

## Concurrent Porcine Reproductive and Respiratory Syndrome Virus, Porcine Circovirus Type 2 and Pseudorabies Virus Infections Associated With 'High Fever Syndrome' in Swine of Northern China

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### Abstract

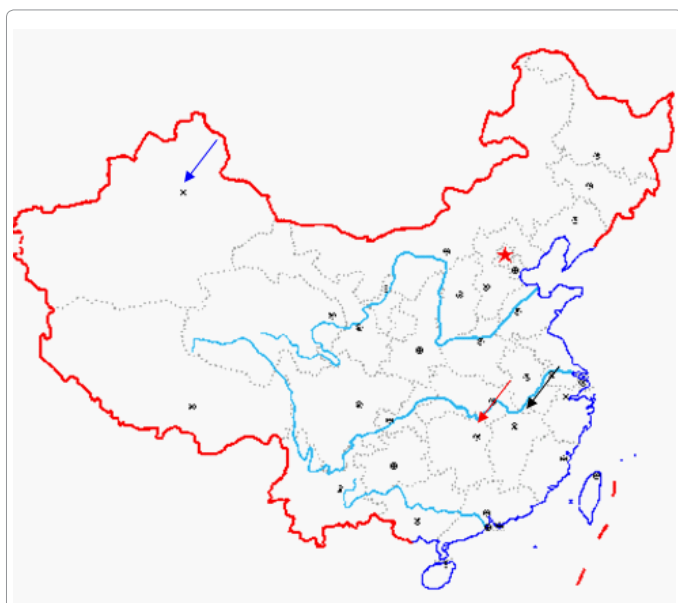
Field and laboratory diagnostic investigation was conducted during recent outbreaks of "High Fever Syndrome (HFS)" in numerous large-sized swine farms in Xinjiang of China which obtained pigs from Jiangxi and Hunan Provinces where outbreaks of HFS coincided. The syndrome occurred in these provinces from April 2006 to June 2006 with a peak of index cases in late May 2006. The most common clinical symptoms of HFS in affected pigs were elevating body temperature and rubefaction. Overall morbidity and mortality ranged from 20 to 80%. In sow farms the abortion rate by HFS reached 65% and many weaned piglets had severe dyspnea. The incidence rate of the syndrome in finishing/marketing pigs (10–15 months of age) was up to 50%. In deceased pigs, organ failure was observed in the lungs, liver, spleen, intestines, and lymph nodes. Animals examined had serological evidence of prior exposure to pseudorabies virus (PRV). Furthermore, laboratory testing by a PCR assay on samples from clinically affected animals demonstrated that the majority of index animals were concurrently infected with PRRSV and porcine circovirus type 2, suggesting that epidemic outbreaks of HFS was attributed to infection by multiple viral agents. Many index cases appeared to be related to the application of multiple live virus vaccines in an uncontrolled manner, raising the necessity for implementation of rigid regulation for vaccination programs.

**Keywords:** Swine High fever syndrome; Co-infection; PCV2; PRRSV; PRV

Wild and domesticated pigs are susceptible to a number of viral infections. Exemplary viral agents of economic importance in the Chinese swine industry are swine influenza (SIV), classical swine fever virus (CSFV), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), and porcine circovirus type2 (PCV2) [5,10,14,16,18,22]. Each agent has been implicated in reproductive, respiratory and/or neurological disease by itself or in combination.

Concurrent infections of PCV2 and PRRSV are common in swine [5,7,8,13,20]. Concurrent infections with PCV2 were linked to the secondary immune suppression noted in the postweaning multisystemic wasting syndrome (PMWS). Lymphoid depletions and immune system dysfunction at both humoral and cellular levels in pigs affected by PMWS may increase the vulnerability of the affected animals to other viruses [21]. Dual infections are not limited to PCV2, Swine can also be concurrently infected with PRRSV and PRV [17]. In general concurrent infections are believed to exacerbate the severity of disease. For example, experimental co-infection of PRRSV and PCV2 [11,24] produced significantly higher rate of mortality than did individual infection.

