An infectious cDNA clone of a highly pathogenic porcine reproductive and respiratory syndrome virus variant associated with porcine high fever syndrome

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Since May 2006, a so-called ‘porcine high fever syndrome’ (PHFS) has spread all over China. The arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) was believed to be the main causative agent, although the involvement of other pathogens was not formally excluded. The genome of a representative Chinese PRRSV strain, named JX143, was sequenced and used to develop infectious cDNA clones, pJX143 and pJX143M, with the latter containing an engineered Mlu I site that served as a genetic marker. In various virological assays, the rescued viruses, vJX143 and vJX143M, were indistinguishable from their parental virus. Animal experiments showed that these recombinant viruses retained the high pathogenicity and induced the typical clinical symptoms observed during PHFS outbreaks. This is the first report describing infectious cDNA clones of this highly pathogenic PRRSV. Our results unambiguously fulfil Koch’s postulates and define highly pathogenic PRRSV as the aetiological agent of PHFS in China.

In the last two years, an epidemic of what is locally called ‘porcine high fever syndrome’ (PHFS) has spread over the Chinese swine industry, resulting in the culling of an estimated 20 million pigs annually. Infected animals suffer from laboured breath, pyrexia, lethargy, anorexia and reproductive failure. Furthermore, the mortality rate of infected piglets is alarmingly high. Following extensive epidemiological investigations, PRRSV was suspected to be the causative agent of the PHFS epidemic (Tian et al., 2007). However, it remained to be further investigated whether PRRSV is the sole aetiologic agent of the disease, since other pathogens like classical swine fever virus (CSFV) and type 2 porcine circovirus (PCV2) were occasionally isolated from PHFS cases (Li et al., 2007; Ning et al., 2006). In this study, we have therefore characterized a PRRSV strain isolated from a typical PHFS case and have investigated its possible role in the PHFS outbreak.

The PRRSV strain used in this study, named PRRSV JX143 strain, was isolated from the serum of a dying piglet displaying the clinical signs of PHFS. The third virus passage (P3) from MA-104 cells was used in the present study. Specific primer pairs (Supplementary Table S1, available in JGV online) were used for RT-PCR amplification and sequence analysis of the PRRSV JX143 genome. Primers were based on genomic sequences (GenBank accession nos: AF184212; DQ176021) of the prototypic type II PRRSV. Viral RNA purification, RT-PCR, and nucleotide sequencing were carried out as previously described (Yuan & Wei, 2008) and the full-length genome sequence was assembled with the Lasergene package (DNASTAR). The genomic RNA of PRRSV JX143 turned out to be 15 320 nt in length, excluding the poly(A) tail (GenBank accession no. EU708726). As summarized in Supplementary Table S2, the JX143 nucleotide sequence shared an overall identity of 61.6 % and 89.5 %, respectively, with the LV strain (Type I, GenBank accession no. M96262) and VR-2332 strain (Type II, GenBank accession no. DQ176021) and was 99.3 % identical to the JXA1 sequence (GenBank accession no. EF112445), another Chinese PRRSV strain isolated during the PHFS outbreak (Tian et al., 2007). Compared to VR-2332 (Nelsen et al., 1999), the PHFS-related PRRSV isolates contained a single nucleotide deletion in both the 5’ UTR and the 3’ UTR. Moreover, these PRRSV isolates shared the same
consecutive deletions of 1 and 29 amino acids in their nsp2-coding region, which is the most variable part of the PRRSV genome (Fang et al., 2004; Gao et al., 2004; Han et al., 2007; Ropp et al., 2004). Curiously, the nsp2 deletion partially overlaps with that in the genome of PRRSV MN184, an atypical high pathogenicity (HP) PRRSV strain from North America, thus promoting the hypothesis that such a deletion may be related to the increased virulence of these viruses (Han et al., 2007). Tian et al. (2007) first demonstrated that this PRRSV variant caused almost 100% mortality in 35-day-old pigs and 57% mortality in 77-day-old growing pigs. Based on these results, Tian and co-workers proposed that the causative agent of PHFS was a HP PRRSV variant. Other groups also confirmed the HP nature of PRRSV isolated from typical PHFS cases by using cell-culture-amplified viruses for challenge studies in animals (An et al., 2007; Tong et al., 2007), although it remained to be proven that the virus inoculum was free of other pathogens. In fact, it has been debated whether other agents or unknown novel pathogens may cause or contribute to PHFS. Therefore, the role of the prevalent HP PRRSV strain in the PHFS outbreak remained to be characterized in more detail.

