Emergence of novel European genotype porcine reproductive and respiratory syndrome virus in mainland China

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Porcine reproductive and respiratory syndrome (PRRS) has a major negative economic impact on the swine industry worldwide. During the investigation of PRRS virus (PRRSV) in mainland China, European genotype (EU, type 1) PRRSV isolates were detected in swine herds both with and without clinical symptoms. Two complete genome sequences for Chinese type 1 PRRSV isolates were identified from viruses isolated from lung tissue and sera. The two viruses, designated BJEU06-1 and NMEU09-1, produced cytopathic effects in primary porcine alveolar macrophages but not in Marc-145 cells, and had a mean diameter of 55 nm, as measured by transmission electron microscopy. Comparative sequence analysis revealed that they shared 87.0–91.5 % and 58.0–58.2 % identity with the EU and North American genotype (NA, type 2) prototypic strains LV and VR-2332, respectively. Remarkably, these isolates, characterized by concomitant deletions within non-structural protein 2 (Nsp2) and ORF3 hypervariable regions, have never been described. Phylogenetic trees showed that all of the novel Chinese isolates of European genotype are in the pan-European subtype 1 that is predominant in Europe. However, they evolved from different ancestors. These novel viruses are predicted to be products of the divergent evolution of ancestor PRRSV isolates introduced from Europe. This is the first report of type 1 PRRSV wild isolates being in mainland China. Our findings confirm that the Chinese type 1 PRRSV isolates originated from diverse progenitors and the type 1 and type 2 PRRSV isolates, having different biological properties, have coexisted on the Chinese mainland for several years.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) emerged in North America in 1987 (Keffaber, 1989) and in western Europe in 1990 (Wensvoort et al., 1991), and is now one of the most economically significant swine diseases worldwide. The aetiological agent of the disease, PRRS virus (PRRSV), was isolated in the Netherlands in 1991 (Wensvoort et al., 1991) and subsequently in the United States (Collins et al., 1992). Even though the PRRSV isolates on the two continents emerged almost simultaneously, caused similar disease symptoms and shared the same virion morphology, European and North American PRRSV isolates were antigenically and genetically very different (Forsberg et al., 2002; Ropp et al., 2004; Rossow, 1998). Thus, two genotypes of PRRSV have been defined: the European (EU genotype, type 1) and the North American (NA genotype, type 2) strains (Meng et al., 1995; Nelsen et al., 1999). Originally, EU PRRSV was restricted to Europe, while NA PRRSV was restricted to North America and Asia. Nowadays however, coexistence of the two genotypes has been identified in Europe, North America and Asia, complicating PRRSV differential diagnosis, disease prevention and control (Amonsin et al., 2009; Balka et al., 2008; Bötner et al., 1997; Dewey et al., 2000; Fang et al., 2007; Kim et al., 2009b; Ropp et al., 2004; Thanawongnuwech et al., 2004; van Vugt et al., 2001).

PRRSV is an enveloped, single-stranded positive-sense RNA virus belonging to the family Arteriviridae within the order Nidovirales (Cavanagh, 1997). The PRRSV genome is approximately 15 kb in length. The genome possesses a 5′-capped and 3′-polyadenylated region, a UTR at both the

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Supplementary tables describing the primers used for the amplification of the complete genomes of EU genotype PRRSV isolates and details of the PRRSV isolates used in this study are available with the online version of this paper.
Snijder, 1998; Snijder and replication (Allende proteolytic cleavage products relate to virus transcription encode the large ORF1ab replicase polyprotein whose UTR, cover more than two-thirds of the genome and

In order to detect PRRSV infection during the seroepidemiological survey of mainland China in 2009, both conventional RT-PCR, capable of detecting the EU and NA PRRSV genotypes, and differential detection real-time RT-PCR, capable of distinguishing between the classical and highly pathogenic NA PRRSV subgenotypes coexisting in the Chinese mainland, were implemented. Intriguingly, five sera samples collected from clinically normal herds in Inner Mongolia province were positive for PRRSV when tested by conventional RT-PCR, but were negative for both classical and highly pathogenic NA PRRSV when tested by differential-detection real-time RT-PCR, indicating the existence of type 1 PRRSV in mainland China (data not shown). Retrospective tests on the clinical samples submitted to the China Animal Disease Control Center (CADC) identified a lung tissue collected from an affected herd from Beijing in 2006 that showed the same result as the Inner Mongolia samples. According to the records of the samples, the Beijing herd presented temporary fever, mild respiratory manifestations and common clinical signs such as lethargy, anorexia, rubefaction and blue ears. In contrast, the Inner Mongolian herds did not show any visible clinical symptoms, indicating that infections of the Inner Mongolian herds were milder than that of the Beijing herd.