From April 2006 to Oct 2006, continuous outbreaks of the disease commonly referred as "Porcine High Fever Syndrome (PHFS)" were reported in numerous large-scale commercial swine farms in Nanchang (Jiangxi province) and Wuzhou (Hunan province) which are located in southern China. Geographic location of each province in China is illustrated in Figure 1. The clinical episode lasted until the spring of 2007. From March to October of 2006, the weather in Jiangxi and Hunan provinces were overcast and rainy. The daily temperature dramatically changed and became abnormally higher by the end of



**Figure 1:** Geographic location of Xinjiang (blue arrow), Hunan province (red arrow) and Jiangxi province (black arrow) in China.

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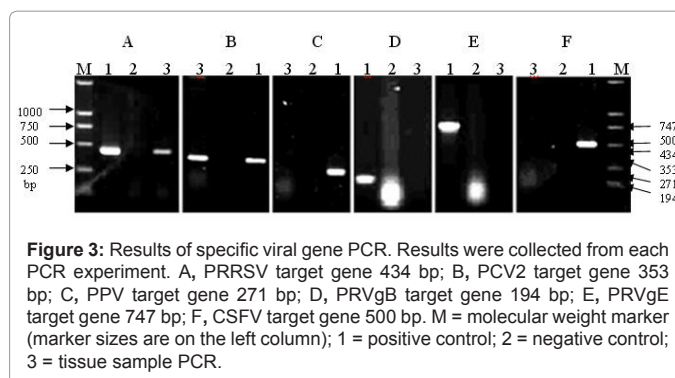
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May 2006 than all previous years. Almost concurrently, a few large-scale swine farms in Hami of Xingjing Autonomous region in northern China which obtained their piglets from the same supplier farms in Nanchang and Wuzhou had disease outbreaks similar to PHFS. In those farms, pregnant sows displayed increased rate of abortions (10%-30%) at early gestation; Postweaning piglets appeared with respiratory distress. The morbidity rate was about 50% and mortality rate ranged from 50% to 90% among the affected animals. Soon after the temperatures rose, there was the increase in outbreaks of PHFS. After local swine producers became aware of the disease outbreak, they fed or administrated various antibiotics (e.g. taimycin, oxytetracycline, cephalixin) without determining the cause of fever or identifying any specific infectious pathogens. As a result, many pigs died of drug toxicity, as well as kidney failure, after the indiscriminant drug treatments. In order to rescue the remaining pigs, some producers simultaneously injected attenuated virus vaccines for CSFV, PRV and/or PRRSV.

High fever (40.5-41.5°C, very few was 42.5°C) was exhibited in affected pigs, and Anorexia, lethargy, emesia, diarrhea, constipation, rhinorrhea, cough and increased ocular discharge were also observed in clinical cases of PHFS; hyperpyrexia and anorexia afflicted about 65% of sows and a high rate of early abortions was noted, whereas a



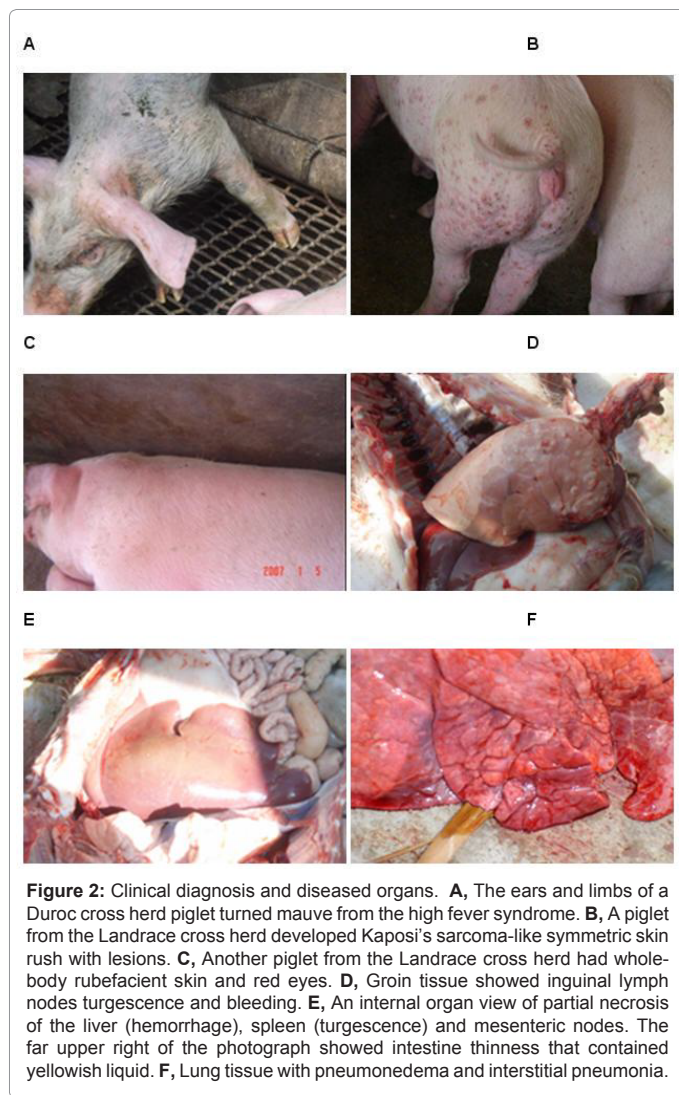
**Figure 3:** Results of specific viral gene PCR. Results were collected from each PCR experiment. A, PRRSV target gene 434 bp; B, PCV2 target gene 353 bp; C, PPV target gene 271 bp; D, PRVgB target gene 194 bp; E, PRVgE target gene 747 bp; F, CSFV target gene 500 bp. M = molecular weight marker (marker sizes are on the left column); 1 = positive control; 2 = negative control; 3 = tissue sample PCR.