Reverse genetic systems are a powerful tool for the molecular dissection of arteriviruses and infectious cDNA clones have been developed for equine arteritis virus and traditional PRRSV strains (Balasuriya et al., 1999, 2007; Meulenberen et al., 1998; van Dinten et al., 1997; Wang et al., 2008). In this study, we set out to construct infectious cDNA clones of HP PRRSV to further clarify the role of this virus as the major aetiological agent of PHFS. The strategy we adopted is shown in Supplementary Fig. S1 and was described previously (Yuan & Wei, 2008). Briefly, cDNA fragments covering the entire JX143 genome were cloned and assembled into full-length cDNA clone pJX143, in which the viral sequence was placed downstream of a T7 promoter. The viral genome sequence was determined and deposited in GenBank (accession no. EF488048). Compared to the parental genome sequence (EU708726), a total of 34 nucleotide variations were identified (Supplementary Table S3). Among these, two substitutions, C328T and C6267T, were attributed to the quasispecies nature of the PRRSV genome, whereas the remaining 32 nucleotide differences might be PCR artefacts. Among these mutations, 15 nucleotide changes were translationally non-silent, almost all of which were located in ORF1 except for nt 12 309 (Asn→Pro) in ORF2, and nt 12 697 (Ser→Arg) in ORF3.

To differentiate the recombinant virus from the parental virus, and to exclude the possibility of a contamination, pJX143M was constructed containing a translationally silent substitution, A14680G, which created a novel MluI restriction site. As described previously (Yuan & Wei, 2008), 3 μg in vitro-transcribed RNA was mixed with 2 μl DMRIE-C (Invitrogen) and transfected into a subconfluent monolayer of MA-104 cells. At 72 h post-transfection (h p.t.), cytopathic effect (CPE) was observed in cells transfected with both pJX143 (Fig. 1a, panel i) and pJX143M (data not shown), while mock-transfected cells remained normal (Fig. 1a, panel ii). To characterize the rescued viruses, immunofluorescence assays (IFA) were conducted using a monoclonal antibody recognizing the viral nucleocapsid (N) protein (a kind gift from Dr Kegong Tian). Briefly, passage 3 material of parental JX143 and the rescued viruses (vX143 and vX143M) was used for inoculation of MA-104 cells at an m.o.i. of 0.1. At 36 h post-infection (p.i.), the infected cells were fixed and used for IFA as previously described (Sun et al., 2007). As shown in Fig. 1(b), at 36 h p.i., about 20% of the cells stained positive, indicating that the rescued viruses displayed infection kinetics similar to those of the parental virus.

The growth kinetics of the rescued viruses were evaluated by inoculating MA-104 cells with P3 viruses and at m.o.i. of 0.1, collecting the supernatant of the infected cells and virus progeny titration by determining the 50% tissue culture infectious dose (TCID50 ml−1) (Pizzi, 1950). As shown in Fig. 1(c), the growth curves of the rescued viruses were similar to those of the parental virus. At 56 h p.i., all three viruses reached their peak titre, which was around 1.5 × 106.5 TCID50 ml−1, indicating that the growth properties of the recombinant viruses on MA-104 cells were unchanged.

To investigate if the rescued viruses remained stable during further passaging, both vX143 and vX143M were serially passaged on MA-104 cells using low infection doses (0.1 m.o.i.). The culture supernatant of the tenth passage was used for viral RNA isolation, followed by single-tube RT-PCR amplification (Tiangen) with primer pair SF13851 and Qst (Supplementary Table S1). The nucleotide sequence of the genomic region from nt 13 851 to the end of the poly(A) tail revealed that the engineered MluI site and its flanking sequences had remained unchanged. We next evaluated the viral RNA profiles in infected cells using Northern blot as described previously (Sun et al., 2007). The rescued viruses displayed the same genomic and subgenomic mRNA profiles as parental JX143 (Fig. 1d), demonstrating that the recombinant viruses had retained the molecular biological properties of the parental JX143 virus. Taken together, our data showed that we developed the first infectious cDNA clones for the PRRSV isolate associated with the PHFS outbreak in China.

The potential role of other unknown pathogen(s) in PHFS pathogenesis has been debated, since other viral and even bacterial pathogens were isolated from PHFS-like cases (Ning et al., 2006). Therefore, the question remained whether PHFS is caused by a single highly virulent pathogen or may result from a co-infection by multiple aetiological agents. To further define the aetiological agent of PHFS, and the pathogenicity of our recombinant PRRSV, we conducted animal challenge experiments. A group of 35-day-old pigs were purchased from a PRRSV-free farm and were further screened using serological tests
and RT-PCR to exclude the presence of PRRSV, PCV2, CSFV, pseudorabies virus (PRV) and the pathogenic bacteria *Streptococcus*, *Staphylococcus* and *Mycoplasma*, as described by Tian et al. (2007). The 12 pigs were randomly divided into four groups and housed in isolation in a BL-2 animal facility (Zhejiang Yebio Biotech). The animals in groups A, B and C were inoculated with 2 ml $3 \times 10^4.5$ TCID$_{50}$ of parental JX143, recombinant vJX143 or recombinant vJX143M, respectively, with group D forming the mock-inoculated control. The injected pigs were observed daily for clinical symptoms and rectal temperatures were recorded until the end of the experiment. Blood samples were collected from all the animals at 0, 3, 5, 7, 14 and 16 days p.i.