Viruses isolated from the Beijing lung tissue and Inner Mongolian sera were used to infect primary porcine alveolar macrophages (PAMs) and Marc-145 cells. All six samples induced cytopathic effects (CPE) in PAMs, but not in Marc-145 cells (data not shown). The results corresponded with a previous report that EU PRRSV could barely adapt to grow in Marc-145 cells without additional passages (Fang et al., 2004). Subsequently, PAM cultures inoculated with the Beijing and Inner Mongolian samples were harvested for further analysis when they reached approximately 70% CPE, at 36 and 72 h post-inoculation, respectively, and were designated BJEU06-1 (Beijing) and NMEU09-1 to NMEU09-5 (Inner Mongolia). Examination of virion particles by using TEM confirmed that the particles within the endoplasmic reticulum in the cytoplasm of PAMs were 45–65 nm in diameter and contained a 25–35 nm nucleocapsid (Fig. 1), consistent with PRRSV described previously (Pol et al., 1997; Wensvoort et al., 1992). All six PAM cultures gave a positive reaction in the IPMA assay using Amervac-specific antiserum as primary antibody, implying that the viruses obtained were PRRSV and probably of the European type (data not shown). Finally, EU and NA PRRSV-specific differential-detection real-time RT-PCR corroborated all of the samples as being type 1 PRRSV (Fig. 2).

Genome analysis of type 1 PRRSV

Complete genome sequences were determined for BJEU06-1 and NMEU09-1 viruses and assembled into contiguous sequences of 15 059 and 15 068 nt, respectively, excluding the 3’ poly(A) tails. The nucleotide sequences and genome organization of the two viruses were similar to other EU PRRSV isolates. Compared with EU PRRSV prototypic

RESULTS

Identification of type 1 PRRSV in mainland China

In order to detect PRRSV infection during the seroepidemiological survey of mainland China in 2009, both

5’- and 3’-ends, and at least nine ORFs (Conzelmann et al., 1993; Meulenberg et al., 1993; Snijder & Meulenberg, 1998). ORFs 1a and 1b, located immediately downstream of the 5’ UTR, cover more than two-thirds of the genome and encode the large ORF1ab replicase polyprotein whose proteolytic cleavage products relate to virus transcription and replication (Allende et al., 1999; Fang & Snijder 2010; Snijder, 1998; Snijder et al., 1995, 1996; van Dinten et al., 1996; Wassenaar et al., 1997; Wootton et al., 2000; Ziebuhr et al., 2000). The remaining ORFs (2a–7) are located upstream of the 3’ UTR and encode the structural proteins of the virion, including four glycoproteins (GP2a, GP3, GP4 and GP5), two unglycosylated envelope-associated proteins (E and M) and a nucleocapsid protein (N) (Meulenberg et al., 1995; Murtaugh et al., 1995; Nelsen et al., 1999).

Since its initial outbreak in mainland China at the end of 1995 PRRS has become widespread, and the infection rate of Chinese swine herds has risen to 90% generally (Guo et al., 1996; Yuan & Wei, 2008). Since June 2006, devastating large-scale outbreaks of PRRS, characterized by high fever and high morbidity and mortality, have overwhelmed nearly all Chinese herds. The disease is caused by a highly pathogenic PRRSV (HP-PRRSV) containing a unique discontinuous deletion of 30 aa in non-structural protein 2 (Nsp2) (Tian et al., 2007). Currently, PRRS has become one of the most significant problems for Chinese swine production, resulting in immense economic losses for pig farmers every year. However, all heretofore described PRRS viruses in China, including the HP-PRRSV isolates, are of the NA genotype (Li et al., 1999; Li et al., 2009b; Zhou & Yang, 2010; Zhou et al., 2009a, b). Although few partial sequences of type 1 PRRSV isolated in mainland China, such as B13, Ningbo42 and FJ0603 (GenBank nos AY633973, EF473137 and EF592535, respectively) have been submitted to GenBank, as yet type 1 PRRSV isolation and analysis have not been reported in the Chinese mainland.