smaller percentage of the animals exhibited abdominal erythema and a significant portion of pigs with PHFS suffered from abdominal respiration and rubefaction over their entire bodies.

Many pigs showed periodic hyperexcitability, tremors, and paddling, and at later stage of the syndrome, their ears, abdomens, and limb terminals became purple (Figure 2A). Others developed skin rash with lesions (Figure 2B) or rubefaction skin over their entire bodies (Figure 2C). The clinical signs observed in field cases were nonspecific and variable. In addition to the clinical signs described above, there was a marked increase in mortality. Grossly, swollen inguinal lymph nodes displayed a mauve color (Figure 2D). Partial necrosis of the liver, hemorrhaged spleens, and swollen mesenteric nodes (Figure 2E) were also observed. Lung lesions included edema, interstitial pneumonia, and bronchiointerstitial pneumonia with moderate to marked multifocal peribronchial and peribronchiolar fibrosis that often extended into the airway lamina propria (Figure 2F).

Clinical specimens (altogether 52 samples) were collected from diseased Duroc and Landrace crossbred herds undergoing PHFS from eight large-scale farms, and tested by polymerase chain reaction (PCR)-based assays for PRRSV, PCV2, PRV, PPV and CSFV, as well as virus isolation technique. For PCR testing, viral nucleic acids (DNA and RNA) were extracted from tissue homogenates (including heart, liver, spleen, lung, kidney, brain and lymph nodes) with Universal Genomic DNA Extraction Kit (Ver.3.0; TaKaRa Bio Inc.) or RNAiso Reagent Extraction Kit (TaKaRa) according to the manufacture's protocol. The reverse transcription reaction was performed in 20µl PCR master mixture consisting of 4µl 1x reaction solution [50 mM Tris-HCl (pH 8.3), 75mM KCl, 3mM MgCl<sub>2</sub>, 10mM DTT], 2µl dNTP, 1µl random 6 mers (50µmol) (TaKaRa), 1µl M-MLV reverse transcriptase (200U/µl) (Promega) and 1µl RNase Inhibitor (40U/µl)(TaKaRa), 7µl of RNA and 4µl DEPC-water(TaKaRa). PCR was carried out in a 25µl mixture containing 1x PCR buffer (500mM KCl, 100mM Tris HCl, pH 8.3), 200µM each dNTP, 10pmol of primer, 1unit of Taq™ DNA Polymerase (5U/µl) (TaKaRa), and 1µl of each virus DNA or cDNA template. All PCR primers were synthesized by a commercial vendor (TaKaRa). Primer sequences, target genes, and sizes of amplification are summarized for each target viral agent in Table 1. PRRSV, CSFV, PRV, PPV, PCV2, SIV single PCR single PCR were performed as previously described [1,3,9,23].