All virus-inoculated groups developed high fever, a typical PHFS sign, with rectal temperatures up to 41°C, starting from 4 days p.i. and sustained for at least 8 days (Fig. 2a). The temperature peak and infection kinetics of the recombinant virus-inoculated groups were similar to those of the parental virus-infected group ($P<0.05$). All virus-inoculated pigs developed PHFS clinical symptoms beginning at 5 days p.i., displaying lethargy, lack of appetite, coughing and often paralysis, as reported by others (An et al., 2007; Li et al., 2007; Tian et al., 2007). Remarkably, all virus-inoculated pigs had died by 13–16 days p.i. Specifically, pigs in group A died at 13, 14 and 16 days p.i., while the pigs of group B and C died at 14–15 days p.i. Post-mortem necropsy revealed pathological changes including interstitial pneumonia with lung hyperplasia, lung oedema, blood spots or ecchymosis, splenitis, sporadic blood spots in the kidneys, perivascularitis, cerebral oedema, meningoencephalitis and suppurative encephalitis, haemorrhagic spots in the lymph nodes and lymphadenectasis (data not shown), as previously described by Tian et al. (2007). Although the numbers of animals used were limited by the availability of qualified pathogen-free pigs, this in vivo study demonstrated that the recombinant viruses retained the in vivo pathogenicity of the parental virus.

The humoral immune response in the infected animals was measured by ELISA (IDEXX HerdChek PRRS 2X52). As shown in Fig. 2(b), the serum antibody level began to rise at 5 days p.i. and all virus-inoculated pigs had seroconverted by 14 days p.i. The overall serological responses of
the virus-inoculated pigs were indistinguishable from each other, demonstrating that the recombinant viruses retained the immunological properties of the parental JX143 isolate.

Virus isolation on MA-104 cells, RT-PCR and nucleotide sequencing were conducted to evaluate virus replication and tissue distribution in the infected animals. Viruses were detected in multiple tissues including lymph nodes, lungs, spleens and kidneys, which were tested by inoculating MA-104 cells with homogenized tissues. There were no significant differences in virus distribution among different tissues and between the recombinant and parental viruses (data not shown). These results established that all inoculated pigs developed a viraemia starting at 3 days p.i. and lasting until the day of the animal’s death, as summarized in Table 1, and that the recombinant viruses displayed virtually the same in vivo infection kinetics as the parental virus.

Taken together, both recombinant viruses (vJX143 and vJX143M) reproduced the clinical, pathological, immunological and virological parameters observed in clinical cases of PHFS, thus fulfilling Koch’s postulates and unambiguously identifying HP PRRSV as the causative agent of the devastating PHFS outbreak in China. However, it should be pointed out that PHFS has remained a poorly defined clinical term and that there is no generally accepted description of the clinical symptoms associated with PHFS, although ‘high fever’ was often observed in field cases. The experiments with the molecularly cloned virus described in this paper demonstrated that PRRSV is the major culprit of the PHFS epidemic, which in the field often presents as a variety of secondary infections due to HP PRRSV-induced immunosuppression. PRRSV shows a high degree of genetic, antigenic and virulence variations (Ellis et al., 1999; Meng, 2000; Mengeling et al., 1998; Sutherland et al., 2007; Yuan et al., 1999), and the cross-protection efficacy of the current vaccines based on classical PRRSV strains needs to be improved (Cano et al., 2007; Charerntantanakul et al., 2006). The development of the HP PRRSV reverse genetics system described in this study may contribute to the improvement of such a live attenuated vaccine.

Table 1. Viraemia in animals infected with recombinant or parental HP PRRSV-inoculated pigs, as analysed by RT-PCR and virus isolation

RT-PCR results using serum samples of the inoculated pigs. Virus isolation results based on the induction by serum samples of CPE on MA-104 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus</th>
<th>Number of RT-PCR positive pigs/total number of pigs* (days p.i.)</th>
<th>Number of virus isolation positive pigs/total number of pigs* (days p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>Parental JX143</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>B</td>
<td>vJX143</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>C</td>
<td>vJX143M</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>D</td>
<td>None†</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

*The numbers of dead pigs at the indicated time points were deducted, and the remaining pigs which died later were all viraemia positive. †Negative control group inoculated with PBS.
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References