Here, we report the first identification of type 1 PRRSV isolates from clinical samples in mainland China from 2006 to 2009. Virus isolation, transmission electron microscopy (TEM) observations, immunoperoxidase monolayer assays (IPMA) and differential-detection real-time RT-PCR for EU and NA PRRSV confirmed that these isolates obtained in the present study are PRRSV and of type 1. Determination of complete genome sequences and phylogenetic analyses show that while the type 1 PRRSV isolates from the Chinese mainland are closely related to the PRRSV isolates that are predominant in Europe, the newly emerging Chinese isolates are unique and originated from different ancestors than did type 1 PRRSV.
strain Lelystad virus (LV), each fragment of these two genomes was of the same length except that the ORF1a, ORF3 and ORF4 genes had novel deletions (Table 1). In addition, the sequences of ORFs 2a–7 for isolates NMEU09-2 to NMEU09-5 were also identified and showed 99.4–99.9 % nucleotide identity with the NMEU09-1 isolate.

In order to evaluate the genomic characteristics of the Chinese type 1 PRRSV isolates, we compared the nucleotide and amino acid sequences of the LV, BJEU06-1 and NMEU09-1 isolates (Table 1). Complete sequence analysis showed that, compared to LV, the genomes of BJEU06-1 and NMEU09-1 exhibited 91.5 and 87.0 % identity, respectively. However, when compared to the NA PRRSV prototypic strain VR-2332 they showed only 58.0 and 58.2 % identity, respectively. Thus, these PRRSV isolates were further confirmed to be of the European genotype. Notably, the degree of complete sequence identity between BJEU06-1 and NMEU09-1 was only 84.9 %, even lower than the identities that resulted from comparisons with LV, indicating that they were probably from different ancestors.

When the Chinese type 1 PRRSV isolates were compared with LV, the Nsp2 protein showed one of the lowest amino acid identities among all of the protein products, consistent with the notion that the Nsp2 gene was the most variable part of the genome (Allende et al., 1999; Fang et al., 2004; Shen et al., 2000). Of all the structural proteins encoded by ORFs 2a–7, GP3 shared the lowest degree of identity with EU PRRSV and was recognized as one of the most heterogeneous proteins (Dea et al., 2000). GP5 was another of the most variable structural proteins, and thus accorded with previous reports (Dea et al., 2000). Intriguingly, we identified a 4 aa deletion between amino acids 741 and 742 and a 1 aa deletion at residue 792 in the Nsp2 protein of BJEU06-1, and a 2 aa deletion between amino acids 742 and 743 in the Nsp2 protein of NMEU09-1 (Fig. 3a).

Meanwhile, an 8 aa deletion was observed between amino acids 60 and 61 within the 84 aa encoded by overlapping regions of ORF3 and ORF4 (Drew et al., 1997; Fig. 3b), which correlates highly with the Danish viruses (such as virus 21191, isolated in 1997) and Hong Kong isolate HKEU16. Remarkably, this is the first report of type 1 PRRSV mutants in mainland China, and concomitant deletions in the Nsp2 and ORF3 hypervariable regions have not been described among all PRRSV isolates. However, we did not observe any unique substitution, insertion or deletion in the GP5 envelope protein (Fig. 3c). In addition, recombination analysis with the SimPlot and Bootscan programs showed that both these viruses are probably not recombinants derived from any known EU PRRSV isolates (data not shown). To further identify whether the wide spread of type 2 PRRSV in mainland China influenced the evolution of Chinese type 1 PRRSV, we compared three variable regions including Nsp2, the overlap of ORF3 and ORF4, and ORF5 (data not shown). We found that amino acid 478 of Nsp2 in both BJEU06-1 and NMEU09-1 was alanine, which was previously specific for type 2 PRRSV (Fig. 3a). Therefore, the evidence supports type 2 PRRSV isolates acting upon the evolution of the novel Chinese type 1 PRRSV.

Fig. 1. Electronmicrograph of PAMs 60 h post-infection with the NMEU09-1 isolate. NMEU09-1 virus particles were visualized within the endoplasmic reticulum in the cytoplasm (indicated by arrows). The micrograph was taken at ×70 000 magnification.