PCR amplification was done in GeneAMP PCR System 9700. Thermocycling conditions were: a) one cycle at 94°C for 5 min, b) 35 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C (PPV), 55°C (PCV2, CSFV), 58.6°C (PRRSV), 61.5°C (PRV) or 48.5°C (SIV) for 1min, respectively, and c) finally amplified at 72°C for 1min. The PCR reaction was completed with a final extension step of 10 min



**Figure 2:** Clinical diagnosis and diseased organs. A, The ears and limbs of a Duroc cross herd piglet turned mauve from the high fever syndrome. B, A piglet from the Landrace cross herd developed Kaposi's sarcoma-like symmetric skin rash with lesions. C, Another piglet from the Landrace cross herd had whole-body rubefaction skin and red eyes. D, Groin tissue showed inguinal lymph nodes turgescence and bleeding. E, An internal organ view of partial necrosis of the liver (hemorrhage), spleen (turgescence) and mesenteric nodes. The far upper right of the photograph showed intestine thinness that contained yellowish liquid. F, Lung tissue with pneumoedema and interstitial pneumonia.

Virus	Primer name and sequence	Target	Annealing temprature	Product size (bp)	Reference virus
PCV2	PCV2F: 5'-AAGGGCTGGGTATGGTATG-3' PCV2R: 5'-CGCTGGAGAAGGAAAAATGG-3'	ORF2	55°C	537	AY660574
PPV	PPV1: 5'-GAATAGGATGCGAGGAAAG-3' PPV2: 5'-GTGGAAATCTGAGAGTCTGT-3'	NS-1	50°C	271	PPU44978
PRV	PRVB1: 5'-GAAGGGGTTGGACAGGAAGGAC-3' PRVB2: 5'-TGGGGAGCGGGTACGTGTA-3'	gB	61.5°C	194	BK001744
PRV	PRVE1: 5'-GCCACCATCGCAGAGGAACAA-3' PRVE2: 5'-CCCACCGCCACAAAGAACAC-3'	gE	61.5°C	747	BK001744
PRRSV	PRRSV1: 5'-GCCAGTCCAGCCAGTCAATCA-3' PRRSV2: 5'-GCCCGATTGAATAGGTGAC-3'	N	58.6°C	434	AF331831 AY032626
CSFV	CSFV1: 5'-CTGGCGGGTGTATAGAGTGC-3' CSFV2: 5'-ATACTCGCCCTTGAGCATAT-3'	C	55°C	500	AY775178
SIV	SIV1: 5'-ACTAAGGGCTTCACTGAGG-3' SIV2: 5'-ACCGTGTACCATTCCATTC-3'	NS	48.5°C	138	AY700216 DQ145543 EF140698
Randrom 6 mers	5'-(p)NNNNNN-3'				

**Table 1:** The primer sequences using in this study.

Samples source	N	Virus		
		PRRSV	PRV	CSFV
Diseased piglets	30	0	18	0
Non-clinical piglets	10	0	0	0

**Table 2:** Summary results of the Dot-immunogold filtration assay.

at 72°C. The presence of target viral gene(s) in samples was detected by electrophoresing 10µl of amplicon through 1% agarose gels in TAE buffer [40mM Tris-Aceate (pH 8.0), 1mM EDTA]. None of the samples were positive for PPV, CSFV and PRV while all of the samples were positive for both PRRSV and PCV2. As shown in [Figure 3](#), each positive control viral agent could be specifically amplified using its defined primer pair.

Virus isolation attempts were performed using PK-15 cell line free of PCV1 (Iowa State University Veterinary Diagnostic Laboratory), primary pig fetal kidney (PFK) cells and porcine alveolar macrophages (PAMs). The PK-15 and PFK cells were maintained in Minimum Essential Medium (Sigma) supplemented with 8% fetal calf serum (GIBCO), 0.15% sodium bicarbonate and 1% antibiotics (BenzylpenicillinSodium and StreptomycinSulfate, Sangon), while PAMs were maintained in RPMI-1640 (Sigma)supplemented with 10% fetal bovine serum (GIBCO) and 1% antibiotics (Sangon) [27]. Virus isolation test for PCV2 was performed as previously described [25]. Inoculated cells were monitored daily for cytopathic effect (CPE) up to 5 days post inoculation. At the completion of 5 days, PCR (PCV2, PPV, PRRSV, CSFV, PRV) was performed on cell culture supernatant and/or cell lysate. Samples were considered negative after two blind passages. No other viral agents except PCV2 and/or PRRSV were isolated from the samples.

