. 1, lines 5, 6).

Our concept to discriminate vaccine strains from European field isolates was obtained from the findings of previous studies on the genetic heterogeneity of CSFV strains. Phylogenetic analysis of the virus genome in the 5'-NC (*Hofmann et al.* 1994; *Stadejek et al.* 1996) and gp55 regions (*Lowings et al.* 1996, *Vilček et al.*

AAATCACACCACGTGATGGG	Consensus
.....	*Vac A (Sal)[a]
.....	*Vac B (US)[a]
.....	*Vac C (Mex)[a]
.....	*PS Porco (Brasil)[c]
.....	*Pestiffa[b]
.....	*Riems[b]
.....	Austria 6639-90[b]
.....TA.T.....	Austria 6879-90[b]
.....A.T.....	France D 1047[b]
.....T.....	Hannover 633/90[b]
.....TT.T.....	Switzerland 1-93[b]
.....TT.T.....	Italy D 4990-83[c]
.....TT.T.....	Netherland, Bergen[c]
.....TT.T.....	Slovakia 9201-94[c]

Figure 2. Nucleotide sequence alignment from the 5'-NC region of the vaccine and field CSFV strains (positions in the genome of CSFV strain Alfort: 273-292). The *Bbr* PI restriction site is underlined.
*Vaccine strains; [a]*Stadejek et al.* 1996; [b]*Hofmann et al.* 1994; [c]*Vilček and Belák,* unpublished results.

1996) revealed that all tested vaccine strains were grouped with old and recent American, Asian isolates, as well as with old European isolates from the fifties, while recent European isolates formed a separate genogroup. These data predicted that the CSFV strains, used for the preparation of vaccines all over the world, were most probably evolving from an ancestor node different from field strains circulating recently in Europe (*Vilček et al.* 1996).

Computer-assisted analysis of published nucleotide sequences of vaccine strains showed that a part of the 5'-NC region, flanked by primers 324 and 326, contains a *Bbr* PI recognition site (CAC GTG). In contrast, the 5'-NC genomic regions of recent European field isolates did not retain this sequence (*Hofmann et al.* 1994, *Stadejek et al.* 1996; see also Fig. 2). We have also found the unique *Bbr* PI recognition site at the same position of the 5'-noncoding region by analyzing the published sequences of the Chinese C strain (*Moormann et al.* 1996) and ALD strain (*Ishikawa et al.* 1995), the ancestor strains of many CSFV vaccines.

Interestingly, *Hofmann et al.* (1994) characterized an Austrian strain (Austria 6639-90; isolated in 1990), which phylogenetically fell into a group of old European CSFV isolates and vaccine strains. As it is illustrated in Fig. 2, this strain contains the *Bbr* PI restriction site. The occurrence of the Bbr PI cleavage site supports the phylogenetic study of *Hofmann et al.* (1994) and indicates that Austria 6639-90 strain is most likely a vaccine virus of unknown origin, which was spreading in the Austrian pig populations. By comparing a 116 nucleotide long section from the 5'NC regions of Austria 6639-90, ALD and Chinese C strains we found complete homology between these strains. This finding supports the concept, that Austria 6639-90 is most likely a strain of vaccine origin (Fig. 3).

The restriction enzyme cleavage experiments of the present study confirmed the theoretical expectations, since each amplicon, obtained from vaccine strains, was cleaved by *Bbr* PI. In contrast, none of the amplified fragments of recent European field isolates was cut by this enzyme. The results indicate that the *Bbr* PI cleavage of the 5'NC amplicons presents a simple and rapid approach of genetic discrimination between vaccine strains and recent wild variants of CSFV in Europe. Regarding CSFV variants occurring on other continents, we have not found sequence differences in the viral genomes, which would allow such discrimination. But in Europe all the 23 tested recent field virus isolates were similarly lacking the *Bbr* PI recognition site in the 5'NC region, and this genetic homogeneity indicates a good confidence for this approach as a diagnostic tool. It has to be mentioned that the PCR used in these studies will not only amplify a fragment from CSFV but also from BVDV and BDV. According to the article of *Hofmann et al.* (1994), none of these fragments would be cut by *Bbr* PI, thus the discrimination of wild type CSFV from BVDV or BDV would not be possible by using the present

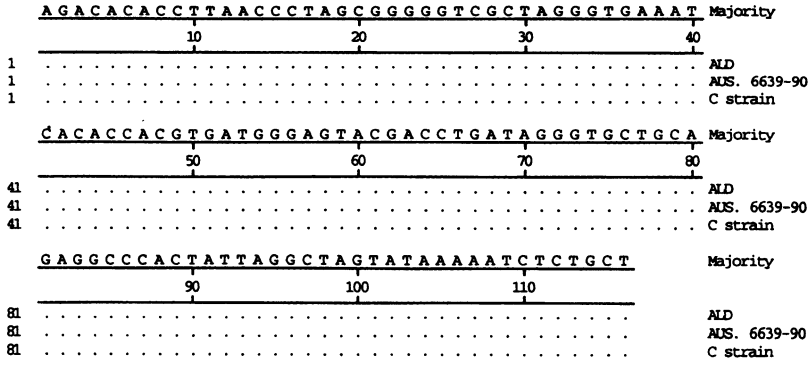

Figure 3. Alignment of 116 nucleotides in the 5'NC region of CSFV strains Austria 6639-90 (*Hofmann et al.* 1994) , ALD (*Ishikawa et al.* 1995) and Chinese C (*Moormann et al.* 1996).

approach. However, the goal of the present study was restricted to differentiate vaccine and wild type strains within CSFV. For differentiation of various pestiviruses, various further approaches have been developed (*Vilček et al.* 1994, 1996, *Lowings et al.* 1996, *Stadejek et al.* 1996).

In conclusion, the present approach provides simple and practical means for the identification of attenuated vaccine strains of CSFV in Europe. However, we have to consider the following risk factors: i) new CSFV strains appear in Europe with Bbr PI recognition site in 5'NC region; ii) new vaccines are developed from recent European strains. The emergence of new genetic variants is a permanent risk, not only from the diagnostic point of view, but also from other aspects of epizootiology. Nevertheless, regarding the *Bbr* PI recognition site in the 5'NC region, the isolates obtained from 9 countries between 1977 and 1994 showed strong evolutionary conservation in this part of the genome. The second hazard, i.e., the appearance of new CSFV vaccines prepared from recent European strains, is very unlikely, considering the above mentioned situation on the continent today.

By keeping all these aspects in evidence, we can regard the assay presented here as a novel method of choice to discriminate vaccine strains and wild viruses in Europe today. Compared to conventional methods of discrimination, the advantage of the new approach is that it does not require cultivation of viruses in cell cultures. The PCR can be performed directly even on clinical specimens (not shown). Furthermore, by this method the virus variants are identified within 1 or 2 days, in contrast to virus isolation, which requires longer time and may yield uncertain results.

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