Fig. 2. Identification of Chinese type 1 PRRSV isolates using a duplex real-time RT-PCR assay. FAM fluorescent signal specific for NA PRRSV was only detected when the NA-genotype positive control (VR-2332) was used as the template. Meanwhile, only HEX signals could be detected when the six clinical samples and type 1-genotype positive control (LV) were detected. Neither FAM nor HEX signals could be detected when assaying the negative control.
Phylogenetic analyses

To gain further insight into the genetic relationship of BJEU06-1 and NMEU09-1 with other PRRSV isolates, four phylogenetic trees were constructed based on complete genome sequences (Fig. 4) and Nsp2, ORF3 and ORF5 nucleotide sequences (Fig. 5a–c). The phylogenetic tree constructed using complete sequences showed that both BJEU06-1 and NMEU09-1 clustered with type 1 PRRSV isolates and were clearly different from the dominant viruses found in China in recent years. To understand the genetic variation and evolution of Chinese type 1 PRRSV, Nsp2-, ORF3- and ORF5-based phylogenetic trees were produced. The trees inferred from all of these genes were in satisfactory agreement and revealed that the newly emerging Chinese type 1 PRRSV isolates belonged to the pan-European subtype 1 that is predominant in Europe (Stadejek et al., 2006, 2008), were unique and were closely related to Danish isolates. Interestingly, although three Chinese type 1 PRRSV viruses, isolated from both temporally and geographically different Chinese regions (HKEU16 was isolated in 2007 in Hong Kong while BJEU06-1 and NMEU09-1 were isolated in 2006 and 2009, respectively, in mainland China) had the same characteristic 8 aa deletion from the ORF3 and ORF4 overlapping region, they probably originated from different ancestors. The trees also showed that the five viruses (NMEU09-1 to NMEU09-5) isolated in the Inner Mongolia province of China represented the latest evolutionary events pertaining to type 1 PRRSV and formed a new subgroup. Moreover, the ORF5 sequence of B13, the earliest Chinese type 1 PRRSV sequence in GenBank, was highly consistent with LV, containing only a two nucleotide difference. The Nsp2 and ORF5 sequences of FJ0603 had the closest identity with the Amervac PRRS strain and belonged to the same subgroup. In addition, the other ORF7 sequences from

Table 1. Detailed comparison of the complete genomes among the LV, BJEU06-1 and NMEU09-1 isolates

<table>
<thead>
<tr>
<th>Region</th>
<th>Length</th>
<th>Identity (%)</th>
</tr>
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<tr>
<td></td>
<td>LV</td>
<td>BJEU06-1</td>
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<tr>
<td>Nucleotides</td>
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<tr>
<td>5' UTR</td>
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<td>221</td>
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<tr>
<td>ORF1a</td>
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<tr>
<td>ORF1b</td>
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<td>4392</td>
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<td>ORFs 2–7</td>
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<td>3165</td>
</tr>
<tr>
<td>3' UTR</td>
<td>114</td>
<td>114</td>
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<tr>
<td>Complete*</td>
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<td>15059</td>
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<tr>
<td>Proteins</td>
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<tr>
<td>Nsp7α</td>
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<td>Nsp12</td>
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<td>ORF2a/GP2</td>
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<tr>
<td>ORF2b/E</td>
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</tr>
<tr>
<td>ORF3/GP3</td>
<td>265</td>
<td>257</td>
</tr>
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<td>ORF4/GP4</td>
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<td>ORF5/GP5</td>
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<tr>
<td>ORF6/M</td>
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<td>173</td>
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<tr>
<td>ORF7/N</td>
<td>128</td>
<td>128</td>
</tr>
</tbody>
</table>

*Complete length is the genome sequence excluding the 3' poly(A) tail.
Fig. 3. The amino acid variants in Nsp2 (a), overlapping region of ORF3 and ORF4 (b) and ORF5 (c) for BJEU06-1 and NMEU09-1 isolates. (a) A 4 aa deletion between amino acids 741 and 742 and a 1 aa deletion at position 792 in Nsp2 of BJEU06-1 are marked with boxes. A 2 aa deletion between amino acids 742 and 743 in Nsp2 of NMEU09-1 is marked with a dotted line box. The alanine at position 478 in Nsp2 of both BJEU06-1 and NMEU09-1 which was previously specific for type 2 PRRSV is marked by a grey background. (b) An 8 aa deletion between amino acids 60 and 61 within overlapping site of ORF3 and ORF4 is marked with a box. (c) No amino acid variant was observed in epitopes at amino acid 24 and between amino acids 189 and 201 of ORF5. Dots in the amino acid sequences identify these residues as being identical to the sequence of LV. Underlined regions are the B-cell epitope sites (ES) identified previously (de Lima et al., 2006; Oleksiewicz et al., 2001; Wissink et al., 2003).
Chinese type 1 PRRSV available from GenBank [Ningbo42, Ningbo45, Ningbo57, N-34 and SS-6 (EF473137, EF473139, EF537859 and GQ183803, respectively)] also closely correlated with the vaccine strain Amervac PRRS and clustered in the same subgroup. These results suggest that these isolates might have evolved from the type 1 prototypic strain and the revertants of the vaccine strain, respectively, and that they share no ancestry with the isolates in the current study. Therefore, although all of the Chinese European-genotype PRRSV isolates were in pan-European subtype 1, they probably evolved from different progenitors, including the LV-like isolate B13, the Amervac PRRS vaccine-related isolate FJ0603 and three diverse isolates represented by deletion variants of HKEU16, BJEU06-1 and NMEU09-1.