Standard aerobic and anaerobic bacterial culture testing was conducted using pre-reduced Columbia agar and MaConkey's agar (Sanland-chem International Inc). After 96-hour incubation, any growth of different colony type was subcultured and then subjected to phenotypic and biochemical identification. *Escherichia coil* (5/52) and *Pasteurella multocida* (2/52) were cultured out a few samples.

Sera (altogether 30 samples) were also collected from 30 diseased piglets pigs and 10 non-clinical piglets from eight large-scale swine farms and tested by dot immunoblot assays for antibodies specific for CSFV, PRRSV and PRV [4,12,19]. Pre-prepared diagnostic strips for the assay were kindly provided by the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (Harbin, Heilongjiang Province, China). Briefly, one drop (about 100µl) of

serum sample which was considered to contain PRRSV-, CSFV- or PRV-specific antibody , was spotted into the center (5-mm square) of the strip, if serum antibody were positive of PRRSV-, or CSFV- or PRV specific antibody , ten minutes later, the Control band (C) and Test band (T) lines which treated with a colloidal gold stain would become visible at ambient temperature, Otherwise, only Control band (C) lines became visible. The detect results of serum sample were demonstrated in [Table 2](#).

The present study reports a novel form of Porcine High Fever Syndrome among swine farms in Hami of Xinjiang Autonomous region in China. The etiological agents of the epidemic outbreaks were identified as PRRSV, PCV2, and PRV, demonstrating multi-factorial nature of the syndrome. As concurrent infections of PRRSV and PCV2 have become a major threat to pig-producing regions throughout the world since the discovery of PRRSV in the 1990s, finding of PCV2 and PRRSV co-infection would not be a novel observation. However, identification of concurrent infection by multiple viral agents is unique and distinct from previous reports [15,16] claiming that PRRSV with Nsp2 gene deletion was the etiology of PHFS. Although PRV DNA was detected in any of the samples tested, 60% of the pigs tested demonstrated serological evidence for the exposure to PRV. Furthermore, PRV infection was not previously found in Hami and vaccines were not used in the area. Thus, the pigs probably recovered from prior PRV infections. Nonetheless, effect of the prior PRV infections on the pathogenesis of, and/or immune response to, PRRSV and/or PCV2 is unknown. The role of interaction among multiple agents in PHFS as a pathogenic mechanism remains to be further studied.

Vaccines for various infectious agents were used in response to outbreaks of HFS. The field epidemiological data suggested that PHFS outbreaks were most likely worsened by the uncontrolled administration of multiple live virus vaccines. Interestingly, CSFV (wild type) was not detected and despite two-thirds of the pigs were vaccinated with CSFV live vaccine before sampling. Perhaps the dual infection of PRRSV and PCV2 may have inhibited the effective immune response to CSFV although it did not inhibit the on-going immune response to PRV. There was no evidence that dual infection of

PRRSV and PCV2 promoted CSF by a wild type. HPRRSV and PCV2 could cause *Escherichia coli* and *Pasteurella multocida* culture, and HPRRSV and PCV2, *Escherichia coli* and *Pasteurella multocida* may cause HFS together. Nonetheless, these observations emphasize that precaution should be taken when applying multiple live vaccines to swine farms in an unregulated manner because multiple agents could act synergistically for a disease even though the vaccine viruses are attenuated.

#### Sources and Manufacturers

TaKaRa Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa Bio Inc., Dalian, Liaoning Province, China)  
TaKaRa RNAiso Reagent Extraction Kit (TaKaRa Bio Inc.)  
M-MLV RT, (Promega Corporation, Beijing, China)  
Taq™ DNA Polymerase (TaKaRa Bio Inc.)  
GeneAMP PCR System 9700 (Applied Biosystems, USA)  
Agarose gels (NuSieve, FMC BioProducts, USA)  
PK-15 (Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa, USA)  
Strips (Harbin Veterinary Research Institute, Harbin, Heilongjiang, China)  
Colloidal gold stain (Aurodyne; Jansen Life Sciences, Piscataway, NJ)  
Fetal bovine serum (Invitrogen, USA)  
Columbia agar and MaConkey's agar (Sanland-chem International Inc, Xiamen, China)

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