DISCUSSION

Research on PRRSV in China has primarily focused on NA PRRSV isolates, and in recent years especially on the highly pathogenic PRRSV isolates (Gao et al., 2004; Li et al., 2008, 2009b; Tian et al., 2007; Zhou et al., 2009a). In contrast, only a few Chinese type 1 PRRSV sequences have been submitted to GenBank and no corresponding research has been reported, probably owing to the difficulties of EU PRRSV isolation and the lack of epidemic diseases directly caused by Chinese type 1 PRRSV. Here, we focus on the detection and identification of Chinese type 1 PRRSV isolates from herds both with and without clinical manifestations, which led to the discovery of type 1 PRRSV field isolates in mainland China. Complete genomic sequences of Chinese type 1 PRRSV isolates were determined and used to analyse the molecular variation and evolution of these viruses. This study led to the first identification of type 1 PRRSV deletion mutants in mainland China.

Previous studies revealed that hitherto fully sequenced type 1 PRRSV isolates, with the exception of 01CB1, contain deletions within the Nsp2 or ORF3 hypervariable regions when compared with LV. EuroPRRSV, SD01-08 and KNU-07 include 17, 17 and 20 aa deletions, respectively, within Nsp2 (Nam et al., 2009; Ropp et al., 2004), while HKEU16 possesses an 8 aa deletion within the overlapping region of ORF3 and ORF4, similar to the Danish viruses isolated in 1997 and 1998 (Oleksiewicz et al., 2000). Noticeably, both BJEU06-1 and NMEU09-1 contain deletions in these two hypervariable regions, including relatively shorter deletions within Nsp2 (5 and 2 aa, respectively) and the same 8 aa deletion within the region of ORF3 overlap with ORF4. These concomitant deletions within Nsp2 and ORF3 have not been described for any other PRRSV isolates and such deletions could be the biological marker of novel Chinese type 1 PRRSV field isolates.

Nsp2, the most variable protein within PRRSV, is tolerant of mutations, including deletions and insertions, within both genotypes of PRRSV (Gao et al., 2004; Han et al., 2006; Nam et al., 2009; Ropp et al., 2004; Shen et al., 2000; Tian et al., 2007). In recent studies, six B-cell epitope sites (ES) have been identified within Nsp2 of type 1 PRRSV, and the deletions of Nsp2 for type 1 PRRSV isolates are located among ES3 and ES4 (Fang et al., 2004, 2008; Kim et al., 2009a). In this study, we identified the 4 aa and 2 aa deletions within Nsp2 of BJEU06-1 and NMEU09-1, respectively, located between the ES3 and ES4, and another 1 aa deletion for BJEU06-1 within ES4. The immunodominant epitopes of Nsp2 are demonstrated to have a great effect upon the modulation of the porcine humoral immune response; the deletions in the Nsp2 hypervariable regions are predicted to be immunologically important and might affect pathogenic mechanisms (Chen et al., 2010; Fang et al., 2004, 2008; Kim et al., 2009a). ORF3, for which a hypervariable region has been identified...
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(Chart depicting the genetic relationship between different strains of PRRSV.)

Subtype 1

Subtype 2

Subtype 3

http://vir.sgmjournals.org
at the carboxy-terminal end overlapping with ORF4, encodes the second most variable protein of PRRSV (Dea et al., 2000; Drew et al., 1997; Katz et al., 1995; Oleksiewicz et al., 2000). Early reports hypothesized that the overlapping regions of ORF3 and ORF4 may be subjected to selective evolutionary pressure (Drew et al., 1997; Katz et al., 1995). The 8 aa deletions of BJEU06-1 and NMEU09-1 are located in ES12 at the overlap, resulting in disruption of ES12 and inducing conformational changes, which could possibly have a role in virus–host cell interactions and therefore on PRRSV protective immunity (Drew et al., 1997; Oleksiewicz et al., 2000; Urniza et al., 1997). Although the exact significance of these deletions cannot be elucidated at present, previous reports found that ES3, ES4 and ES12 exhibited low levels of sequence conservation and induced high levels of antibodies, and proposed that these hypervariable epitopes could act as decoys, luring the host antibody responses away from the conserved neutralizing epitopes nearby (Fang et al., 2004; Oleksiewicz et al., 2001; Ostrowski et al., 2002). GP5, encoded by ORF5, is another of the most heterogeneous PRRSV structural proteins (Dea et al., 2000). Two immunodominant epitopes, one at amino acid 24 and another between amino acids 189 and 201, have been identified in this envelope protein (de Lima et al., 2006; Wissink et al., 2003). However, these epitopes are conserved in both BJEU06-1 and NMEU09-1. In conclusion, we predict that the emergence of Chinese type 1 PRRSV deletion mutants is probably due to huge immunological pressures within Chinese herds, which are probably caused by the high infection ratio and wide immunity towards genotype II PRRSV. The phylogenetic trees based on the most hypervariable genes, Nsp2, ORF3 and ORF5, show that the majority of type 1 PRRSV isolates from the same countries in Europe and North America are similar and belong to the same or adjacent branches. However, Chinese type 1 PRRSV isolates are remarkably different and can be clustered into diverse branches. These trees reveal that Chinese type 1 PRRSV isolates evolve more quickly, and Chinese type 1 PRRSV infections can be more complicated, than in many European countries and in North America, which also have closely related special selective pressures to China owing to the prevalence of the highly pathogenic PRRSV from 2006. Furthermore, the coexistence of both NA PRRSV (such as CH-1a and JXA1) and type 1 PRRSV (such as B13 and FJ0603) may accelerate the genetic variation and evolution of PRRSV in China. Also, the fact that Chinese type 1 PRRSV isolates have the same amino acid that was previously specific to type 2 PRRSV indicates that type 2 PRRSV acts upon the evolution of type 1 isolates. The adaptive PRRSV evolution would be presumed to generate a gain in viral fitness. The direct origin of Chinese type 1 PRRSV has not yet been elucidated. However, the LV-like type 1 PRRSV isolates from China are probably the decedents of the EU PRRSV prototypic strain introduced to mainland China for research. Nowadays, although the EU PRRSV live-attenuated vaccine (Amervac PRRS) is not commercially available in the Chinese mainland, Amervac PRRS vaccine-related isolates probably originate from the vaccine that comes from intercommunication with neighbouring Asian countries, such as Thailand, where this vaccine is available. Furthermore, there is evidence to indicate that ORF3/4 deletion mutants mostly evolved from deletion mutant progenitors that existed at the start of the Danish epidemic (Oleksiewicz et al., 2000). Together with our findings, it is likely that the novel Chinese type 1 PRRSV isolates (BJEU06-1 and NMEU09-1) are the products of ancestral PRRSV isolates introduced from herds from the European continent that experienced gradual variation and accumulation of genomic changes in mainland China. The low identity between BJEU06-1 and NMEU09-1 indicates that they are probably from two different introductions. In addition, the different activities of replication in cell culture shown by BJEU06-1 and NMEU09-1 probably correlate with the different deletions observed within Nsp2 (Chen et al., 2010; Fang & Snijder, 2010; Han et al., 2007). Furthermore, the different clinical manifestations could be closely related to the distinction between genomes and the difference in growth kinetics. However, further studies need to be performed to define the relationships between the genomic distinctions, different grown kinetics and differences in pathogenicity among Chinese type 1 PRRSV isolates. So far no information about the origin of HKEU16 has been reported; however, it could be proposed that it was introduced from a Danish strain because the same deletion is within the overlap region of ORF3 and ORF4 (Oleksiewicz et al., 2000).

Even though the isolates discovered in the present study are probably not recombinant viruses, recombination is a common evolutionary mechanism (another is mutation) implicated in the generation of nidovirus genome diversity (Gorbalenya et al., 2006). The coexistence of the EU and NA PRRSV genotypes within Chinese herds creates a suitable environment for PRRSV intra-type and inter-type recombination. Intra-type recombination of NA and EU PRRSV has been described previously (Forsberg et al., 2002; Kapur et al., 1996; Li et al., 2009a; van Vugt et al., 2001; Yuan et al., 1999). Inter-type recombination between EU and NA PRRSV is unlikely to occur and has not been reported to date (van Vugt et al., 2001). However, toroviruses appear to undergo non-homologous recombination during viral evolution (Snijder et al., 1991);
were resuspended in Roswell Park Memorial Institute 1640 medium resulting cells were mixed together and washed twice in PBS. Cells from the pigs were in accordance with the regulations of the People’s Republic of China on the administration of laboratory animals. The lung tissues were washed 3–5 times with PBS (pH 7.2).

1993). Briefly, PAMs were obtained from the lungs of 4–6-week-old pigs and one lung tissue sample from Beijing were used for virus isolation and further identification of type 1 PRRSV. A total of 100 PRRSV isolates in Chinese swine herds provides a suitable environment for developing new viral biological characteristics. Meanwhile, it complicates the prevention and control of PRRSV in China. Available strategies should be deployed to constrain the prevalence of PRRSV in Chinese swine herds, not only for type 2 PRRSV but also for type 1 PRRSV. Strategies such as the introduction of an effective type 1 PRRSV vaccine should be considered, especially when the prevalence of pathogenic type 1 PRRSV in Chinese herds has been determined. However, according to the weak cross immunoprotection of different PRRSV isolates, the diversity of type 1 PRRSV may lead to insufficient protection being provided by the current type 1 genotype live-attenuated vaccines (Labarque et al., 2004). Therefore, preparation of a highly efficient live vaccine based on the novel Chinese type 1 PRRSV isolates should also be considered.

METHODS

Sample collection and routine detection. Samples were obtained from clinical samples submitted to the CADC from various Chinese provinces from 2006 to 2009 and collected during the molecular epidemiology investigation of PRRSV in China in 2009. Conventional RT-PCR for both genotypes of PRRSV (Oleksiewicz et al., 1998) and the differential-detection real-time RT-PCR for classical and highly pathogenic NA PRRSV coexisting in China (Chen et al., 2009) were carried out at the CADC. All samples infected by other swine viruses, such as classical swine fever virus, Japanese encephalitis virus, pseudorabies virus, porcine parvovirus and porcine circovirus type 2 were excluded from this study.

Virus isolation. Five serum samples from Inner Mongolia province and one lung tissue sample from Beijing were used for virus isolation in PAMs and Marc-145 cells. Marc-145 cells were obtained from the China Institute of Veterinary Drug Control. PAM preparation and inoculation from sera and tissue homogenate have been described previously (Mengeling et al., 1995; Ropp et al., 2004; Zeman et al., 1993). Briefly, PAMs were obtained from the lungs of 4–6-week-old specific-pathogen-free pigs, and the procedures for collecting lung tissues from the pigs were in accordance with the regulations of the People’s Republic of China on the administration of laboratory animals. The lung tissues were washed 3–5 times with PBS (pH 7.2). Each aliquot of lavage fluid was centrifuged for 10 min at 800 × g. The resulting cells were mixed together and washed twice in PBS. Cells were resuspended in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Gibco) supplemented with 10% irradiated PBS (Biochorm), 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin. Macrophages were aliquoted at 3 × 10⁶ cells ml⁻¹ into plastic flasks and inoculated with the positive samples. The same samples were propagated on Marc-145 cells cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS as previously described (Han et al., 2009; Kim et al., 1993). The inoculated cells were maintained at 37 °C in a 5% CO₂ atmosphere. The cultures were observed daily for CPE and were frozen at −70 °C when 70% CPE was reached, or after 5 days of culturing (Wensvoort et al., 1991).

Further identification of type 1 PRRSV. One of the Chinese type 1 PRRSV pathogens was subjected to electron microscopic observation (Tian et al., 2007). Briefly, PAMs were infected with NMEU09-1 at an m.o.i. of approximately 0.01 and infected cells were collected at 60 h post-infection. The cells were fixed overnight with 3% glutaraldehyde, washed with PBS, treated with 1% osmic acid, dehydrated in a graded ethanol series, and infiltrated and embedded in 618 epoxy resin (Zhongjing). Ultrathin sections, 50–100 nm in thickness, of NMEU09-1-infected PAMs were cut from embedded blocks and mounted onto 200-mesh copper grids. The grids were then stained with uranyl acetate and Reynolds’ lead citrate and examined using a Hitachi-7500 transmission electron microscope. Furthermore, cultures that showed CPE were cultured in 36-well microtitre plates and stained with IPMA as described by Wensvoort et al., (1991), using an EU PRRSV antiserum prepared by Amervacc PRRS live-vaccine inoculation (Yoon et al., 1996). In addition, the isolates of five serum samples from Inner Mongolia (NMEU09-1 to NMEU09-5) and of lung tissue from Beijing (BJEU06-1) were further identified by a differential-detection real-time RT-PCR assay for EU and NA PRRSV (Kleiboeker et al., 2005), which used a one step PrimeScript RT-PCR kit (TaKaRa).

RNA extraction, primer design and DNA sequencing. To preclude the possibility of sequence changes when adapting the viruses to growth on PAMs, viral RNA was extracted directly from the sera and lung tissue samples using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Viral RNA was eluted from the filter column with 50 µl of nuclease-free double-distilled water and stored at −80 °C. A total of 16 primer pairs were designed from the complete genomic sequences of LV and HKEU16 (GenBank accession nos M96262 and EU076704, respectively). The 16 RT-PCR amplified products overlapped each other, and these spanned the entire genome. The list of primers is provided in Supplementary Table S1 (available in JGV Online). Two complete genomes of BJEU06-1 and NMEU09-1 and four partial genomes of NMEU09-2 to NMEU09-5, each comprising ORFs 2a–7, were sequenced. The RT-PCR system and amplification conditions have been described previously by Han et al. (2009). The amplicons were purified with an EZ.N.A. Gel Extraction kit (OMEGA) and cloned into pGEM-T Easy (Promega). DNA sequencing of the recombinant clones was carried out by a commercial laboratory (Invitrogen) using primers specific for the T7 and SP6 promoters in both directions. The nucleotide sequences were determined for at least three independent cDNA clones.

Sequence and phylogenetic analyses. A total of 100 PRRSV isolates, including 18 completely sequenced isolates, were independently used in sequence and phylogenetic analyses (Supplementary Table S2, available in JGV Online). Sequence identities were calculated with DNAMAN (Lynnon Biosoft Company). Multiplex sequence alignments were generated using CLUSTAL X version 1.83 (Jeanmougin et al., 1998). Recombination identifications were analysed with the SimPlot and Bootscan programs (Li et al., 2009a). Phylogenetic analyses were conducted with the MEGA4 program (Tamura et al., 2007). Phylogenetic trees were constructed from aligned nucleotide sequences using the neighbour-joining method and maximum composite likelihood model, which included thereby, inter-type recombination might also emerge in Chinese herds coinfected with both virus genotypes and become a threat. Intra-type recombination is probably an important genetic mechanism contributing to PRRSV evolution and could potentially generate a virus with new biological properties. The inter-type recombination could be presumed to serve a similar function, especially considering the recent confirmation of PRRSV natural recombination in China (Li et al., 2009a). Recombination should be seriously taken into account when studying the epidemiology of PRRSV, including NA and EU PRRSV in mainland China.

The coexistence of type 1 and type 2 genotype PRRSV isolates in Chinese swine herds provides a suitable environment for developing new viral biological characteristics. Meanwhile, it complicates the prevention and control of PRRSV in China. Available strategies should be deployed to constrain the prevalence of PRRSV in Chinese swine herds, not only for type 2 PRRSV but also for type 1 PRRSV. Strategies such as the introduction of an effective type 1 PRRSV vaccine should be considered, especially when the prevalence of pathogenic type 1 PRRSV in Chinese herds has been determined. However, according to the weak cross immunoprotection of different PRRSV isolates, the diversity of type 1 PRRSV may lead to insufficient protection being provided by the current type 1 genotype live-attenuated vaccines (Labarque et al., 2004). Therefore, preparation of a highly efficient live vaccine based on the novel Chinese type 1 PRRSV isolates should also be considered.
transitions and transversions, substitutions, homogeneous patterns among lineages and uniform rates among sites. Also, we chose the complete deletion option to treat gaps and missing data. The robustness of the phylogenetic constructions was evaluated by bootstrapping using 1000 replicates.

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